distinct types of CSF was also observed (Wu & Yunis, 1980). The CSFs from human lung and placenta share all the properties we have described above.

Perhaps the most interesting aspect of the two types of CSF are their species specificities and the type of cell differentiation they promote. It is not clear at present why a given CSF from a human source should exhibit activity in mouse but not in human marrow. Neither is the biochemical basis for stimulating the formation of granulocyte or macrophage colonies apparent. Detailed biochemical and functional studies on homogeneous preparations of these two types of CSF should help provide some answers to these important questions.

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p-Isothiocyanatophenyl 6-Phospho-α-D-mannopyranoside Coupled to Albumin. A Model Compound Recognized by the Fibroblast Lysosomal Enzyme Uptake System. 1. Chemical Synthesis and Characterization†

Gloria N. Sando* and Evelyn M. Karson

ABSTRACT: We have developed a simple synthesis for a conjugate of albumin and *p*-aminophenyl 6-phospho-α-D-mannopyranoside to study the requirements of the fibroblast lysosomal enzyme recognition system. *p*-Aminophenyl 6-phospho-α-D-mannopyranoside was prepared in two ways: (1) phosphorylation of *p*-nitrophenyl α-D-mannopyranoside and subsequent reduction of the nitro group by catalytic hydrogenation and (2) direct phosphorylation of *p*-aminophenyl α-D-mannopyranoside. Mannosides were phosphorylated in a reaction with phosphoryl chloride, pyridine, and water at 0 °C for 1 h, by a procedure selective for primary hydroxyl groups. Purified *p*-aminophenyl 6-phospho-α-D-mannopyranoside was characterized by chromatographic, enzymatic, and ^{13}C nuclear magnetic resonance spectroscopic methods. *p*-Isothiocyanatophenyl 6-phospho-α-D-mannopyranoside and the *p*-isothiocyanatophenyl glycosides of α-mannose, α-glucose, α- and β-galactose, and α-L-fucose were formed by reaction of the respective *p*-aminophenyl glycosides with thiophosgene. Incubation of the *p*-isothiocyanatophenyl glycosides with bovine serum albumin at pH 8.5, 25 °C, for 18 h generally resulted in the coupling, primarily through lysine residues, of up to 20-30 mol of glycoside per mol of protein. Biological properties of the conjugates in the fibroblast lysosomal enzyme recognition system are described in the accompanying paper.

The potential of synthetic sugar-protein conjugates (neoglycoproteins) as mechanistic probes and as models for the design of specific cell-directed substances has been demonstrated in studies of carbohydrate recognition systems for receptor-mediated endocytosis. Following the discovery by Morell et al. (1971) that mammalian hepatocytes recognized and removed from the circulation a large series of galactose-terminated glycoproteins, Rogers & Kornfeld (1971) showed that the uptake of certain nonglycosylated proteins by the liver was enhanced when they were chemically coupled to fetuin asialoglycopeptides. More recently, chemical coupling

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of lactose to two enzymes with potential chemotherapeutic value, asparaginase (Marsh et al., 1977) and ribonuclease dimer (Wilson, 1978), resulted in rapid removal of these compounds from circulation by the liver. Much information concerning the requirements for recognition by the hepatic galactose receptor was obtained from the study of a series of synthetic protein–thioglycoside conjugates which displayed carbohydrate-specific, high-affinity binding properties similar to those of serum asialoglycoproteins in a galactose-binding membrane receptor system (Lee et al., 1976; Krantz et al., 1976). The same receptor system was found to recognize thioglucosyl in addition to thiogalactosyl derivatives (Stowell & Lee, 1978). The use of neoglycoproteins has contributed to the characterization of another distinct carbohydrate recognition system for receptor-mediated endocytosis, which is present on reticuloendothelial cells. Isolated alveolar macrophages recognized and efficiently took up radiolabeled neoglycoproteins that contained thiomannosyl, *N*-acetylthioglucosaminyl, and thioglucosyl, but not thiogalactosyl derivatives (Stahl et al., 1978). An approach to the study of multivalent interactions at carbohydrate recognition sites has recently been taken by Lee & Kawaguchi (1979) with the development of procedures for synthesis and coupling of "cluster glycosides" (two or three glycosides linked to a low molecular weight carrier) to proteins.

Evidence for phosphorylated mannose as the signal by which lysosomal enzymes are recognized by receptors on human fibroblasts (e.g., Kaplan et al., 1977a,b; Sando & Neufeld, 1977; Ullrich et al., 1978) prompted us to prepare a conjugate of mannose 6-phosphate with bovine serum albumin. In this paper we report on the chemical synthesis of such a neoglycoprotein; the accompanying paper describes the interaction of the mannose 6-phosphate–albumin conjugate with the lysosomal enzyme uptake system (Karson et al., 1980). A preliminary report of these results has been presented (Sando, 1978).

Experimental Procedures

Materials. *p*-Aminophenyl α-D-mannoside was purchased from Calbiochem. Other aminophenyl glycosides, *p*-nitrophenyl α-D-mannoside, and methyl α-D-mannoside were from Sigma Chemical Company.

Bovine serum albumin (fatty acid free), *Escherichia coli* alkaline phosphatase (EC 3.1.3.1, type III-R), jack bean α-D-mannosidase (EC 3.2.1.24, type III), glucosephosphate isomerase (EC 5.3.1.9, type X), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, type XXII) were products of Sigma Chemical Company; mannosephosphate isomerase (EC 5.3.1.8) was from Boehringer-Mannheim.

Phosphoryl chloride was purchased from Aldrich Chemical Company or Matheson Coleman and Bell; thiophosgene was from Aldrich or Pfaltz and Bauer, Inc.; and *N*-ethylnaphthylamine was from Eastman. Charcoal (acid washed with HCl), diatomaceous earth (grade I), and poly(ethylenimine)-cellulose were obtained from Sigma Chemical Company; AG 50W-X8 (100–200 mesh) and Bio-Gel P-2 were from Bio-Rad.

All other chemicals were of the highest quality available from commercial suppliers.

Analytical Procedures. Hexose was determined by the phenol–sulfuric acid procedure (Dubois et al., 1956), scaled down to one-fifth the original volumes. Phosphate was measured by the method of Ames & Dubin (1960). Ammonia was determined colorimetrically (Fawcett & Scott, 1960) using a modification of the procedure in Sigma Technical Bulletin No. 640: to 0.10 mL of sample which contained 10–200 nmol of ammonia were added 0.25 mL of phenol nitroprusside reagent, 0.25 mL of alkaline hypochlorite reagent, and 1.25 mL of H_2O; absorbance was read at 570 nm after 30 min.

Glycosides of *p*-aminophenol were quantitated for aromatic amine content by a modification of the method of Levvy & Storey (1949). To 350 μL of sample and 50 μL of 2 M sodium phosphate, pH 2.25, 100 μL of 0.1% $NaNO_2$ was added with mixing. After 3 min, diazotization was stopped with 100 μL of 0.5% ammonium sulfamate; then 100 μL of 0.1% *N*-α-naphthylethylenediamine·2HCl was added. The mixtures were protected from light and incubated at room temperature for 2 h; absorbance at 565 nm was compared to that obtained with *p*-aminophenyl α-D-mannoside.

For chromatographic analyses, Whatman No. 1 paper was used in a descending system, or aluminum-backed 0.1-mm cellulose thin-layer sheets (E. Merck Co.) were used in an ascending system. The solvent was 95% ethanol–1 M ammonium acetate, pH 3.8 (75:30), which contained 1 mM EDTA[1] (Paladini & Leloir, 1952). Products were detected with ultraviolet light and by reaction with *N*-ethylnaphthylamine (Ekman, 1948); staining procedures were carried out in a fume hood, and skin contact with reagents or with dyed chromatograms was avoided.

Alkaline phosphatase and α-mannosidase treatments of products were carried out at 37 °C under the following conditions. Samples (200 nmol) were incubated for 4 h with alkaline phosphatase (1.4 U) in 90 μL of buffer, 0.05 M $NaHCO_3$, pH 8.0, which contained 0.5 mM $MgCl_2$. A portion of this incubation mixture (10 μL, 20-nmol sample) was then incubated for 6 h with α-mannosidase (0.02 U) in 30 μL of 0.2 M sodium citrate buffer, pH 4.6, which contained 1 mM $ZnCl_2$. Aliquots from the incubation mixtures were applied to Whatman No. 1 paper and chromatographed as described above. Incubations in which one or the other enzyme treatment was omitted served as controls. Standards, *p*-aminophenol, and *p*-aminophenyl α-D-mannoside were treated in parallel with samples.

Quantitation of the mannose 6-phosphate content of products was accomplished using a coupled enzyme system (Slein, 1957) under the following conditions. The sample was hydrolyzed in a sealed ampule at 100 °C with 1 N HCl for 1 h or 2 N HCl for 2 h; 0.05 μmol was added to a solution at pH 7.6, 25 °C, which contained mannosephosphate isomerase (1.2 U), glucosephosphate isomerase (15 U), glucose-6-phosphate dehydrogenase (0.5 U), $MgCl_2$ (6 μmol), $NADP^+$ (0.5 μmol), and Tris-Cl (320 μmol) in a final volume of 1.0 mL. The extent of $NADP^+$ reduction, determined by absorbance at 340 nm, was compared to that of a control incubation in which mannosephosphate isomerase was omitted.

The ^{13}C NMR spectra were obtained at 25 °C with broad-band proton decoupling on a Bruker HX-90E pulse Fourier transform NMR spectrometer (22–63 MHz) interfaced with a Nicolet 1080 computer and disk unit. The spectral accumulations for the samples, 0.1 M *p*-aminophenyl 6-phospho-α-D-mannopyranoside (**4**), 0.1 M *p*-aminophenyl α-D-mannopyranoside (**3**), and 1.0 M methyl α-D-mannopyranoside were 28 976, 1696, and 1110, respectively. Aqueous solutions of the mannosides, pH ~6, contained 50% D_2O, which served as an internal deuterium lock. Methanol standard was included in the 10-mm sample tube at the same concentration as the glycoside. Chemical shifts are given in parts per million (ppm) downfield from tetramethylsilane.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced $NADP^+$; NMR, nuclear magnetic resonance.

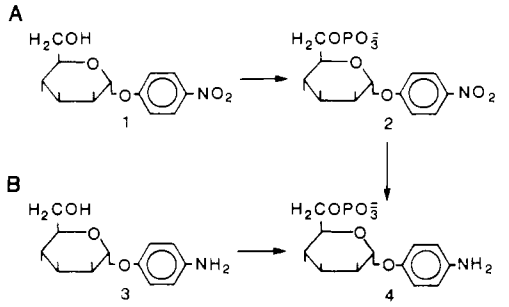

FIGURE 1: Synthesis of *p*-aminophenyl 6-phospho-α-D-mannopyranoside by methods A and B (see Results).

Amino acid analyses were performed with a Durrum Model 500 amino acid analyzer. Samples (~100 μg of protein) were hydrolyzed in 6 N HCl, 22 h, 100 °C.

Results

Synthesis of p-Aminophenyl 6-Phospho-α-D-mannopyranoside. The two approaches used for the synthesis of **4** are outlined in Figure 1. In A, *p*-nitrophenyl α-D-mannoside (**1**) was phosphorylated to give *p*-nitrophenyl 6-phospho-α-D-mannoside (**2**); reduction of the nitro group gave the aminophenyl glycoside. In B, *p*-aminophenyl α-D-mannoside (**3**) was phosphorylated directly. Method A was used when a larger amount of well-characterized material was required because the starting material was much cheaper, and because **2** was purified more easily and at a higher yield than **4** from the phosphorylation reaction mixture.

Method A. The phosphorylation procedure was adapted from that described by Sowa & Ouichi (1975) for the direct conversion of ribonucleosides to 5′-ribonucleotides. Phosphoryl chloride (0.41 mL; 4.4 mmol) pyridine (0.39 mL; 4.8 mmol) acetonitrile (1.0 mL; 19 mmol), water (0.04 mL; 2.2 mmol), and *p*-nitrophenyl α-D-mannoside (**1**) (0.30 g; 1.0 mmol) were stirred for 1 h at 0 °C in a stoppered 2-mL flask. The reaction mixture was added to 30 mL of ice water and adjusted to pH 3 with NH_4OH. After 30 min the solution was neutralized and mixed with 5 g each of charcoal and Celite. The adsorbent was washed with water until ammonia and inorganic phosphate were barely detectable in the eluate. Elution with 500 mL of 0.2% concentrated NH_4OH (v/v) in 50% ethanol resulted in a 50–60% recovery of the hexose-containing material applied.

The ethanol eluate was concentrated in vacuo at 30 °C to 50 mL and passed through a bed (4.5 × 1 cm) of AG 50W-X8 (H^+) to remove NH_4^+. After addition of 0.1 g of 10% Pd on charcoal, the mixture was subjected to hydrogenation for 1 h at 25 °C, 28 psi, on a Parr Model 3911 apparatus. Catalyst was removed by filtration with Whatman No. 1 paper.

The reaction mixture was applied to a column of poly(ethylenimine)-cellulose (Figure 2). Fractions from the major peak were pooled, concentrated to 10 mL, and desalted on a column of Bio-Gel P-2 (Figure 3A). This column also separated out a minor component (450–520 mL), which gave positive sugar and aromatic amine reactions and eluted at the same position as authentic **3**. The major peak from the Bio-Gel column contained the final product in a yield of 30–40% (0.3–0.4 mmol).

Method B. *p*-Aminophenyl α-D-mannoside (**3**) was phosphorylated as described in method A for the *p*-nitrophenyl derivative, except that the molar ratio of the less soluble aminophenyl mannoside to the other reagents was reduced in half, and at the end of the reaction, to minimize the sample volume for the subsequent chromatographic step, the mixture was diluted with only 1 mL of ice water prior to adjusting the pH to 3. After 15 min, the solution was neutralized and chromatographed on a column of Bio-Gel P-2 (Figure 3B). Fractions (300–325 mL) which contained hexose and aromatic amine and had the chromatgraphic mobility of **4** were pooled. The product migrated more slowly than the purer material in Figure 3A and was not separated from salts. The problem of retardation of heteroaromatic anions by salts in gel filtration

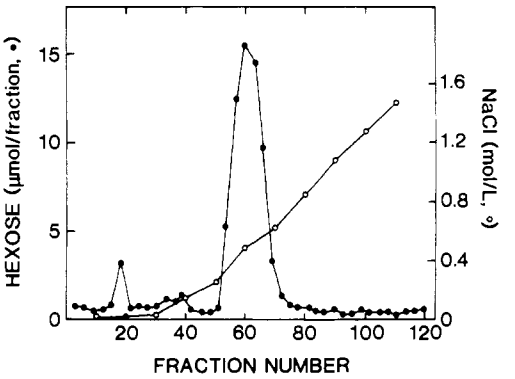

FIGURE 2: Poly(ethylenimine)-cellulose chromatography of **4**. Approximately 200 μmol of the hydrogenation reaction product from method A in 50 mL of water was applied to a column (1.9 × 14 cm) which was prepared and equilibrated with water as described by Pflüger (1977). The column was washed with water (65 mL), and a gradient, consisting of 2 M NaCl (250 mL) and water (250 mL), was applied. Fractions (4.6 mL) were monitored for hexose (●) and conductivity (○) (expressed as NaCl concentration); aromatic amine content was determined qualititatively by treating sample aliquots on filter paper with Ekman's reagent (results not shown). All fractions which contained hexose also gave a positive Ekman test.

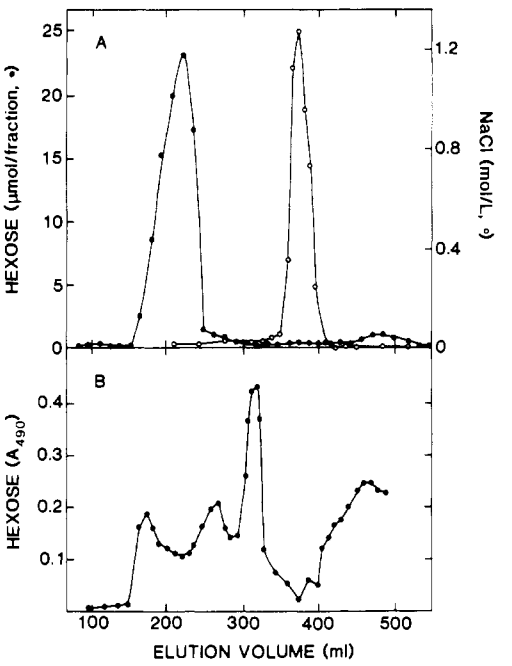

FIGURE 3: (A) Bio-Gel P-2 chromatography of **4**. A concentrate (10 mL) of the major peak from the poly(ethylenimine)-cellulose column was applied to a column (2.5 × 90 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated with water. Fractions (6.9 mL) were analyzed for neutral sugar (●), conductivity (○), and aromatic amine content as described in Figure 2. (B) Bio-Gel P-2 chromatography of phosphorylated product from method B. The quenched, neutralized reaction mixture (~3 mL) was applied to a column (2.5 × 92 cm) of Bio-Gel P-2 (100–200 mesh), equilibrated with water. Fractions (4.6 mL) were collected and assayed for hexose and aromatic amine content as described in Figure 2; results of hexose determinations (10-μL aliquots of fractions) are indicated by absorbance at 490 nm.

Table I: ^{13}C NMR Chemical Shifts of Mannosides[a]

compound	carbon position (ppm)[b]					
	C-1	C-2	C-3	C-4	C-5	C-6
methyl α-D-mannopyranoside	101.6	70.7	71.3	67.5	73.3	61.7
p-aminophenyl α-D-mannopyranoside (3)	100.3	70.8	71.3	67.4	74.1	61.6
p-aminophenyl 6-phospho-α-D-mannopyranoside (4)	100.6	70.9	70.9	66.6	73.6[c]	63.3[c]

[a] Tentative assignments are based on those of Walker et al. (1976) for methyl α-D-mannopyranoside. [b] Chemical-shift values are given in parts per million from tetramethylsilane. The internal methanol standard was assigned a value of 49.7 ppm. [c] Those peaks were doublets.

has been considered by Engel (1977). Further purification was achieved by adsorbing the prodcut to a cation exchange resin. Dry AG 50W-X8 (H^+) was added with mixing to the pooled fractions, 1 g at a time, until the test for aromatic amine was negative. The resin was collected by gravity in a coarse scintered glass funnel and washed with water (200 mL) until no more NH_4^+ was detectable. The phosphorylated product **4** was eluted with 20-mL portions of $NaHCO_3$ ranging from 0.2 to 1.0 M. Fractions which contained aromatic amine were pooled, concentrated, and desalted as in method A (Figure 3A); the product was now eluted at the same position as the product from method A, in a yield of 15–25%, based on starting material.

Characterization of p-Aminophenyl 6-Phospho-α-D-mannopyranoside. The final product from both methods contained equal molar amounts of mannose, organic phosphorus, and aromatic amine, and migrated as a single component (R(*p*-aminophenyl α-D-mannoside) = 0.69) on cellulose thin-layer and paper chromatograms. For the conditions used, a contaminant would have been detected if it had an intensity of 1–2% that of the major component by the Ekman test or ~5% by ultraviolet light. Treatment of the product **4** with alkaline phosphatase gave an aromatic amino compound with a mobility on paper (R_f 0.68) identical with that of authentic **3**. Whereas mannosidase treatment had no effect on **4**, such treatment of the dephosphorylated product resulted in an aromatic amine with a mobility (R_f 0.76) identical with that of *p*-aminophenol.

The mannose 6-phosphate content of product **4** was quantitated using a coupled enzyme system in which free mannose 6-phosphate is eventually converted to 6-phosphogluconate, with a stoichiometric production of NADPH. To hydrolyze the glycosidic bond, the sample was treated at 100 °C in 1 N HCl for 1 h or 2 N HCl for 2 h; a mannose 6-phosphate standard was exposed to the same conditions. Identical values for the ratio of NADPH generated/sugar phosphate were found for product **4** and for mannose 6-phosphate (0.73 after 1 h and 0.67 after the 2-h hydrolysis period, respectively). Since the expected ratio of 1.0 was obtained with unhydrolyzed mannose 6-phosphate, the reduced, but identical ratios found for the hydrolyzed samples suggested that hydrolysis affected both compounds similarly, consistent with both having the same sugar phosphate composition.

Fourier transform ^{13}C NMR spectroscopy was used to verify the position of the phosphate ester group without prior hydrolysis. Spectra of **4** and **3** were identical with respect to the aminophenyl group (118.5, 119.7, 142.4, and 149.6 ppm). However, the spectrum of **4** differed from that of **3** and of methyl α-D-mannopyranoside in the signals derived from the mannosyl residues (Table I). Most notably, the signal corresponding to C-6 in **4** was shifted 1.7 ppm downfield from that in **3**, and peaks assigned to C-5 and C-6 were doublets, with values for coupling constants of 8 and 4 Hz, respectively. The only extraneous signals noted in the spectrum of **4** were minor peaks (area = 5–10% that of major peaks) at 61.6 and 67.4 ppm attributable to **3**, which was generated from **4** during the 12-h sampling period, as was demonstrated by chromatography of the sample before and after NMR examination. Under the conditions used, enzymatic hydrolysis seems a more likely explanation for this observation than chemical instability of the phosphomonoester linkage. Occasionally we discovered a solution of the mannose 6-phosphate–albumin conjugate with a reduced organic phosphorus content following storage, chromatography, or dialysis; although we did not detect phosphatase activity in such preparations with *p*-nitrophenyl phosphate as a substrate, we found that hydrolysis in other samples could be minimized by adding inorganic phosphate, keeping samples cold, and avoiding possible sources of phosphatase contamination.

Synthesis of p-Isothiocyanatophenyl 6-Phospho-α-D-mannopyranoside–Albumin Conjugates. A modification of the procedure of Smith et al. (1978) was used to couple **4** to albumin. In a typical preparation, 15 μmol of **4** in 1.0 mL of 0.1 M $NaHCO_3$, pH 8, and 10 μL (132 μmol) of thiophosgene in 1.0 mL of chloroform were stirred vigorously for 20 min in a tightly capped vessel in a fume hood. The aqueous phase was extracted twice with 1.5 mL of chloroform to remove unreacted thiophosgene, placed briefly under a stream of nitrogen to remove excess chloroform, and then combined with fatty acid free bovine serum albumin, 15 mg (0.22 μmol) in 1.0 mL of buffer, 0.1 M $NaHCO_3$, pH 9, and 0.15 M NaCl. The reaction mixture was kept at room temperature for 18 h and then applied to a column (1 × 10 cm) of Sephadex G-25, previously equilibrated with 0.1 M sodium phosphate–0.15 M NaCl, pH 6.0. Fractions which contained the protein conjugate were dialyzed extensively in the same buffer at 4–6 °C and stored at −20 °C. The same reaction volumes were used for preparations in which the ratio of **4** to albumin was varied, with concentrations of up to 30 μmol/mL of **4** and 15 mg/mL of albumin.

Synthesis of Nonphosphorylated p-Isothiocyanatophenyl Glycoside–Albumin Conjugates. A coupling procedure identical with that described for *p*-aminophenyl 6-phospho-α-D-mannopyranoside was used to attach commercially obtained aminophenyl derivatives of mannose, galactose, glucose, and fucose to albumin, without prior purification.

Characterization of p-Isothiocyanatophenyl Glycoside–Albumin Conjugates. The degree of substitution in products from several preparations derived from **4** as well as from the nonphosphorylated aminophenyl glycosides is given in Table II. Good agreement was found between the number of glycosyl residues bound and unmodified lysine lost per mole of albumin for most derivatives, which argues for covalent binding of the sugars. The ε-amino group of lysine appears to be the major site for coupling, a finding predictable from the reactivity of phenyl isothiocyanate (Edman, 1950; Buss & Goldstein, 1968). Significant alterations in the levels of other amino acids were not apparent following conjugation, but the possibility that other sites were modified cannot be ruled out, since those amino acids which contain hydroxyl or thiol groups and are likely to be modified under more rigorous conditions (Fraenkel-Conrat, 1959) are also the most difficult to measure accurately. The extent of substitution obtained with the phosphorylated derivative was generally lower than that with

Table II: Sugar Content of Albumin Conjugates

glycoside	*p*-aminophenyl-glycoside/albumin in reaction mixture (mol/mol)	residues/albumin in product (mol/mol)[a]	
		sugar[b]	lysine lost[c]
α-Man 6P	34	6	5
	68	16	16
	123	16	18
	164	24	23
α-Man	34		16
	68	29	30
	137	31	32
	411	33	36
α-Gal	68	23	25
β-Gal	68	21	27
α-Glc	68	29	26
α-L-Fuc	68		14

[a] Protein concentration was estimated by amino acid analysis and was based on a value of 46 mol of alanine/mol of albumin (Hunt et al., 1976). [b] Sugar content was determined by the phenol–sulfuric acid method, using as a standard the corresponding free sugar or sugar phosphate. [c] The difference between the lysine found in underivatized albumin (57 mol/mol) and that found in a conjugate was calculated using the criteria given in footnote *a*.

derivatives of several simple sugars, including mannose, at the same initial ratio of *p*-aminophenyl glycoside to albumin in the reaction mixture.

Discussion

Many approaches have been taken for the synthesis of sugar–protein conjugates. Diazonium salts derived from *p*-aminophenyl glycosides have been widely used as coupling intermediates (Goebel & Avery, 1929; McBroom et al., 1972). Oligosaccharides have been attached to proteins directly by reductive amination (Gray, 1978) or through phenethylamine (Zopf et al., 1978), aminoflavazole (Himmelspach & Kleinhammer, 1972), or aldonic acid derivatives (Ashwell, 1972; Lönngren & Goldstein, 1978). King et al. (1977) have described the cyanuric trichloride coupling of 2-aminoethyl glycosides as well as mixed anhydride coupling of carboxyoctyl glycosides to protein. Lee et al. (1976) coupled a series of 2-amino-2-methoxy-1-thioglycosides to several proteins by amidination. Lee & Lee (1979) have recently developed synthetic procedures for ω-aldehyde thioglycosides which can be attached to proteins by reductive amination; this method has been applied to the synthesis of a conjugate of albumin and mannose 6-phosphate (R. T. Lee and Y. C. Lee, personal communication). Mannopentaose monophosphate has been utilized as a coupling intermediate in another current approach to the synthesis of mannose 6-phosphate–protein conjugates (Youle et al., 1979). Our approach to the synthesis of a mannose 6-phosphate–protein conjugate was influenced by the low cost of *p*-nitrophenyl α-D-mannopyranoside, the commercial availability of analogous glycosides, and the convenience of the phosphorylation and reduction procedures.

Many different macromolecules have been chemically glycosylated, including those proteins chosen for a specific functional property, such as enzymatic activity (Krantz et al., 1976; Marsh et al., 1977; Wilson, 1978), immunogenicity (Himmelspach & Kleinhammer, 1972; Zopf et al., 1978), or utility as a cytochemical marker (Kieda et al., 1977). We chose albumin primarily because of its availability and high derivatizable lysine content, and because numerous reports of sugar–albumin preparations were available for comparison (Krantz et al., 1976; Gray, 1978 McBroom et al., 1972; Ashwell, 1972; Smith et al., 1978; King et al., 1977).

p-Aminophenyl glycosides have most often been coupled to proteins via diazonium salt derivatives. We adopted instead a procedure in which *p*-aminophenyl glycosides were converted to isothiocyanate derivatives prior to coupling (McBroom et al., 1972; Smith et al., 1978). This method gave much higher, reproducible levels of substitution, and was more specific than diazo coupling, with a preferential modification of lysines in bovine serum albumin.

Catalytic hydrogenation was the method of choice for the reduction of *p*-nitrophenyl 6-phospho-α-D-mannopyranoside; conversion to the amine was efficient, convenient, and free from detectable side reactions. Use of another reducing agent, sodium hydrosulfite (Mapes & Sweeley, 1973), resulted in modification of the nitrophenyl moiety to give an unidentified, Ekman-positive compound.

Most procedures for the phosphorylation of the primary hydroxyl group of a sugar require prior protection of the secondary hydroxyls and removal of the protective group after phosphorylation. We chose a method which was specific for the primary hydroxyl, thus eliminating the need for protection procedures. When this method was originally applied to the phosphorylation of ribonucleosides by Sowa & Ouichi (1975), greater than 90% conversion to the corresponding 5′-ribonucleotides was reported. The yield of phosphorylated mannosyl glycoside which we obtained was considerably lower (~60% after charcoal treatment) but acceptable in view of the selectivity of the methods, low cost of the starting material, and convenience of the procedures. Enzymatic analysis showed that mannose 6-phosphate was liberated from the product following hydrolysis. The specificity of the phosphorylation was confirmed by ^{13}C NMR analysis of the unhydrolyzed product. The chemical-shift difference between the peak assigned to C-6 in the product and the corresponding peak from *p*-aminophenyl α-D-mannopyranoside was consistent with the conversion of a primary alcohol to a phosphomonoester (Rosenthal & Fendler, 1976; Stothers, 1972). In addition, the peaks assigned to C-6 and C-5 were doublets, indicating spin-sin interactions between ^{13}C and ^{31}P nuclei. The coupling constants we observed, C-6 ~ 4Hz and C-5 ~ 8 Hz, with the smaller ^{31}P–^{13}C interaction involving the carbon bound to the phosphate oxygen and the larger involving the adjacent carbon, are similar to those found with several other phosphomonoesters, including mannose 6-phosphate (Koerner et al., 1973; Gorin, 1973; Mantsch & Smith, 1972; Serianni et al., 1979). Since none of the other glycosyl resonances was detected as a doublet, we concluded that the product was phosphorylated significantly only at position 6.

The mannose 6-phosphate conjugate of albumin interacts with the fibroblast lysosomal enzyme recognition system (Karson et al., 1980). The facile synthesis of *p*-aminophenyl 6-phospho-α-D-mannopyranoside thus provides a reagent that may be used to modify a variety of substances such as drugs, for introduction into cells that have the mannose 6-phosphate receptor. The promise of such an approach is indicated by the uptake of ricin coupled to mannopentaose monophosphate, under conditions such that the usual mechanism of uptake of the toxin had been blocked (Youle et al., 1979).

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