Preparation of Some New Neoglycoproteins by Amidination of Bovine Serum Albumin Using 2-Imino-2-methoxyethyl 1-Thioglycosides†

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ABSTRACT: The cyanomethyl 1-thioglycosides of β-D-galactose, 6-*O*-methyl-β-D-galactose, α-L-arabinose, β-D-fucose, β-L-fucose, β-D-glucose, β-D-xylose, β-D-allose, α-D-mannose, 2-acetamido-2-deoxy-β-D-glucose, 2-acetamido-2-deoxy-β-D-galactose, 2-deoxy-β-D-glucose, and 3-*O*-methyl-β-D-glucose were prepared from the respective pseudothiourea derivatives and chloroacetonitrile. The nitrile function in the aglycon of the cyanomethyl 1-thioglycosides was converted to a methyl imido ester by treatment with sodium methoxide in methanolic solutions, thereby affording the 2-imino-2-methoxyethyl 1-thioglycosides [Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963]. The stability of these reagents was investigated. The 2-imino-2-methoxyethyl 1-thioglycosides were then used to attach carbohydrates to bovine serum albumin. Amidination could be accomplished within a few hours in a pH range of 7–10. The extent of amidination could be controlled by varying the ratio of imido ester to protein amino group. These new neoglycoproteins were used to determine stereospecificity of the rabbit hepatic carbohydrate-binding system [Stowell, C. P., Lee, R. T., & Lee, Y. C. (1980) *Biochemistry* (following paper in this issue)].

Neoglycoproteins, proteins to which carbohydrates have been covalently attached by chemical means, have long been used to study the biological role of the carbohydrate prosthetic group of glycoproteins. The methods which have been developed for attaching carbohydrates to proteins for various purposes have been reviewed (Stowell & Lee, 1980).

One of the effective methods for attaching carbohydrates to proteins under mild conditions is to use the 2-imino-2-methoxyethyl 1-thioglycosides (IME-thioglycosides)[1] (Lee et al., 1976). These reagents contain a methyl imido ester function in the aglycon which reacts with the amino groups of proteins to form amidines. The IME-thioglycosides have also been used to amidinate enzymes with no loss of activity (Lee et al., 1976; Stowell, 1978). The neoglycoproteins of several proteins to which β-D-galactopyranose, β-D-glucopyranose, α-D-mannopyranose, and 2-acetamido-2-deoxy-β-glucopyranose had been attached by using these reagents have been used to characterize the specificity of several glycoprotein-binding systems (Krantz et al., 1976; Stowell & Lee, 1978; Stahl et al., 1978). We now report the preparation of a number of new IME-thioglycosides, as well as the results of an investigation of their stability, the conditions for amidination, and the stability of the resultant amidinoneoglycoproteins.

Experimental Section

Materials. All chemicals mentioned in this report, obtained from the indicated sources, were reagent grade and used without further purification. Bovine serum albumin (BSA), D-fucose, L-fucose, 2-acetamido-2-deoxy-D-galactose, 2-deoxy-D-glucose, and 3-*O*-methyl-D-glucose were from Sigma Chemical Co.; D-xylose and L-arabinose were from Pfanstiehl Laboratories, Inc., Waukegan, IL; chloroacetonitrile was from Aldrich Chemical Co.; Bio-Gel P-2, 200–400 mesh, was from Bio-Rad Laboratories; Sephadex G-25 (medium) was from Pharmacia; Jeffamine ED 2001, poly(oxyethylene)diamines of average molecular weight of 2001 was from Jefferson Chemical Co., Houston, TX; thiourea (A grade) was from Calbiochem. The per-O-acetylated sugars were either purchased or prepared from the parent sugar by treatment with a mixture of acetic anhydride and pyridine as described by Wolfrom & Thompson (1963).

Methods. All evaporations were carried out in vacuo with a rotary evaporator. D-Allose was prepared by the method of Stevens (1972) and 6-*O*-methyl-D-galactose was prepared by the method of Brimacombe (1972). Thin-layer chromatography (TLC) was carried out on silica gel F-254 precoated on aluminum sheets (E. Merck). Solvent systems used were (1) 1:1 (v/v) benzene–diethyl ether and (2) 8:2:1 (v/v/v) ethyl acetate–acetic acid–water. Different components on TLC were visualized as follows: carbohydrates, spray with 10% sulfuric acid in 50% ethanol and then heat at 130–140 °C; thiourea derivatives, spray with an aqueous solution containing 1.6% sodium periodate, 0.2% potassium permanganate, and 0.5% sodium carbonate and then heat at 130–140 °C; thiols, spray with 0.4% 5,5′-dithiobis(2-nitrobenzoic acid) in 0.01 M sodium phosphate, pH 7.5 (Ellman, 1959).

Melting points (uncorrected) were determined on a Fisher-Johns appparatus; ^{1}H NMR spectra were taken on a JEOL JNM-MH-100 spectrometer; optical rotation was measured with a Cary 60 polarimeter.

Total neutral sugar was determined by a modification (McKelvy & Lee, 1969) of the phenol–sulfuric acid assay, and ammonia was measured by using the trinitrobenzenesulfonic acid assay (Lee et al., 1976).

Imido esters were determined by two methods previously described in detail (Lee et al., 1976) with a few modifications. *Method A* (by hydrolysis): Imido esters can be quantitatively hydrolyzed to esters and ammonia by acid (Bayliss et al., 1956), and the ammonia can be determined by the trinitrobenzenesulfonic acid assay. *Method B* (by reaction with amines): A sample containing 1–10 μmol of IME-thioglycoside (in 0.01 M sodium methoxide in methanol) was

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[1] Abbreviations used: IME, 2-imino-2-methoxyethyl; CNM, cyanomethyl; BSA, bovine serum albumin; TLC, thin-layer chromatography; Glyc$_n$-AI-BSA, a preparation of BSA to which *n* moles of thioglycosides had been attached by amidination with IME-thioglycoside.

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Table I: Synthesis of Cyanomethyl Per-*O*-acetyl-1-thioglycosides

glycose	steps[a] I[b]	steps[a] III[c]	% yield[d]
β-D-Gal	i	1	55
6-*O*-Me-β-D-Gal	i	3	26
α-L-Ara	ii	3	42
β-D-Fuc	ii	3	15
β-L-Fuc	i	1	50
β-D-Glc	i	1	54
β-D-Xyl	i	1	50
β-D-All	i	2	40
α-D-Man	i	1	50
2-deoxy-β-D-Glc	iv	2	50
3-*O*-Me-β-D-Glc	i	2	28
β-D-GlcNAc	iii	1	45
β-D-GalNAc	iii	2	15

[a] See Results for a description of synthetic methods. [b] Synthesis of per-*O*-acetyl-1-haloglycoses. [c] Synthesis of cyanomethyl per-*O*-acetyl-1-thioglycosides. [d] Yield from per-*O*-acetyl glycose.

brought to 0.4 mL with methanol and diluted with 1.4 mL of 0.25 M sodium borate, pH 8.5. To this mixture was added 0.2 mL of Jeffamine ED 2001. After 2 h at room temperature, a sample containing 0.1–20 μmol of sugar was applied to a water-jacketed (55 °C) column (0.6 × 70 cm) of Bio-Gel P-2 (200–400 mesh) which was eluted at 0.30 mL/min with 0.1 M sodium sulfate into an automated neutral sugar analyzer (Lee, 1972). The ratio of the areas of the void-volume peak (thioglycoside coupled to amine) to the included peak (unreacted carbohydrate) was used to determine the concentration of IME-thioglycoside in the reaction mixture. One analysis could be performed in 75 min. Although both methods gave essentially identical results, method B is more sensitive, whereas method A allows handling of many samples at the same time.

The extent of amidination (incorporation of thioglycosides) was determined by two methods. *Method a*: Proteins which had been amidinated were purified by gel filtration on Sephadex G-25 as described (Lee et al., 1976). Protein was determined by a microbiuret technique (Zamenof, 1957) and carbohydrate was determined by hydrolysis of the 1-thioglycosidic linkage with mercuric acetate (Krantz & Lee, 1976), followed by quantitation of the released sugar by automated analysis as described (Lee et al., 1976). *Method b*: The extent of amidination could also be determined by applying samples of neoglycoprotein of known protein concentration (microbiuret method) to a water-jacketed (55 °C) column (0.6 × 70 cm) of Bio-Gel P-2 (200–400 mesh) which was eluted at 0.4 mL/min with 0.1 M sodium sulfate into the automated neutral sugar analyzer (Lee, 1972). The extent of amidination could be determined by comparing the area of the void-volume peak with neoglycoprotein standards whose thioglycoside contents had been determined by method a. Peak area was a linear function of the amount of sugar (in neoglycoprotein) applied in the range 0.1–1.0 μmol. This method cannot be used for neoglycoproteins containing 2-acetamido-2-deoxy sugars, which do not react with the orcinol reagent used in automated analysis.

Results

Synthesis of Cyanomethyl 1-Thioglycosides. The general scheme of reactions as published before (Lee et al., 1976) was followed. However, the conditions for the reaction or the procedure for working up the product was varied, depending on the sugar. The particular procedure used is indicated in Table I and a description of each procedure is provided below.

Synthesis of Per-O-acetyl 1-Halosugars (Step I). Method i: Per-O-acetylated sugars were treated with HBr in acetic acid, and the products were isolated by extraction and crystallization (Weigel et al., 1979). *Method ii*: Per-O-acetylation and bromination could be accomplished in one flask using red phosphorus and bromine in acetic anhydride according to the method of Lemieux (1963). *Method iii*: Both GlcNAc and GalNAc were per-O-acetylated and chlorinated in one step by the method described by Horton (1972). *Method iv*: A solution of per-O-acetylated 2-deoxy-D-glucose in acetyl chloride was saturated with dry HCl gas at 0 °C and was kept in the cold for 20 h. Solvent was evaporated at room temperature, and the resulting syrup was used immediately for the next reaction.

The yields from methods i and ii were about the same (75–95%); however, the products from method i were purer than those from method ii and reacted more smoothly in subsequent steps. The per-O-acetylation and chlorination of GalNAc (method iii) was accomplished in 50–60% yield.

Synthesis of 2-S-(Per-O-acetylglycopyranosyl)-2-thiopseudourea Hydrohalides (Step II). These compounds were prepared essentially as described previously (Chipowsky & Lee, 1973; Lee et al., 1976). The reaction times were as follows: pentoses and 6-deoxysugars, 20 min; neutral hexoses, 1 h, GalNAc, 2 h. Crystalline products were usually obtained by storage of the reaction mixture in the cold. Occasionally, however, storage in the cold did not produce crystals, in which case the reaction mixture was evaporated to a syrup. This syrup was usually greater than 80% pure as judged by TLC and was used directly in step III. All the thiopsuedourea derivatives were obtained in 75–95% yield, except for the GalNAc derivative, which was obtained in yields of 50–60%.

Synthesis of Cyanomethyl Per-O-acetyl-1-thioglycopyranosides (Step III). The conversion of the hydrohalide salts of 2-*S*-(per-*O*-acetylglycopyranosyl)-2-thiopseudourea to the 1-thiosugars and the formation of the CNM-per-*O*-acetyl-1-thioglycosides were accomplished in one step as described (Lee et al., 1976) with a few variations. *Method 1*: 2-*S*-(2,3,4-Tri-*O*-acetyl-β-D-xylopyranosyl)-2-thiopseudourea hydrobromide (3.2 g, 7.6 mmol) was mixed with 20 mL of acetone to which were then added 20 mL of water, sodium bisulfite (2.0 g, 38 mmol), potassium carbonate (1.6 g, 11.5 mmol), and chloroacetonitrile (2.5 mL, 40 mmol) in order. The reaction was stirred at room temperature for 1.5 h and then poured into ice water (40 mL) and stirred rapidly at 4 °C for 2 h. The precipitate was collected by vacuum filtration, washed with cold water, and dried in a vacuum desiccator. This precipitate was recrystallized from 10 mL of hot methanol, affording 1.8 g (5.4 mmol, 70% yield) of pure cyanomethyl 2,3,4-tri-*O*-acetyl-1-thio-β-D-xylopyranoside. *Method 2*: In some instances, little or no solid material was obtained by stirring the crude product in ice water, in which case the following workup procedure was followed instead. 2-*S*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-2-thiopseudourea hydrochloride (2.5 g, 5.7 mmol), in a mixture of 5 mL of acetone and 5 mL of water, was reacted with sodium bisulfite (1.0 g, 10.1 mmol), potassium carbonate (0.8 g, 5.8 mmol), and chloroacetonitrile (1.25 mL, 19.8 mmol). The reaction was stirred at room temperature for 3 h and then poured into 40 mL of ice water and stirred rapidly at 4 °C for 3 h, by which time a small amount of gummy precipitate had formed. The ice water was extracted twice with 40 mL of chloroform, and the chloroform solutions were combined, washed 3 times with 40 mL of cold 1 M NaCl, dried with anhydrous sodium sulfate, decolorized with acti-

Table II: Physical Constants of Cyanomethyl 1-Thioglycosides

glycose	mp (°C)	R_f[a]	$M_r[\alpha]_D/100$ (deg)[c]	anomeric ^{1}H NMR[d] δ	anomeric ^{1}H NMR[d] J (Hz)
β-D-Gal (acetate)	95–97	0.26	−121 (M)	4.90, C′	10.0
6-O-Me-β-D-Gal (acetate)	syrup	0.32	−190 (M)	4.56, C′	8.6
6-O-Me-β-D-Gal	95–99	0.43[b]	−108 (W)	4.57, D′	10.0
α-L-Ara (acetate)	syrup	0.31		4.71, C′	8.0
α-L-Ara	110–111	0.45[b]	−90 (W)	4.84, D′	7.0
β-D-Fuc (acetate)	92–93	0.37	−213 (M)	4.59, C′	8.4
β-L-Fuc (acetate)	109–110	0.36	+213 (M)	4.58, C′	8.2
β-D-Glc (acetate)	98	0.26	−252 (M)	4.93, C′	10.0
β-D-Xyl (acetate)	109–110	0.31	−278 (M)	5.04, C′	7.8
β-D-All (acetate)	syrup	0.24	−86 (M)		
α-D-Man (acetate)	130–131	0.23	+305 (C[e])	5.51, C′	10.4
2-deoxy-β-D-Glc (acetate)	68–70	0.37			
2-deoxy-β-D-Glc	165–166			4.95, W′	11.5, 2.8
3-O-Met-β-D-Glc (acetate)	93	0.43			
β-D-GlcNAc (acetate)	181–182	0.22	−325 (M)	4.83, C′	10.0
β-D-GalNAc (acetate)	182–183	0.22	−162 (M)	4.77, C′	10.0

[a] TLC on silica gel solvent 1, unless otherwise noted. [b] TLC on silica gel solvent 2. [c] Solvents used: M, methanol; W, water; C, chloroform. Ca. 5% solutions were used. [d] Signal for the anomeric proton by using tetramethylsilane as the standard. Solvents used: C′, $CDCl_3$; D′, dimethyl sulfoxide-d_6; W′, D_2O. [e] A 0.2% solution was used.

vated charcoal, and filtered through Celite. The filtrate was evaporated to a solid which was crystallized from hot 95% ethanol. Pure cyanomethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-β-D-galactopyranoside was obtained in two crops (3.2 mmol total, 56% yield). *Method 3*: When the desired product failed to crystallize, it was purified by silica gel chromatography using a Jobin-Yvon Chromatospac Prep 100. The column was packed with 200 g of silica gel 60 H (50–250 μm, Merck) under 9 bars of pressure and eluted at 2–3 bars. The solvent system used was 2:1 (v/v) toluene–diethyl ether for all compounds.

The yields for this reaction step ranged from 50 to 80% for the neutral monosaccharides and from 50 to 60% for the 2-acetamido-2-deoxysugars. The reactions with the pentoses and 6-deoxysugars were complete within 30 min, while a reaction time of 1 h was required for the neutral hexoses and 3 h was required for the 2-acetamido-2-deoxysugars.

De-O-acetylation of Cyanomethyl Per-O-acetyl-1-thioglycosides (Step IV). For the purposes of characterization, some of the CNM-per-O-acetyl-1-thioglycosides were de-O-acetylated by using catalytic amounts of sodium methoxide in methanol as described (Lee et al., 1976). If the de-O-acetylated glycosides did not crystallize out of the reaction mixtures, sodium was removed with cation-exchange resin, and the solution was evaporated to a syrup and then crystallized from methanol–ether.

Characterization of Cyanomethyl 1-Thioglycosides. The procedures whereby each of the CNM-1-thioglycosides were prepared are listed in Table I. The overall yields from per-O-acetylglycose to CNM-per-O-acetyl-1-thioglycoside (three steps) ranged from 15 to 55%.

The physical constants for these compounds are shown in Table II. All of the CNM-1-thioglycosides or their acetates could be crystallized and stored for at least several years at room temperature as crystals. The ^{1}H NMR data are consistent with the structures assigned and confirm the anomeric configurations proposed. Where they could be adequately resolved, the methylene protons in the aglycon appeared as a widely separated quartet at ~3.4–3.5 ppm ($CDCl_3$). The elemental analyses of several of these compounds were reported previously (Lee et al., 1976).

Synthesis of the 2-Imino-2-methoxyethyl 1-Thioglycosides. The conditions for the synthesis of the IME-thioglycosides of β-D-galactopyranose, β-D-glucopyranose, and α-D-mannopyranose have been extensively studied (Lee et al., 1976; Stowell, 1978). Optimal yields (~60%) were routinely obtained by treating 0.1 M solutions of the per-O-acetylated CNM-1-thioglycosides in dry methanol with 0.01 M sodium methoxide for 36–48 h at room temperature. Yields of 50–60% of the IME-thioglycosides of all the neutral sugars were also achieved by this procedure in the same period of time. Due to its limited solubility in methanol, the IME-thioglycoside of GalNAc was prepared by treating a 0.05 M solution of the per-O-acetylated CNM-1-thioglycoside in methanol with 0.01 M sodium methoxide. Yields of 40% were obtained as had been the case with the GlcNAc analogue (Lee et al., 1976).

Storage of IME-thioglycosides. Although it was convenient to generate the IME-thioglycosides for each experiment as needed, it was considered desirable to be able to prepare large batches and store them for future use. Since the IME-thioglycosides resisted efforts to crystallize them, it was necessary to find some other way to store them. It was shown before that the IME-thioglycosides were stable in the reaction mixture (typically 0.1 M in methanol and 0.01 M sodium methoxide) for up to 7 days at room temperature (Lee et al., 1976). The stability of several IME-thioglycosides in the reaction mixture for more prolonged periods was determined by using method A and correcting for free ammonia. These imido esters were found to be completely stable in the reaction mixture for up to 33 days, although by 138 days their levels were quite low (10–20% of the original imido ester concentration).

IME-thioglycosides were also stored as dry powders after lyophilization. A mixture of CNM- and IME-thio-β-D-galactosides in the reaction mixture was evaporated to dryness and then dissolved in 0.025 M sodium borate, pH 8.5, and frozen at −70 °C. This aqueous solution was lyophilized and stored in a vacuum desiccator at room temperature. About 10% of the original imido ester content was lost in the process of dissolving the evaporated reaction mixture in buffer, freezing it, and lyophilizing it. The imido ester content of the lyophilized powder was stable for at least 60 days and has been used to amidinate proteins. In terms of the extent of modification, lyophilized imido ester was as effective, mole for mole, as freshly generated imido ester. By 11 months, the level of lyophilized imido ester had fallen to 30% of the original content.

Stability of IME-thioglycosides. Since the IME-thio-

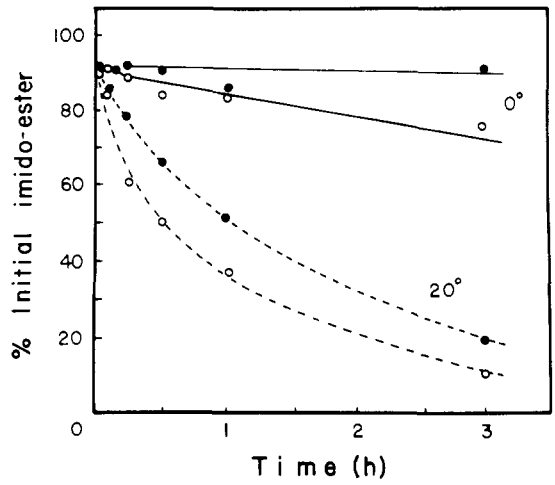

FIGURE 1: Stability of IME-thio-β-D-galactoside in aqueous solutions. Samples of the reaction mixture (initially containing 0.1 M CNM-per-*O*-acetyl-thio-β-D-galactoside and 0.01 M sodium methoxide in methanol and kept for 48 h at 20 °C) containing IME-thio-β-D-galactoside were evaporated and then dissolved in 0.1 M sodium phosphate, pH 7.6 (○), or 0.1 M sodium borate, pH 8.5 (●), at 0 °C (solid line) or 20 °C (dotted line). At various times, samples were removed and assayed for imido ester by method B.

glycosides were prepared with the intention of amidinating proteins in aqueous solutions, it was imperative to determine their stability in water. These experiments were carried out by evaporating the reaction mixture containing IME-thioglycoside to dryness under reduced pressure and then adding the appropriate buffer. At various times the imido ester content of the aqueous solution was determined by method B.

The kinetics of the decomposition of IME-thio-β-D-galactoside in solutions buffered to pH 7.6 or 8.5 or 0 or 20 °C are shown in Figure 1. Decomposition was more rapid at the higher temperature and the lower pH. At 0 °C, decomposition was very slow; 80–90% of the original imido ester was still reactive at 180 min at both pH values. At 20 °C decomposition was considerably more rapid. At pH 8.5, half of the original imido ester was still present at 60 min, and at pH 7.6, half remained at 30 min. By 24 h the level of imido ester under all four sets of conditions was down to 11%. The same kinetics were observed with the IME-thioglycosides of β-D-Glc and β-D-GlcNAc.

The effect of pH on the stability of IME-thio-β-D-galactoside was determined by incubating it in solutions buffered to pH values between 3 and 11 at 20 °C for 30 min. The results are shown in Figure 2. The imido ester was clearly very labile at pH values below 7 but very stable between pH 8 and pH 11.

Solubility of IME-thioglycosides. The IME-thioglycosides were found to be soluble in 0.25 M sodium borate, pH 8.5, up to a concentration of 0.25 M, the highest concentration tested. At this level, the total glycoside concentration, including the CNM-thioglycoside which had not been converted to the imido ester, was ~0.5 M.

Amidination of BSA. Generally speaking, amidination was carried out by evaporating the methanolic solution containing IME-thioglycoside to dryness under reduced pressure. A solution of protein in buffer was added, and the IME-thioglycoside was dissolved in the solution. The extent of amidination was determined by either method a or method b.

The amidination of BSA with ~5 equiv of IME-thioglycoside/protein amino group at pH 8.5 and 37 °C was complete within 1 h while at 20 °C it took 2 h. The temperature did not affect the extent of the reaction. Amidination

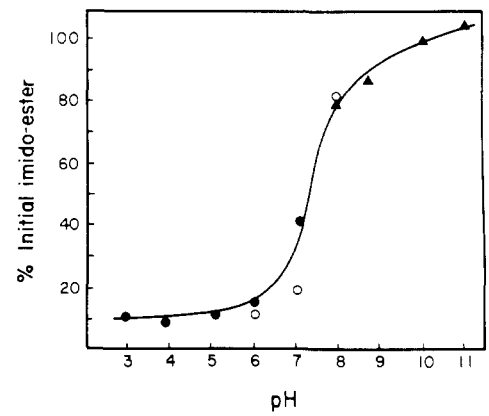

FIGURE 2: Stability of IME-thio-β-D-galactoside at various pH values. Samples of IME-thio-β-D-galactoside in the reaction mixture (initially containing 0.1 M CNM-per-*O*-acetyl-thio-β-D-galactoside and 0.01 M sodium methoxide in methanol and kept for 48 h at 20 °C) were evaporated and then dissolved in 0.25 M citrate–phosphate (●), 0.25 M sodium phosphate (○), or 0.25 M sodium borate (▲). After 30 min at 20 °C, samples were removed and assayed for imido ester by method B. The pH of the mixture was measured before and after incubation.

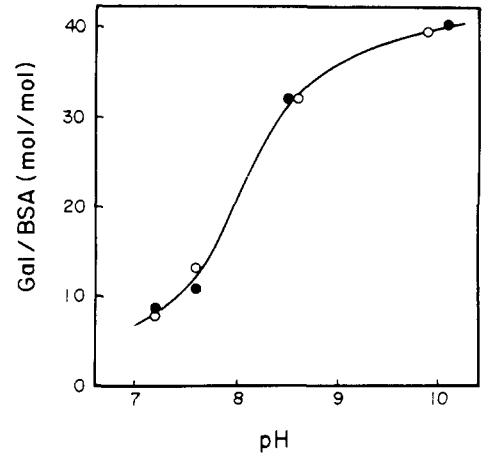

FIGURE 3: Effect of pH on the extent of amidination of BSA with IME-thio-β-D-galactoside. Solutions of 5 mg of BSA in 0.5 mL of 0.25 M sodium borate at various pH values were treated with 20 μmol of IME-thio-β-D-galactoside at 20 (●) and 37 °C (○) for 2 h. The extent of amidination was determined by method b. The pH of the complete amidination reaction mixture was measured at 20 or 37 °C.

reactions were therefore routinely carried out at 20 °C for at least 2 h. The effect of pH on the extent of amidination is shown in Figure 3.

The dependence of the extent of the amidination of BSA on the ratio of IME-thioglycoside to protein amino group is shown in Figure 4. Using a 20-fold excess of any of the IME-thioglycosides listed in Table I to protein amino groups, it was possible to attach 25–40 mol of sugar to 1 mol of BSA which corresponds to the modification of 46–68% of the 59 amino groups (α and ϵ). However, a high number of thioglycosides could be incorporated efficiently at lower concentrations of IME-thioglycoside. For example, by using a twofold excess, between 13 and 24 mol of sugar could be attached to 1 mol of BSA, a modification of 22–41% of the amino groups. The maximum extent of amidination was not changed by adding the IME-thioglycoside over a period of 2 h in four equal portions rather than all at once or by using IME-thioglycoside which had been stored as a dry, lyophilized powder at room temperature.

Using these data, it was possible to calculate the percentage of IME-thioglycosides utilized in the amidination of BSA

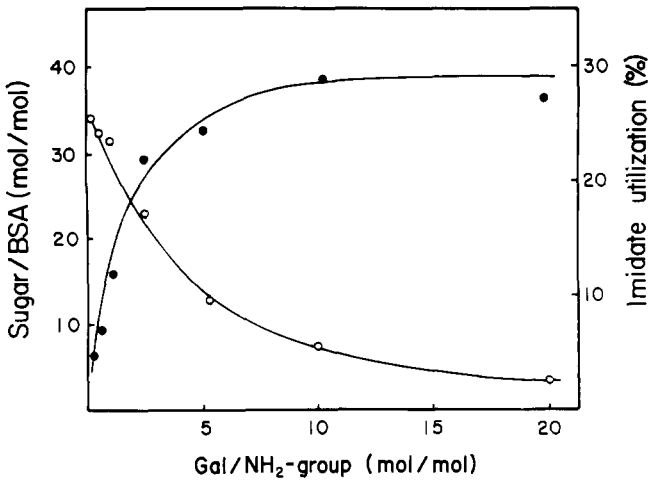

FIGURE 4: Effect of the ratio of IME-thioglycoside to protein amino group on the extent of amidination of BSA. Solutions of 10 mg of BSA/mL of 0.25 M sodium borate, pH 8.5, were incubated with various amounts of the IME-thioglycosides listed in Table I (●). The data from the curve were used to calculate the percentage of IME-thioglycoside which coupled to BSA (○). The extent of amidination was determined by method a.

(Figure 4). At the highest concentrations, only 1–3% of IME-thioglycoside was coupled to BSA, but at the lower concentrations, 25–30% of the reagent was utilized.

On the basis of these experiments, standard conditions for the amidination of proteins were established as follows: 10 mg of protein/mL of 0.25 M sodium borate, pH 8.5, was added to predetermined amounts of IME-thioglycoside at 20 °C for 2 h. As needed, the reaction could be carried out between 4 and 37 °C in a pH range of 7–10. The extent of the reaction could best be controlled by varying the ratio of IME-thioglycoside to protein amino group, rather than by controlling the reaction kinetically.

Properties of Amidinoneoglycoproteins. Amidinoneoglycoproteins of BSA were homogeneous as determined by electrophoresis in 7% polyacrylamide gels at pH 8.3, and their mobilities were the same as that of unmodified BSA.

The amidinoneoglycoproteins were routinely stored at −20 °C at a concentration of 1–2 mg/mL in 0.1 M sodium chloride. The stability of the amidino linkage to these storage conditions was determined by method a after repurification by chromatography on Sephadex G-25 as described under Methods. Several amidinated derivatives of BSA which had been stored up to 2 years retained at least 95% of the attached carbohydrate. Amidinoneoglycoproteins were also successfully stored as lyophilized powders.

The stability of Gal_{34}-AI-BSA in 0.1 M sodium phosphate, pH 7.5, at 20 and 37 °C was determined by the automated gel filtration method (method b for determining the extent of amidination). There was no change in the sugar content of Gal_{34}-AI-BSA at either temperature after 6 h.

Discussion

We originally reported (Lee et al., 1976) the synthesis of the CNM-thioglycosides of β-D-Gal, β-D-Glc, α-D-Man, and β-D-GlcNAc by the reaction of chloroacetonitrile and 1-thioglycose per-*O*-acetate. The reactions have now been applied successfully to the preparation of analogous derivatives of pentoses (α-L-Ara; β-D-Xyl), hexoses (β-D-allose; 6-*O*-Me-β-D-Gal; 3-*O*-Me-β-D-Glc), deoxysugars (β-L-Fuc; β-D-Fuc; 2-deoxy-β-D-Glc), and β-D-GalNAc, with only minor modifications in the procedures. The properties of the IME-thioglycosides and their efficiency as amidination reagents, as discussed below, are independent of the nature of the sugars.

The amidination of proteins using the IME-thioglycosides is a rapid reaction, usually a matter of a few hours at room temperature, and can be carried out under a wide variety of conditions (temperatures ranging from 4 to 37 °C and pH values from 7–10). The extent of amidination is greater at higher pH values although at pH 8.5 the extent of amidination is already 75% of that obtained at the highest pH tested. The dependence of the extent of amidination on pH reflects in part the stability of the IME-thioglycosides in aqueous solutions which is greater at pH values above neutral, a characteristic of imido esters (Roger & Neilson, 1961).

The extent of amidination of proteins can be controlled reproducibly by varying the ratio of imido ester to protein amino group. To date, four different proteins have been extensively amidinated (50–60% of amino groups modified) by using a fivefold ratio of IME-thioglycoside to protein amino group (Lee et al., 1976; Stowell, 1978). None of these proteins showed any decrease in solubility, enzymatic activity, or change in electrophoretic mobility. The amidino linkage has proven to be stable to long-term storage and, more importantly, to physiological conditions.

The neoglycoproteins prepared by the new thioglycosides described in this paper, together with the original derivatives (Lee et al., 1976), have proven to be useful for probing the specificity of several carbohydrate-binding systems (Krantz et al., 1976; Stowell & Lee, 1978; Stahl et al., 1978, 1980; Kuhlenschmidt & Lee, 1980; Stowell et al., 1980).

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Studies on the Specificity of Rabbit Hepatic Carbohydrate-Binding Protein Using Neoglycoproteins†

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ABSTRACT: The binding of amidinoneoglycoproteins of bovine serum albumin to rabbit liver membranes was measured. Derivatives of bovine serum albumin to which equivalent amounts of β-D-Gal, 6-*O*-Me-β-D-Gal, β-D-Fuc, α-L-Ara, β-D-Glc, β-D-Xyl, and β-D-GalNAc had been attached bound to the membranes equally well. The attachment of α-D-Man, β-L-Fuc, β-D-GlcNAc, β-D-allose, 3-*O*-Me-β-D-Glc, and 2-deoxy-β-D-Glc did not promote strong binding. The specificity of binding to the membranes was confirmed by measuring the binding of neoglycoproteins to the purified rabbit hepatic carbohydrate-binding protein immobilized on Sepharose 4B. The results indicate that, for binding, (1) neither the 6-OH (D-Fuc) nor the 5-CH_2OH (L-Ara; D-Xyl) is required, (2) the 4-OH can be axial (D-Gal; L-Ara) or equatorial (D-Glc; D-Xyl), (3) the 3-OH must be equatorial (D-Glc) not axial (D-All) nor may it be substituted (3-*O*-Me-D-Glc), (4) the 2-OH must be equatorial (D-Glc) not axial (D-Man) and must be present (2-deoxy-D-Glc), and (5) the 2-OH can be replaced by an equatorial acetamido group if the 4-OH is axial (D-GalNAc) but not if it is equatorial (D-GlcNAc).

The carbohydrate prosthetic group has long been implicated as having a role in the recognition and binding of many glycoproteins by cells (Roseman, 1970; Ashwell & Morell, 1974; Stahl et al., 1978; Kaplan et al., 1977; Neufeld et al., 1977). Of particular interest is the observation that the requirements for binding with respect to carbohydrate structure are quite stringent. In order to approach the general problem of defining the structural requirements for the binding of glycoproteins to cells and receptors, we have prepared a series of synthetic glycoconjugates (neoglycoproteins) by covalently attaching carbohydrates to proteins using the 2-imino-2-methoxyethyl 1-thioglycosides (Lee et al., 1976). In particular, we have examined the binding of these neoglycoproteins to the rabbit hepatic carbohydrate-binding protein (Krantz et al., 1976; Stowell & Lee, 1978) which has been extensively characterized (Ashwell & Morell, 1974; Hudgin et al., 1974; Kawasaki & Ashwell, 1976a,b; Sarkar et al., 1979). As would have been predicted on the basis of the behavior of deglycosylated serum-type glycoproteins (Ashwell & Morell, 1974), β-D-galactosylneoglycoproteins bound to the binding protein in rabbit liver membranes whereas 2-acetamido-2-deoxy-β-D-glucosyl- and α-D-mannosylneoglycoproteins did not (Krantz et al., 1976; Stowell & Lee, 1978). Interestingly, β-D-glucosylneoglycoproteins were also found to bind to the binding protein as strongly as β-D-galactosylneoglycoproteins or asialoorosmucoid whose oligosaccharide chains are terminated in β-D-galactosyl residues. The evidence of the somewhat relaxed specificity of this binding protein prompted us to examine the structural requirements for binding using some new amidinoneoglycoproteins (Stowell & Lee, 1980). The use of (neo)glycoproteins rather than simple oligosaccharides or glycosides in the present study is imperative because the binding of low molecular weight carbohydrates to the binding protein is many orders of magnitude weaker than the binding of macromolecules (Stowell & Lee, 1978; Sarkar et al., 1979) and may not accurately reflect the specificity of the binding site. In addition, the neoglycoproteins offer the advantage of providing large amounts of chemically homogeneous probes which are not readily available from natural sources, thereby permitting a detailed study of the binding site.

Experimental Section

Materials. Amidinoneoglycoproteins of bovine serum albumin (BSA)[1] were prepared as described (Lee et al., 1976; Stowell & Lee, 1980). Orosomucoid (α_1-acid glycoprotein) was a gift of the American Red Cross National Fractionation Center, Bethesda, MD. Asialoorosomucoid (ASOR) was prepared by treatment with *Clostridium perfringens* neuraminidase (Boehringer Mannheim) and radioiodinated by using sodium [^{125}I]iodide (carrier free, in 0.1 M NaOH from New England Nuclear) and Chloramine T (Aldrich Chemical Co.) as described (Krantz et al., 1976).

Rabbit liver membranes were prepared by a modification (Morell & Scheinberg, 1972) of the method of Ray (1970) which does not include the final sucrose gradient centrifugation. Binding protein was isolated from Triton X-100 extracts

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[1] Abbreviations used: BSA, bovine serum albumin; ASOR, asialoorosomucoid; RIP, relative inhibitory power; $Glyc_n$-AI-BSA, neoglycoproteins of BSA to which *n* moles of thioglycosides had been attached by amidination using the 2-imino-2-methoxyethyl 1-thioglycosides (Krantz et al., 1976).

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