# *p*-Isothiocyanatophenyl 6-Phospho-α-D-mannopyranoside Coupled to Albumin. A Model Compound Recognized by the Fibroblast Lysosomal Enzyme Uptake System. 2. Biological Properties†

Evelyn M. Karson, Elizabeth F. Neufeld, and Gloria N. Sando*

ABSTRACT: A conjugate of *p*-aminophenyl 6-phospho-α-D-mannopyranoside and bovine serum albumin was shown to interact with the uptake system for lysosomal enzymes in cultured human diploid fibroblasts. Radioiodinated conjugate containing 20 mol of mannose 6-phosphate/mol of albumin was taken up by the cells and degraded to trichloroacetic acid soluble fragments which were released into the medium. Unlabeled conjugate, mannose 6-phosphate, and a lysosomal enzyme, L-iduronidase, inhibited the uptake of the $^{125}$I-labeled conjugate ($K_i$ = $2 \times 10^{-8}$, $5 \times 10^{-6}$, and $1.5 \times 10^{-9}$ M, respectively). Conversely, the uptake of L-iduronidase was competitively inhibited by the mannose 6-phosphate conjugate as well as by free mannose 6-phosphate; however, higher concentrations of these compounds were required ($K_i$ = $10^{-6}$ and $5 \times 10^{-5}$ M, respectively). These results suggest that although L-iduronidase and the conjugate are bound to the same receptor by mannose 6-phosphate residues, the uptake of the enzyme involves some additional structure that is not shared by the conjugate. Internalization of the radiolabeled mannose 6-phosphate albumin conjugate was observed only in human diploid fibroblast strains. An SV-40 transformed line of human fibroblasts as well as three permanent rodent fibroblast lines (CHO, NRK, and L cells) failed to take up the conjugate, presumably because they were deficient in receptors or in the ability to internalize receptor–conjugate complexes.

The existence of a receptor recognizing some structural feature, or recognition marker, on certain lysosomal enzymes was initially deduced from the selectivity and efficiency of the uptake of these enzymes (Neufeld, 1974). A carbohydrate structure for the recognition marker was suggested by Hickman et al. (1974) because of susceptibility to periodate oxidation, but the relevant carbohydrate was not identified. Hieber et al. (1976) focused attention on mannose residues. Kaplan et al. (1977a) showed that mannose 6-phosphate was 1000 times more potent than mannose as an inhibitor of uptake of β-D-glucuronidase, and that uptake was diminished if the enzyme was first pretreated with phosphatase. Similar kinetic evidence was provided for mannose 6-phosphate participation in the recognition of other lysosomal enzymes: L-iduronidase (Sando & Neufeld, 1977), β-*N*-acetyl-D-hexosaminidase (Kaplan et al., 1977b; Ullrich et al., 1978), α-*N*-acetyl-D-glucosaminidase (Ullrich et al., 1978), and β-D-galactosidase (Distler et al., 1979). Mannose 6-phosphate has recently been identified in hydrolysates of a number of enzymes and other glycoproteins: β-glucuronidase (Natowicz et al., 1979), β-galactosidase (Sahagian et al., 1979), a testicular glycoprotein fraction which inhibits the uptake of β-galactosidase (Distler et al., 1979), α-*N*-acetylglucosaminidase (von Figura & Klein, 1979), α and β chains of β-*N*-acetylhexosaminidase, and cathepsin D (Hasilik & Neufeld, 1980b).

The value of synthetic carbohydrate–albumin conjugates (neoglycoproteins) in exploring pinocytosis systems has been amply demonstrated in studies of the hepatocyte galactose receptor (Krantz et al., 1976; Stowell & Lee, 1978) and of the mannose receptor of macrophages (Stahl et al., 1978). On the basis of these precedents, we prepared a conjugate of *p*-aminophenyl 6-phospho-α-D-mannoside and bovine serum albumin (Sando & Karson, 1980) and are reporting here on its recognition by the lysosomal enzyme uptake system.

## Experimental Procedures

*Materials.* Bovine serum albumin (fatty acid free), mannose 6-phosphate, and mannose 1-phosphate were purchased from Sigma Chemical Company. Sephadex products were obtained from Pharmacia; DEAE Bio-Gel A and Bio-Gel HT (hydroxylapatite) were from Bio-Rad Laboratories. Carrier-free Na$^{125}$I (pH 8–10) was purchased from New England Nuclear and chloramine-T was from Eastman Chemicals. Phenyl α-L-idopyranosiduronic acid and 4-methylumbelliferyl α-L-idopyranosiduronic acid were provided by Dr. B. Weissmann, University of Illinois, Chicago, IL.

The preparation of conjugates of albumin with *p*-aminophenyl glycosides or *p*-aminophenyl 6-phosphomannoside is described in the accompanying manuscript (Sando & Karson, 1980). A conjugate of bovine serum albumin coupled by reductive amination with *N*-[*S*-(6-*O*-phospho-α-D-mannopyranosyl)thioacetyl]aminoacetaldehyde was a gift from Drs. Y. C. Lee and R. Lee, Johns Hopkins University, Baltimore, MD. Mannopentaose monophosphate and proteins to which this oligosaccharide had been coupled were provided by Drs. D. Neville and G. Murray, National Institute of Mental Health, Bethesda, MD.

Human urinary L-iduronidase (EC 3.2.1.76) of the high uptake form was partially purified on heparin–Sepharose (Sando & Neufeld, 1977) and on hydroxylapatite (Rome et al., 1979a); its concentration was calculated on the assumption that it has the same specific activity as the kidney enzyme (Rome et al., 1978).

Other reagents were of the best grade available from commerical suppliers.

*Preparation of Radiolabeled BSA(PhMan-6-P)$_n$.*[1] A

† From the Genetics and Biochemistry Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20205, and the Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242 (G.N.S). *Received January 4, 1980.* This work was supported in part by the Arteriosclerosis/Specialized Center of Research, Grant HL 14230 from the National Heart, Lung and Blood Institute (G.N.S). E.M.K. was a recipient of a fellowship from the American Cancer Society.

[1] Abbreviations used: BSA(PhMan-6-P)$_n$, bovine serum albumin substituted with *n* *p*-isothiocyanatophenyl 6-phospho-α-D-mannopyranoside residues; BSA(PhMan)$_m$, the same, but with unphosphorylated mannoside. The number of substituents, *n*, is defined for each experiment.

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conjugate with 20 or more sugar phosphate substituents was purified on an anion exchange gel prior to iodination as a precautionary measure to remove poorly substituted albumin, which, although present as a small impurity, might be iodinated to a much greater extent than the bulk of highly substituted material. All procedures were carried out at 4 °C. A solution of BSA(PhMan-6-P)$_{20}$ was dialyzed against 0.01 M sodium phosphate, pH 6.0, and applied to a column, 2 × 5.5 cm, of DEAE Bio-Gel A equilibrated with the same buffer. After a 35-mL wash, a linear gradient was applied; it was prepared from 100 mL each of 0 and 0.4 M NaCl in 0.01 M sodium phosphate, pH 6.0. Fractions of 4 mL were collected. The conjugate was eluted in a sharp peak about midway in the gradient, well separated from albumin which would emerge much earlier. Pooled fractions were concentrated in dialysis tubing placed into dry Sephadex G-200, dialyzed against 0.15 M NaCl–0.01 M sodium phosphate, pH 6.0 (buffer A), and stored at −20 °C.

Iodination was performed by a modification of the method of Hunter & Greenwood (1962). Conjugate, approximately 1.2 nmol in 50 μL of buffer A, was mixed with 20 μL of 0.5 M sodium phosphate, pH 7.5. In an appropriate hood, 25 μL of Na$^{125}$I (2.5 mCi, 1.25 nmol) was added. Five aliquots, 10 μL each of freshly dissolved chloramine T (2 mg/mL of 0.05 M sodium phosphate, pH 7.5), were added at 2.5-min intervals. The reaction was terminated with 50 μL of $Na_2S_2O_5$, 2 mg/mL in 0.05 M sodium phosphate buffer, pH 7.5. After 2 min, the mixture was layered onto a disposable Pharmacia P10 column of Sephadex G25M that had been equilibrated with buffer A containing 1 mg/mL of bovine serum albumin. The tube was rinsed with 0.3 mL of this solution and the wash added to the column. The column was then washed with an additional 1 mL of buffer A, and the protein was eluted with another 3 mL. Unbound iodine remained in the column, which was capped and discarded. Subsequent procedures were carried out at 4 °C. The eluate was dialyzed against 3 changes, 1 L each, of buffer A followed by 1 L of 0.01 M sodium phosphate, pH 6.0. The dialyzed iodinated BSA-(PhMan-6-P)$_{20}$ was purified on a column of DEAE Bio-Gel A as described above for the parent compound. Because the iodinated conjugate was adsorbed to uncoated glass or plastic, fractions were collected into Falcon plastic tubes that had been rinsed with bovine serum albumin, 1 mg/mL, and which contained an additional 0.1 mL of that solution. The radioactive peak of conjugate, monitored using 25-μL aliquots, was usually broader than that of the unlabeled material; the center fractions were pooled but the leading and trailing edges were not included. Except for inactive material at the leading edge, samples of conjugate from different points across the peak had similar uptake properties.

The pooled fractions of radioactive BSA(PhMan-6-P)$_{20}$ were concentrated 10-fold in dialysis tubing placed into dry Sephadex G-200 and then dialyzed against several changes, 1 L each, of buffer A. This stock solution, ca. $10^8$ dpm/mL, was sterilized by filtration through nitrocellulose membranes (Millipore 0.45 μm) and stored at 4 °C. Immediately after preparation, the specific radioactivity was 0.2–0.5 atom of $^{125}$I/mol of conjugate, and over 96% of the radioactivity was precipitable by trichloroacetic acid. Labeled preparations of conjugate were used for no more than 3 weeks.

*Cell Culture.* Cells were maintained at 35 °C in Eagle's minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and antibiotics, as previously described (Hall et al., 1978). Media were prepared by the Media Supply unit of the National Institutes of Health. Human diploid fibroblasts deficient in L-iduronidase, from patients with the Hurler syndrome, were derived from samples submitted for diagnosis. Cells from patients with mucolipidosis II (GM 2938) were from the Human Genetic Mutant Cell Repository, Institute of Medical Research, Camden, NJ. The following cell lines were obtained from investigators at the National Institutes of Health: WI-38, a human diploid embryonic lung fibroblast strain, and VA-13B, an SV-40 transformed subline thereof, from Dr. M. Bradley; rat kidney fibroblasts (NRK clone 2) from Dr. I. Pastan; mouse L cells (LTK$^-$) from Dr. R. D. Camerini-Otero; and a mutant line of Chinese hamster ovary (CHO) cells, deficient in β-galactosidase, N211-1-8, from Dr. A. R. Robbins.

*Uptake Experiments.* The experiments described in the first section under Results, in which only inhibition of L-iduronidase was measured, were carried out exactly as described by Sando & Neufeld (1977); cells were incubated in 100-mm petri dishes and L-iduronidase activity was measured with phenyl-α-L-iduronide as the substrate.

In subsequent experiments, the procedure was scaled down to conserve radiolabeled conjugate. Cells were plated into 35-mm petri dishes and used at confluence. Stock solutions were sterilized by filtration through 0.45-μm Millipore membranes. A mixture consisting of 0.8 mL of growth medium and 0.2 mL of the desired components in buffer A was applied to each dish. Cells were incubated at 35 °C in an atmosphere of 5% $CO_2$; addition of test solution was staggered when necessary, so that all incubations within one experiment could be terminated at the same time and all the plates processed identically.

At the end of the incubation, medium was collected and chilled. The cells were rinsed with 0.15 M NaCl, detached from the dish by trypsinization, and collected by centrifugation at 4 °C for 2 min in a Eppendorf 5412 centrifuge. After two washes with 0.15 M NaCl, each cell pellet was suspended in 100 μL of 0.15 M NaCl containing 0.8% Triton X-100, and aliquots were taken for determination of protein and of L-iduronidase activity. Protein was measured by the method of Lowry et al. (1951) except that sodium dodecyl sulfate was added to a final concentration of 1% in order to prevent precipitation of the phenol reagent by the Triton X-100 (Karson & Ballou, 1978). Enzyme activity was measured fluorometrically (Rome et al., 1979a), but the substrate concentration was reduced fivefold and the incubation time increased appropriately. The samples used for protein or enzyme assay were placed directly into a Beckman Gamma 8000 counter for determination of radioactivity.

Degradation of radiolabeled conjugate was determined by measuring the radioactivity that remained soluble in trichloroacetic acid. An aliquot of chilled medium was mixed with an equal volume of 30% (w/v) cold trichloroacetic acid in a 1.5-mL conical plastic tube. After 30 min at 4 °C, the tubes were centrifuged in a Beckman Microfuge B, and the ratio of soluble to total radioactivity was determined. The "fraction degraded" is that ratio corrected for a reagent blank incubated under similar conditions but without cells.

Except for the experiment presented in Figure 1, presumptive $K_i$ values were calculated from the equation:

$$K_i = i\left(\frac{100}{\%\ \text{inhibition}} - 1\right)$$

where $i$ is the concentration of inhibitor in the medium; this equation is applicable if the inhibition is competitive and the concentration in the medium of the substance to be taken up is much less than $K_{uptake}$ (Sando & Neufeld, 1977).

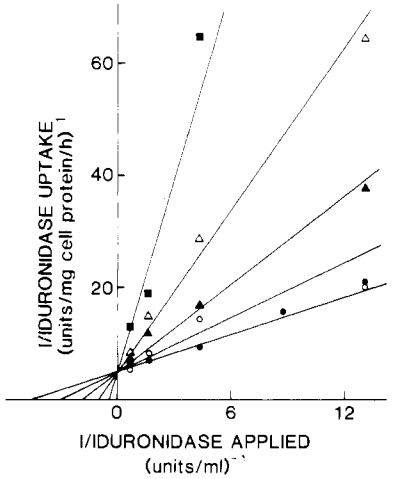


FIGURE 1: Inhibition of uptake of L-iduronidase by BSA(PhMan-6-P)$_{24}$. Concentrations of derivatized albumin, in nmol/mL of medium, were as follows: 0 (●); 0.47 (○); 1.4 (▲); 3.5 (△); 9.3 (■). Rates of internalization were calculated from 2-h uptake measurements (Sando & Neufeld, 1977). A nonlinear least-squares fit of the velocity data to the equation for competitive inhibition:

$$v = \frac{VE}{K_{uptake}(1 + i/K_i) + E}$$

was used to obtain values for $V$, $K_{uptake}$, and $K_i$, which were, in turn, used to position the lines in the reciprocal plot.

## Results

*Inhibition of* L-*Iduronidase Uptake by BSA(PhMan-6-P)$_n$ and Related Compounds.* The conjugate BSA(PhMan-6-P)$_{24}$ was found to inhibit the uptake of L-iduronidase competitively, with a $K_i$ of 0.98 ± 0.02 μM with respect to the derivatized protein or 24 μM with respect to sugar phosphate (Figure 1). Similar conjugates with a lower degree of substitution, 6 or 16 mannose 6-phosphate groups per protein, showed a similar degree of inhibition ($K_i$ = 20–30 μM) calculated on the basis of sugar phosphate. So did ribonuclease derivatized with 5 chains of mannopentaose monophosphate, low-density lipoprotein derivatized with 50 chains of mannopentaose monophosphate/mol, or bovine serum albumin modified with 20 residues of *N*-[*S*-(6-phospho-α-D-mannosyl)thioacetyl]-aminoacetaldehyde. Small phosphorylated molecules—mannose 6-phosphate itself, *p*-aminophenyl 6-phosphomannoside, and mannopentaose monophosphate—inhibited to the same extent ($K_i$ = 40 μM).

By contrast, nonphosphorylated conjugates showed little inhibition. Calculated on the basis of sugar concentration, the $K_i$ of albumin derivatized with 20 or more residues of *p*-aminophenyl glycosides (α-D-mannoside, α- or β-D-galactoside, α-D-glucoside, or α-L-fucoside) was 1 mM or higher.

*Uptake of [$^{125}$I]BSA(PhMan-6-P)$_{20}$ into Diploid Human Fibroblasts.* The mannose 6-phosphate inhibitable internalization of the radiolabeled conjugate is shown in Figure 2 for three different strains of diploid human fibroblasts: WI-38 from normal embryonic lung, and skin fibroblasts from a patient affected with the Hurler syndrome and from one affected with mucolipidosis II. In contrast to the uptake of L-iduronidase, which proceeded almost linearly for several hours (Sando & Neufeld, 1977), the intracellular accumulation of $^{125}$I-labeled conjugate appeared to level off in 1 h in WI-38 and Hurler cells and in 5 h in mucolipidosis II cells. The plateau of intracellular radioactivity was accompanied by the appearance of trichloroacetic acid soluble radioactivity in the medium. The degradation of the conjugate was probably not

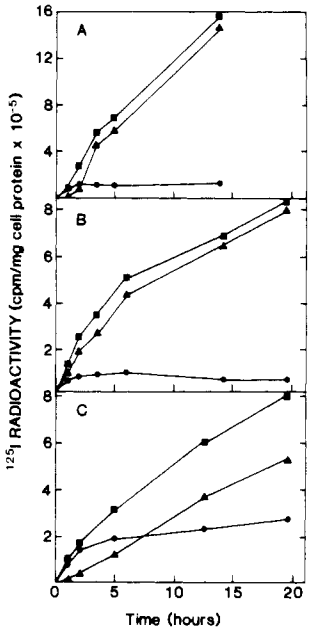


FIGURE 2: Internalization of [$^{125}$I]BSA(PhMan-6-P)$_{20}$ by human diploid fibroblasts. Dishes of cells containing 60–120 μg of protein were incubated with 1.8 × 10$^6$ cpm of the radioactive conjugate as described under Experimental Procedures. Cell-associated radioactivity (●), extracellular trichloroacetic acid soluble radioactivity (▲), and the sum of the two (■) are plotted as a function of time for three cell types: (A) Hurler; (B) WI-38; (C) mucolipidosis II. Values obtained in the presence of 2 mM mannose 6-phosphate have been subtracted, and all figures have been normalized to cell protein. Each point is the average of duplicate determinations.

due to extracellular proteolysis, because (1) it did not appear when conditioned medium was incubated with radioactive conjugate in the absence of cells, and (2) it was inhibited by the presence of mannose 6-phosphate in the medium. The total amount of conjugate that had been internalized by the cells was therefore represented by the sum of cell-associated radioactivity and extracellular, trichloroacetic acid soluble radioactivity. The reduced rate of degradation in cells from the mucolipidosis II patient made these cells particularly useful for kinetic studies, since there was no substantial breakdown of the conjugate in the first hour of incubation.

*Absence of Uptake of [$^{125}$I]BSA(PhMan-6-P)$_{20}$ into Permanent Fibroblast Lines.* Several other types of cultured fibroblasts were tested for their ability to internalize and degrade the radiolabeled conjugate: a virally transformed line derived from WI-38, and three lines of rodent origin. As shown in Table I, the established cell lines failed to take up the iodinated conjugate, in marked contrast to the diploid human fibroblasts.

*Inhibition of Uptake of [$^{125}$I]BSA(PhMan-6-P)$_{20}$.* The uptake of radiolabeled conjugate was inhibited by L-iduronidase with a $K_i$ of 1.5 × 10$^{-9}$ M (Table II). It was also inhibited by mannose 6-phosphate ($K_i$ = 0.5–1 × 10$^{-5}$ M) and by millimolar mannose 1-phosphate. Finally, the uptake of the iodinated compound was inhibited by unlabeled conjugate ($K_i$ = 2 × 10$^{-8}$ M, calculated as protein). If one assumes that the unlabeled conjugate is functionally equivalent to the iodinated species and merely reduces the specific radioactivity, then the $K_i$, 2 × 10$^{-8}$ M, is also the $K_{uptake}$ of the conjugate. An unphosphorylated conjugate of mannose and bovine serum albumin did not inhibit at the highest concentration tested.

Table II also shows that the uptake of L-iduronidase, determined under the same conditions as the uptake of radioiodinated conjugate, was much less sensitive to inhibition by unlabeled conjugate and by mannose 6-phosphate ($K_i$ = 10$^{-6}$

Table I: Uptake of [$^{125}$I]BSA(PhMan-6-P)$_{20}$ by Fibroblasts of Different Origin

| cultured fibroblasts | conjugate taken up[a] (cpm/mg of protein, × $10^{-3}$) cell associated | extracellular degraded |
|---|---|---|
| human, diploid | | |
| WI-38 | 66; 77 | 330; 414 |
| Hurler | 64; 108 | 590; 680 |
| mucolipidosis II | 200; 100 | 160; 370 |
| human, SV-40 transformed | <1 | NS[b] |
| mouse (LTK$^-$) | 9 | NS |
| rat (NRK) | <1 | NS |
| CHO (N211-1-8) | 3 | NS |

[a] Determination of radioactivity in cells and medium was performed as described under Experimental Procedures. Each value is the average of duplicate plates; for human diploid fibroblasts, results of two separate experiments are reported. Data were normalized to the following standard conditions: $1.8 \times 10^6$ cpm of radiolabeled conjugate/mL of medium, 6 h of incubation, and 1 mg of cell protein. The actual amount of cell protein varied from 45 to 180 μg/dish. Values obtained in the presence of 2 mM mannose 6-phosphate have been subtracted. For cell-associated material, blanks ranged from 1000 to 5000 cpm/mg of protein, with the higher values shown by those cells which had the highest uptake values. [b] NS = not significant. Because of the presence of some trichloroacetic acid soluble material in the solution of iodinated conjugate (approximately 5% of the total radioactivity, giving a blank of about 900 000 cpm/mg of protein), values of less than 100 000 cpm/mg of protein over that background were judged to be below the level of accurate measurement.

Table II: Inhibition of Uptake of [$^{125}$I]BSA(PhMan-6-P)$_{20}$ and of L-Iduronidase[a]

| inhibitor | cells | $K_i$[b] (M) for uptake of $^{125}$I-labeled conjugate | L-iduronidase |
|---|---|---|---|
| L-iduronidase | mucolipidosis II | $1.5 \times 10^{-9}$ | |
| BSA(PhMan-6-P)$_{20}$ | mucolipidosis II | $2 \times 10^{-8}$ | $1 \times 10^{-6}$ |
| | Hurler | $2 \times 10^{-8}$ | $2 \times 10^{-6}$ |
| BSA(PhMan)$_{29}$ | mucolipidosis II | NI at $3 \times 10^{-6}$ | |
| | Hurler | | NI at $10^{-5}$ |
| mannose 6-phosphate | mucolipidosis II | $0.5 \times 10^{-5}$ | $6 \times 10^{-5}$ |
| | Hurler | $1 \times 10^{-5}$ | $5 \times 10^{-5}$ |
| mannose 1-phosphate | mucolipidosis II | $2 \times 10^{-3}$ | NI at $10^{-3}$ |

[a] Uptake was measured as described under Experimental Procedures for radiolabeled conjugate (present in the medium at a concentration of $3 \times 10^{-10}$ M) or for L-iduronidase (present at a concentration of $6 \times 10^{-10}$ M). Incubations were generally for 1 h, in which case only the cell-associated radioactivity was measured; in some experiments using Hurler cells, incubation was for 10 h, in which case it was necessary to sum the intracellular radioactivity and the trichloroacetic acid soluble radioactivity in the medium. Concentrations of conjugates are given as molarity of protein. NI = not inhibitory. [b] These are presumptive $K_i$ values, calculated from the equation given under Experimental Procedures.

and $5 \times 10^{-5}$ M, respectively.)

## Discussion

The chemical coupling of mannose 6-phosphate to an inert protein such as bovine serum albumin was undertaken for several reasons, including the need to confirm the recognition of mannose 6-phosphate residues by an independent method. The albumin–mannose 6-phosphate conjugate was recognized by fibroblasts both as a competitive inhibitor of the uptake of L-iduronidase and as a substance that was itself internalized by a mannose 6-phosphate inhibitable mechanism. Unlike urinary L-iduronidase, which has a half-life of 9 days within fibroblasts (Hickman & Neufeld, 1972; Rome et al., 1979b), the conjugate was rapidly degraded and fragments released into the medium. It is likely that this degradation occurred within lysosomes. The rate of degradation was markedly reduced in cells from a patient with mucolipidosis II, a pleiotropic disorder of lysosomal function (Neufeld et al., 1975). In addition, a preliminary experiment (E. M. Karson and L. H. Rome, unpublished results) showed that internalized [$^{125}$I]BSA(PhMan-6-P)$_{20}$ had the bimodal distribution characteristic of lysosomal markers on a colloidal silica gradient (Rome et al., 1979b).

BSA(PhMan-6-P)$_{24}$ and other synthetic conjugates of mannose 6-phosphate with proteins inhibited the uptake of L-iduronidase to approximately the same extent as an equivalent concentration of mannose 6-phosphate, regardless of the nature of the protein backbone, the connecting arm, or the degree of substitution. This result differs from the finding of Krantz et al. (1976) that inhibition of binding of glycoproteins to the hepatocyte galactose receptor was an exponential function of the galactose or glucose content of neoglycoproteins.

A reciprocal inhibition of uptake of L-iduronidase and BSA(PhMan-6-P)$_n$ should be observed if the two macromolecules bind to the same receptor. Indeed, each one was found to inhibit the uptake of the other, with a linear competitive pattern shown for inhibition of L-iduronidase uptake by the conjugate. Since L-iduronidase and the mannose 6-phosphate conjugate can be internalized, they are not dead-end inhibitors, but should behave in the uptake system as does an alternate substrate in the steady-state enzyme-catalyzed process from which our kinetic model of enzyme uptake was derived (Sando & Neufeld, 1977). An alternate substrate in a uni-uni mechanism acts as a competitive inhibitor, for which $K_i$ and $K_m$ are given by the same combination of rate constants (Fromm, 1975). Thus, in the corresponding model for uptake, the inhibition constant and $K_{uptake}$ for such a ligand should be equivalent ($K_i$ represents a dissociation constant only if the rate constant for internalization is small compared to that for dissociation of the receptor–ligand complex). Unexpectedly, the inhibition constant of the conjugate for uptake of L-iduronidase ($10^{-6}$ M) was nearly two orders of magnitude higher than the $K_{uptake}$ of the conjugate. By contrast, the $K_i$ of L-iduronidase for uptake of the conjugate ($1.5 \times 10^{-9}$ M) was similar to the $K_{uptake}$ and $K_d$ previously determined for that enzyme (Sando & Neufeld, 1977; Rome et al., 1979a). The uptake of the radiolabeled BSA(PhMan-6-P)$_{20}$ was significantly more sensitive to inhibition by mannose 6-phosphate than the uptake of L-iduronidase. The $K_{uptake}$ and $K_d$ for low-density lipoprotein derivatized with 50 mannopentaose monophosphate residues/mol ($<2 \times 10^{-9}$ M, G. J. Murray and D. M. Neville, Jr., personal communication) were 200 times less than the $K_i$ of that compound for L-iduronidase. Thus, free and conjugated mannose 6-phosphate appear to be relatively poor inhibitors of L-iduronidase uptake. These results, which were not predicted by the simplest kinetic model, suggest that the uptake of the lysosomal enzyme is mediated not only by mannose 6-phosphate residues but by some additional structural feature(s) which influences the rate of ligand binding, internalization, or both. Arginine residues have been implicated in the binding of L-iduronidase to the receptor (Rome & Miller, 1980). A specific phosphorylated oligosaccharide (Distler et al., 1979) or a particular arrangement of mannose 6-phosphate residues on the enzyme so as to promote multivalent interactions with the cell receptors (Fischer et al., 1980) has been proposed. A likely candidate for a second recognition marker is the polypeptide extension

on the precursor and secreted forms of lysosomal enzymes (Hasilik et al., 1979; Hasilik & Neufeld, 1980a; Skudlarek & Swank, 1979).

An additional goal in the synthesis of the albumin–mannose 6-phosphate conjugate was to facilitate the identification of cells that have the mannose 6-phosphate dependent uptake system. Studies of lysosomal enzyme uptake have generally been restricted to mutant cells that have a low endogenous level of the enzyme under consideration, whereas the uptake of [$^{125}$I]BSA(PhMan-6-P)$_n$ can be examined in any cell type. Thus, it was shown that normal human fibroblasts, WI-38, have the same uptake system for the conjugate as the mutant skin fibroblasts used in previous studies (Sando & Neufeld, 1977). On the other hand, an SV-40 transformed cell line derived from WI-38 failed to take up significant amounts of the conjugate, as did three permanent lines of rodent fibroblasts.

Except for the case of one cell line, whether this failure of uptake was due to a deficiency of receptors and/or of subsequent internalization is not known, since binding in the absence of internalization was not studied. CHO cells were shown to take up $\alpha$-mannosidase (Robbins, 1979) and $\beta$-galactosidase by a mannose 6-phosphate inhibitable system; in the latter case, the kinetics of uptake as well as of inhibition by mannose 6-phosphate and by BSA(PhMan-6-P)$_{20}$ were similar to those found for diploid human skin fibroblasts (R. Myerowitz and A. R. Robbins, personal communication). Thus, the inability of CHO cells to internalize BSA(PhMan-6-P)$_{20}$ cannot be attributed to an absence of receptors and suggests that there is some step in the internalization pathway (for instance, aggregation of bound receptors) that in these cells is affected differently by the binding of conjugate and of lysosomal enzymes.

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