2-Imino-2-methoxyethyl 1-Thioglycosides: New Reagents for Attaching Sugars to Proteins†

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ABSTRACT: Cyanomethyl 1-thioglycosides of D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and D-mannose were prepared from the respective pseudothiourea derivatives and chloroacetonitrile. The nitrile group in these cyanomethyl thioglycosides can be converted to a methyl imidate group by treatment with sodium methoxide or HCl in dry methanol to yield 2-imino-2-methoxyethyl 1-thioglycosides (IME-thioglycosides). The factors influencing the yield of IME-thioglycosides were investigated. The most convenient method of preparing IME-thioglycosides was by treating 0.1 M cyanomethyl thioglycoside peracetate in dry methanol with 0.01 M sodium methoxide at room temperature for 24–48 h (50–60% yield). These IME-thioglycosides reacted readily with simple amines, amino acids, and proteins in mildly alkaline buffer solutions. α-Amylase and lysozyme modified with these reagents under appropriate conditions retained full activities. Thus the IME-thioglycosides constitute a new group of reagents for attaching sugars to proteins.

Although the role of carbohydrate groups in glycoproteins and glycolipids is not well understood, the prevalence of glycoprotein hormones and enzymes as well as the abundance of cell-surface glycoconjugates has led to several hypotheses about the role of the carbohydrate moiety in a variety of biological functions (Eyler, 1965; Roseman, 1970; Winterburn and Phelps, 1972; Roth, 1973). These hypotheses have stimulated the use of synthetic glycoconjugates to study the contribution of carbohydrates to the biological function of these molecules. Some of the earliest efforts were directed toward attaching carbohydrate haptens to proteins for immunological studies (Goebel and Avery, 1929).

Since then a number of techniques have been developed for attaching sugars to proteins. The diazonium salts derived from *p*-aminophenyl glycosides, which react with tyrosyl, histidyl, lysyl, tryptophanyl, and arginyl residues in proteins, have been widely used for such purposes (McBroom et al., 1972). Increased specificity can be obtained by converting the diazonium salts to isothiocyanates which react preferentially with amino groups (McBroom et al., 1972). *S*-Methylglycosylthiourea has also been used as a water-soluble reagent for modifying ε-amino groups (Maekawa and Liener, 1960a,b). The development of an efficient method of obtaining *N*-alkyl-1-amino-1-deoxyalditols with terminal *p*-aminophenyl groups by reductive amination of oligosaccharides (Jeffrey et al., 1975) may be useful for attaching more complex carbohydrates to proteins. Reducing oligosaccharides have been attached to proteins by reduction of the Schiff's base formed between the sugar carbonyl group and the protein amino group with cyanoborohydride (Gray, 1974).

Amide bond formation is also frequently used for attaching sugars to proteins. Sugar acids react with amino groups in proteins using the mixed anhydride method (Ashwell, 1972) while synthetic glycosylamines have been attached to the carboxyl groups of proteins using coupling agents (Moczar, 1973; Moczar and Le Boul, 1975). Elaborately synthesized ω-carboxyoctyl glycosides of trisaccharides having blood group activities have been attached to bovine serum albumin (Lemieux et al., 1975) by the acyl azide method.

Although the methods described above have been shown to be useful, they all have certain shortcomings. The use of aryl functional groups may result in a high degree of nonspecific hydrophobic interaction between the aryl group and the protein that may obscure the effects of the carbohydrate ligand. Other techniques do not show a high degree of specificity in reacting with particular amino acid functional groups. Coupling methods that convert the reducing terminal sugar to an acyclic derivative suffer not only from the loss of the cyclic structure from the terminal sugar residue of the oligosaccharide, but also from the complications introduced by the presence of an acyclic sugar residue. Attachment of sugars by forming an amide bond often causes drastic changes in the electrostatic charge of the protein molecule resulting in decreased solubility and partial or total inactivation. The use of organic solvents or extreme conditions required by some techniques may denature the protein and reduce enzymatic activity. Although *S*-methyl glycosylthiourea reacts with proteins under mild conditions, it is difficult to prepare.

In seeking effective methods for attaching carbohydrates to proteins under mild conditions, we have prepared several 2-imino-2-methoxy 1-thioglycosides (IME-thioglycosides) from cyanomethyl thioglycosides (CNM-thioglycosides). The use of an imidate as the reactive functional group in the aglycon has many advantages. Imidates are water-soluble reagents that react rapidly with primary amino groups to produce amidines (Roger and Neilson, 1961; also see Diagram III). Since there is no gross change in the net charge of the protein after such modification, many proteins, including antibodies and antigens (Wofsy and Singer, 1963), enzymes (Hunter and Ludwig, 1972), and hormones (Zull and Chuang, 1975) have been extensively amidinated with little or no change in tertiary structure. Full activity is retained if critical lysine residues are not modified. The reaction appears to be specific for primary amino groups since imidates do not react with sulfhydryl, phenolic, hydroxyl, imidazole, or guanidino groups in small

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[1] Abbreviations used are: IME-, 2-imino-2-methoxyethyl; TLC, thin-layer chromatography; CNM-, cyanomethyl; TNBS, 2,4,6-trinitrobenzenesulfonic acid; GABA, γ-aminobutyric acid.

test compounds. At higher pHs ϵ-amino groups are relatively more reactive than α-amino groups. The amidino linkage formed is stable to neutral or acidic pH.

In this report we describe the synthesis of several IME-thioglycosides which appear to react readily with simple amino compounds and proteins. The amidino enzymes retained virtually complete activity even at the highest levels of modification.

Materials and Methods

2-*S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea hydrobromide (**1**), 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-thiopseudourea hydrobromide, 2-*S*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-thiopseudourea hydrochloride, and 2-*S*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-2-thiopseudourea hydrobromide were prepared as previously described (Chipowsky and Lee, 1973). Jeffamine ED-600, poly(oxyethylene)diamines of average molecular weights of 600, was a gift from Jefferson Chemical Co. (Houston, Texas).

Thin-layer chromatography (TLC) was carried out on silica gel F-254, precoated on aluminum sheets (E. Merck Co.). Solvents used were (a) 1:1 (v/v) benzene–ether, (b) 8:2:1 (v/v/v) ethyl acetate–acetic acid–water, and (c) 3:2:1 (v/v/v) ethyl acetate–acetic acid–water. Components on TLC were located by spraying with 10% sulfuric acid in 50% ethanol followed by heating at 130–140 °C. Optical rotation was measured with a Cary 60 spectropolarimeter. Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tenn.).

α-Amylase (*Aspergillus oryzae*) was prepared as previously described (McKelvy and Lee, 1969). Chicken egg white lysozyme and bovine serum albumin were from Sigma Chemical Co. Enzymatic activities of α-amylase were measured by the starch–iodine method (McKelvy and Lee, 1969) or by the 3,5-dinitrosalicylic acid method (Bernfeld, 1951). Lysozyme activity was measured by the turbidometric method, using a suspension of *Micrococcus lysodeikticus* cells (Shugar, 1952) Protein concentrations were calculated from the measurement of absorbance at 280 nm ($E_{280}^{1\%}$ = 16.7 for α-amylase, 26.0 for lysozyme, and 6.6 for bovine serum albumin), or by a microbiuret method (Zamenof, 1957).

Measurement of Ammonia. Ammonia produced from imidates (see below) was measured with the trinitrobenzenesulfonic acid (TNBS) reagent (Okuyama and Satake, 1960) by the following procedure. The sample solution (usually 10 μl) was diluted to 1 ml with 0.2 M sodium borate buffer (pH 8.5), mixed with 0.5 ml of 0.2% TNBS in water, and incubated at 55 °C for 30 min. The reaction mixture was immediately diluted with 2 ml of water and the absorbance at 420 nm was determined. Using ammonium sulfate as the standard, the Beer–Lambert law was observed within the range of 15–800 nmol corresponding to A_{420nm} of 0.025–1.300. The color was found to be stable at least overnight in the cold.

Determination of Imidates. (A) By Hydrolysis. Imidates can be quantitatively hydrolyzed to esters and ammonia in the presence of strong acid (Bayliss et al., 1956). The imidate content of the sample hydrolyzed can be determined by measuring the ammonia produced using the TNBS method described above. Optimal conditions for hydrolysis of imidate were determined by measurement of ammonia produced at 37 °C in 0.5 M sulfuric acid. In the finally adapted procedure, 50 μl of sample (in 0.01 M sodium methoxide in dry methanol) containing 30–450 nmol of imidate was incubated with 100 μl of 0.5 M sulfuric acid for 30 min at 37 °C. The ammonia produced was measured by the method described above. CNM-Thioglycosides did not produce any detectable amount of ammonia under the same conditions.

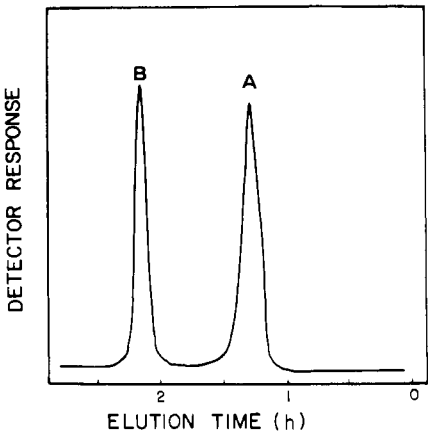


FIGURE 1: Imidate determination by gel filtration (method B). A sample containing about 0.3 μmol of IME-thiogalactoside coupled to Jeffamine ED 600 and 0.2 μmol of CNM-thiogalactoside was chromatographed as described in Materials and Methods. The amidino compound (peak A) appears in the void volume (76 min) and the CNM-thiogalactoside (peak B) at 129 min.

(B) By Reaction with Amines. In this method, the ability of imidate to couple readily to primary amines was used to estimate its concentration. A sample containing 1 to 10 μmol of imidate (in 0.01 M sodium methoxide in methanol) was brought to 0.4 ml with methanol and diluted with 1.5 ml of 0.25 M sodium borate buffer, pH 8.5. To this mixture was added 100 μl (about 175 μmol) of Jeffamine ED-600. Amidination of Jeffamine was complete by 2 h at which time 200 μl of 5 M hydrochloric acid was added to stop the reaction. The amidinothioglycosides (the sugars coupled to Jeffamine) had to be separated from the unreacted CNM-thioglycosides by gel filtration before quantitation. Aliquots of the reaction mixture (usually 50–200 μl containing 0.1–1.0 μmol of sugar) were applied to a column (1.2 × 67 cm) of Bio-Gel P-2 which was eluted with 0.1 M acetic acid at 0.63 ml/min at room temperature. The column effluent was led into the orcinol–sulfuric acid system of an automated sugar analyzer (Lee, 1972). The chromatographic profile is shown in Figure 1. The amidinothioglycosides appeared in the void volume while the CNM-thioglycosides were retained. The ratio of the areas under the two peaks was used to determine the relative concentration of imidate. The area of the first peak was proportional to the amount of imidate added within the range of 1 to 10 μmol of imidate. Jeffamine did not react with **2** or **3**.

Reaction of the Imidates with Proteins. Based on the results described below, imidate formation from cyanomethyl thioglycosides was routinely carried out by treatment of 0.1 M CNM-thioglycoside in absolute methanol with 0.1 equiv of sodium methoxide for 24–48 h at room temperature. Under these conditions, yields of the imidates were consistently between 50 and 60%.

For reaction with proteins, an aliquot from such a reaction mixture (containing 5–500 μmol of imidate) was evaporated to dryness in vacuo and then dissolved in 1 ml of a protein solution (5–10 mg/ml) in the appropriate buffer. After a predetermined time, the reaction was stopped with 100 μl of 1 M acetic acid, applied to a column (2 × 35 cm) of Sephadex G-25, and eluted with 0.1 M sodium acetate buffer, pH 5.5, or 0.1 M sodium chloride to isolate the products.

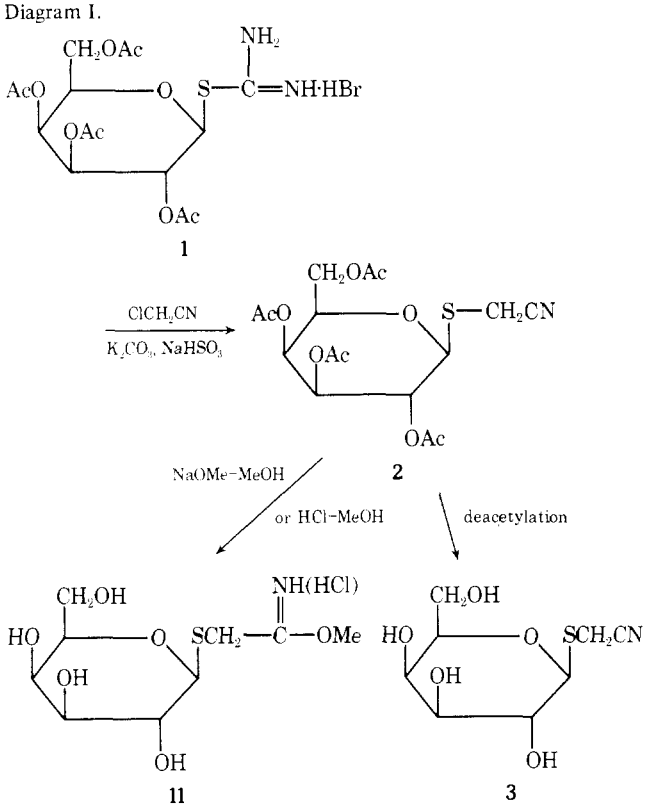
Measurement of Thioglycosides Bound to Proteins. Hy-

drolysis of the 1-thioglycosidic linkage was accomplished by the method of Krantz and Lee (1976). To an aliquot of solution containing 0.2 to 0.5 mg of protein with 10–100 nmol of covalently linked thioglycoside, 10 μl of 0.2 M mercuric acetate in 0.1 M acetic acid was added. The mixture was heated at 100 °C for 5 min, and liberated sugar determined by a modified version of automated borate chromatography (Lee, 1972). An aliquot of the hydrolysate containing 2 to 30 nmol of sugar was applied to a water-jacketed (55 °C) column (270 × 3 mm) of Rexyn 201 (20–40 μm). The column was eluted at 0.5 ml/min with 0.31 M sodium borate, pH 8.7, which was 0.01% (v/v) in Brij 35 into an automated sugar analyzer.

Results

Cyanomethyl 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-galactopyranoside (**2**). 2-*S*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea hydrobromide (**1**), 9.74 g (20 mmol), and chloroacetonitrile (5 ml, 79 mmol) were stirred with a 1:1 (v/v) mixture of water and acetone (40 ml) until a nearly complete solution was obtained. Potassium carbonate (3.2 g, 23.2 mmol) and sodium bisulfite (4.0 g, 40.4 mmol) were added to the stirred solution. Stirring was continued for 30 min at room temperature, at which point the reaction mixture was added to an ice-water mixture (160 ml) and stirred for 2 h. The precipitated products were suction-filtered and washed with cold water. The air-dried precipitate was stirred for a few minutes in boiling methanol (80 ml), and the insoluble material was removed by filtration. Storage of the methanolic filtrate in the cold yielded crystalline **2**: 5.8 g (72% yield); mp 95–97 °C; $[\alpha]_D^{25}$ − 30.0° (*c* 5.02, methanol).

Diagram I.



Anal. Calcd for $C_{16}H_{21}NO_9S$ (403.4 daltons): C, 47.63; H, 5.24; N, 3.47; S, 7.94. Found: C, 47.70; H, 5.53; N, 3.43; S, 7.49.

Cyanomethyl 1-Thio-β-D-galactopyranoside (**3**). (a) A solution of **2** (0.605 g, 1.5 mmol) in methanol (5 ml) was mixed with 1 ml of anhydrous triethylamine and allowed to stand at room temperature for 24 h when TLC revealed complete deacetylation. The crystals (needles) which formed after standing at room temperature overnight were filtered and washed with dry methanol: yield, 0.27 g (80%); mp 169 °C; $[\alpha]_D^{22}$ −51.5° (*c* 5.05, water).

Anal. Calcd for $C_8H_{13}NO_5S$ (235.3 daltons): C, 40.84; H, 5.56; N, 5.56; S, 13.64. Found: C, 41.00; H, 5.44; N, 5.88; S, 13.49.

(b) A solution of **2** (5.69 g, 14.1 mmol) in methanol (54 ml) was mixed with 0.079 mmol of sodium methoxide and kept at room temperature overnight, when TLC indicated complete deacetylation. Crystals formed from the deacetylation mixture overnight at 4 °C. Recrystallization from boiling methanol yielded 2.8 g (84%) of pure **3.** The product obtained by method b was indistinguishable from that obtained from method a described above.

Cyanomethyl 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranoside (**4**) *and Cyanomethyl 1-Thio-β-D-glucopyranoside* (**5**). These compounds were prepared from a pseudothiourea derivative of D-glucose (Chipowsky and Lee, 1973) as in the case of D-galactose analogues described above. **4** was obtained in 92% yield: mp 98 °C; $[\alpha]_D^{22}$ −62.2° (*c* 5.04, methanol).

Anal. Calcd. for $C_{16}H_{21}NO_9S$ (403.4 daltons): C, 47.63; H, 5.24; N, 3.47; S, 7.94. Found: C, 47.70; H, 5.26; N, 3.45; S, 7.74.

Deacetylation of **4** by either method a or b yielded syrupy **5,** which was homogeneous by TLC: $[\alpha]_D^{22}$ −60.2° (*c* 5.06, water).

Cyanomethyl 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-β-D-glucopyranoside (**6**) *and Cyanomethyl 2-Acetamido-2-deoxy-1-thio-β-D-glucopyranoside* (**7**). These compounds were prepared from a pseudothiourea derivative of 2-acetamido-2-deoxy-D-glucose (Chipowsky and Lee, 1973) as in the case of D-glucose analogues described above.

6 was obtained in 78% yield: mp 181–182 °C; $[\alpha]_D^{22}$ −80.8° (*c* 5.05, methanol).

Anal. Calcd for $C_{16}H_{22}N_2O_8S$ (402.34 daltons): C, 47.75; H, 5.51; N, 6.96; S, 7.95. Found: C, 47.66; H, 5.57; N, 6.89; S, 7.89.

De-*O*-acetylation of **6,** resulting in **7,** was accomplished with either sodium methoxide (57% yield) or triethylamine (78% yield): mp 176–177 °C; $[\alpha]_D^{22}$ −81.0° (*c* 5.00, water).

Anal. Calcd for $C_{10}H_{16}N_2O_5S$ (276.32 daltons): C, 43.46; H, 5.83; N, 10.14; S, 11.58. Found: C, 43.61; H, 5.64; N, 10.01; S, 11.75.

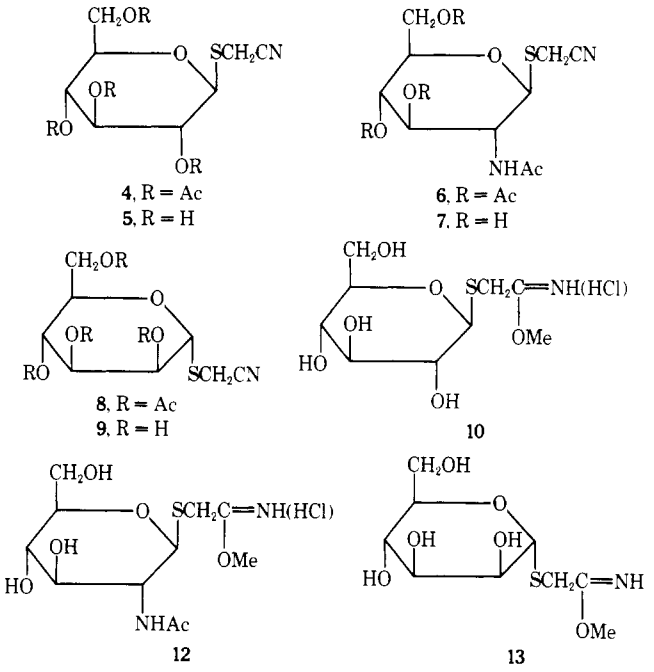
Cyanomethyl 2,3,4,6-Tetra-O-acetyl-1-thio-α-D-mannopyranoside (**8**) *and Cyanomethyl 1-Thio-α-D-mannopyranoside* (**9**). 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl bromide was converted to 2-*S*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-2-thiopseudourea hydrobromide by refluxing in acetone with excess thiourea as in the case of the other sugars (Chipowsky and Lee, 1973). The crude crystalline pseudothiourea derivative was converted to **8** by the same method as described above for **2** (74% yield): mp 130–131 °C; $[\alpha]_D^{22}$ 74.5° (*c* 0.2, chloroform).

Anal. Calcd for $C_{16}H_{21}NO_9S$ (403.4 daltons): C, 47.63; H, 5.24; N, 3.47; S, 7.94. Found: C, 47.60; H, 5.16; N, 3.47; S, 8.06.

Deacetylation of **8** with sodium methoxide gave syrupy **9,** which was homogeneous by TLC.

Sodium Methoxide Catalyzed Formation of 2-Imino-2-methoxyethyl 1-Thio-β-D-glucopyranoside (**10**) *and Its Analogues.* In the presence of sodium methoxide in methanol, **2, 4, 6,** and **8** are de-*O*-acetylated and form imidates. Only

Diagram II.



catalytic amounts of sodium methoxide are necessary for de-*O*-acetylation, while much higher concentrations are required for imidate formation. In order to study the dependence of the rate and extent of imidate formation on the sodium methoxide concentration, methanolic solutions of **4** (0.1–0.25 M) were treated with varying amounts of sodium methoxide (1–100 mM final concentration) at room temperature (20 ± 2 °C). Aliquots from the reaction mixtures were withdrawn at appropriate time intervals for measurement of imidate content by method A. The results are shown in Figure 2. Even with an equimolar concentration of sodium methoxide (curve A), conversion of 0.1 M CNM-thioglucoside did not go to completion, but leveled off within 6 h at about a 60% yield of imidate. The same level of imidate was also attained but more slowly (48 h) when the sodium methoxide concentration was reduced to 0.01 M (curve B). Reduction of the sodium methoxide concentration to 0.001 M further slowed the reaction and only about a 10% yield of imidate was attained by 96 h (curve D). When the sodium methoxide concentration was adjusted to 0.04 M at this point, however, the imidate content rose rapidly to about a 60% yield (curve D).

The rate of the reaction, but not the extent, is also dependent on the ratio of base to nitrile. Although the sodium methoxide concentrations were the same in reactions B and C (0.01 M), the concentration of CNM-thioglucoside was increased to 0.25 M in reaction C. The rate of reaction C, where the molar ratio of CNM-thioglucoside to base was 25:1, was slower than that of reaction B, where the ratio was 10:1, despite the increased concentration of reactants in C. However, the same yield (about 60%) was obtained in reaction C.

Effect of Temperature on the Sodium Methoxide Catalyzed Formation of **11.** A solution of **2** (0.1 M in methanol) was treated with 0.01 M sodium methoxide (see Figure 2B) at about 4, 20, 37 and 55 °C, and the rates of formation of **11** were measured by method A described above. The results shown in Figure 3 indicate that, while the initial rates of formation of **11** were greater at the higher temperatures, the final yields were lower. The highest yield of **11** was obtained when the reaction was carried out at 20 °C. At 4 °C, the rate of imidate formation was impractically slow.

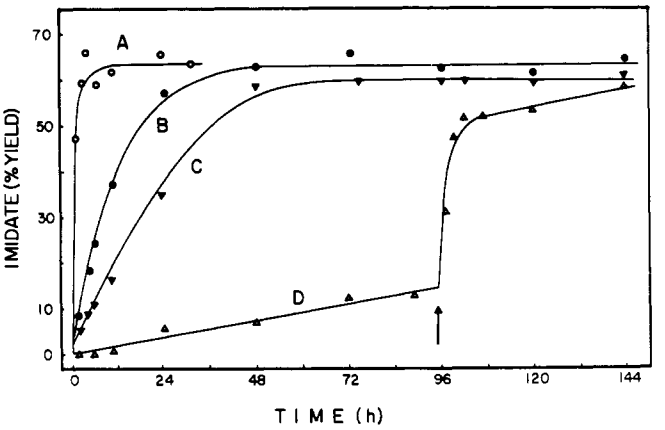


FIGURE 2: Base-catalyzed imidate formation—effect of reactant concentrations. Solutions of **4** in different concentrations were incubated with various concentrations of sodium methoxide in methanol at room temperature. The imidate concentration was estimated by the hydrolysis-TNBS method. (curve A (○)) 0.1 M **4**, 0.1 M NaOMe; (curve B (●)) 0.1 M **4**, 0.01 M NaOMe; (curve C (▼)) 0.25 M **4**, 0.01 M NaOMe; (curve D (▲)) 0.1 M **4**, 0.001 M NaOMe. At 96 h, the concentration of NaOMe in reaction D was adjusted to 0.04 M.

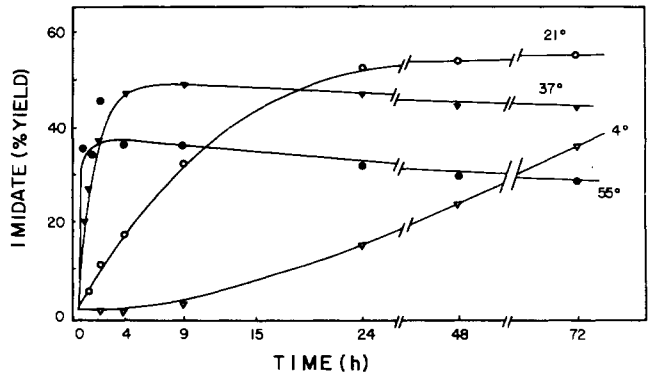


FIGURE 3: Base-catalyzed imidate formation—effect of temperature. Solutions of 0.1 M **2** were treated with 0.01 M NaOMe in methanol at 4 (▽), 21 (○), 37 (▼), or 55 °C (●). The imidate concentration was determined by the hydrolysis-TNBS method.

Sodium Methoxide Catalyzed Formation of Imidates (**11** *and* **12**) *from* **2** *and* **6.** Under the favorable conditions determined above (0.1 M CNM-thioglycoside, 0.01 M sodium methoxide, 20 °C), the rates of conversion of **2** and **4** to their respective imidates were identical. However, because of the relative insolubility of **6** in methanol, 0.05 M **6** and 0.005 M sodium methoxide were used in the corresponding experiment. Consequently, the rate of imidate formation from **6** was considerably slower than those from the D-*gluco*- and D-*galacto*-analogues (**4** and **2**). The final yield was also lower (about 30%).

Acid Catalyzed Formation of Imidates. Using HCl in methanol as the catalyst, similar yields of each of the hydrochloride salts of **10, 11,** and **12** were obtained from **4, 2,** and **6,** respectively. Although under the conditions described in Figure 4 the total ammonia production (method A) reached 50–60% of the starting CNM-thioglycoside, a considerable portion of that was present prior to acid hydrolysis. The net yield was about 20% at 1 day, but by 3 days, virtually no imidate remained.

Stability of the Imidates. The imidates formed by the action of sodium methoxide in absolute methanol could be stored in the same reaction mixture over 2–3 months without any apparent deterioration as determined by method A or by TLC

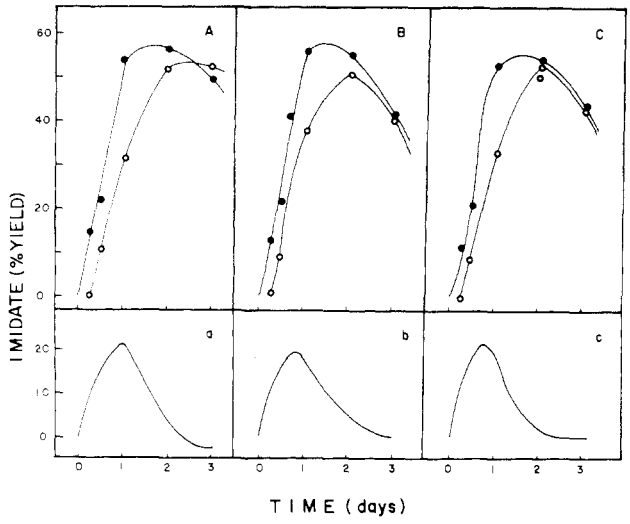


FIGURE 4: Acid-catalyzed imidate formation. Solutions of 0.1 M **2** (A), **4**, (B), or **6** (C) in 5 N HCl in methanol were maintained at 4 °C. At timed intervals the free ammonia (○) and acid-produced ammonia (method A) (●) were determined by the TNBS method. The difference, or net imidate formed from **2**, **4**, and **6**, is shown below in curves a, b, and c, respectively.

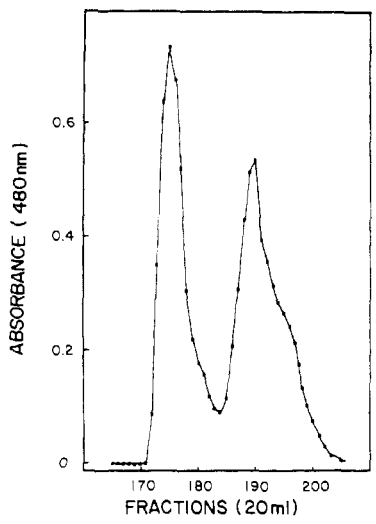
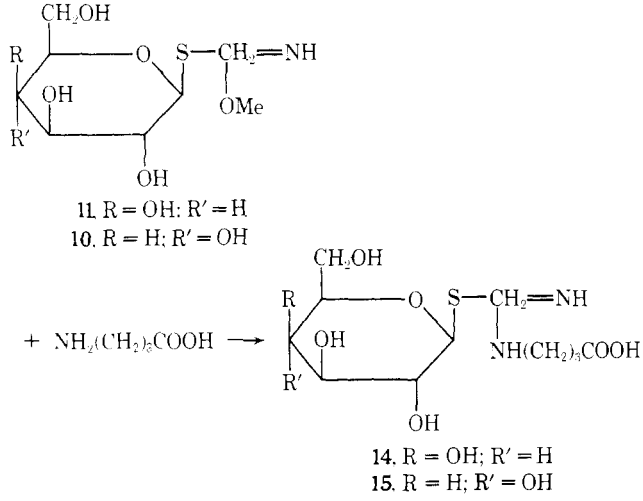


FIGURE 5: Gel filtration (Sephadex G-25, 5 × 220 cm) of the reaction products between IME-thiogalactoside and GABA. The sugar concentration of each fraction was determined by the phenol-sulfuric method (McKelvy and Lee, 1969).

(solvents b and c). However, in aqueous solution, the imidates were relatively unstable, and rates of degradation depend on pH and temperature (Hunter and Ludwig, 1972).

Reaction of IME-Thioglycosides with γ-Aminobutyric Acid (GABA). **2** (4.03 g, 10 mmol) was treated with sodium methoxide under the conditions of Figure 2B. After 17 h at 20–22 °C, the solvent was evaporated in vacuo and the resultant syrup added to GABA (2.06 g, 20 mmol) in 15 ml of 0.4 M sodium borate buffer, pH 10. After 5 h at 20–22 °C, the reaction mixture was directly applied to a column (5 × 220 cm)

Diagram III.



11. R = OH; R′ = H
10. R = H; R′ = OH

14. R = OH; R′ = H
15. R = H; R′ = OH

of Sephadex G-25 (fine), equilibrated in water, to separate the coupled product from the unreacted compounds. The elution profile is shown in Figure 5. The first peak was the amidino compound (**14**) and the second peak was mostly **3** (shown by TLC, solvents b and c). Untreated GABA was found to be present between these peaks.

Similar results were obtained when **4** was used in the same sequence of reactions. In this case, the fractions of the front peak from the gel filtration (similar to Figure 5), were examined by TLC. The pure fractions of **15** were pooled and lyophilized, further dried in vacuo at 20–22 °C for 2 days, and analyzed for elemental composition.

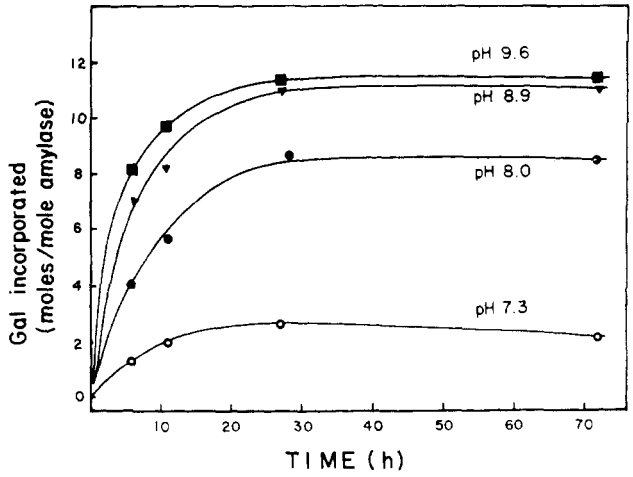


FIGURE 6: Effect of pH on amidination of α-amylase by IME-thiogalactoside. α-Amylase (25 mg in 2.5 ml) was reacted with approximately 70 μmol of IME-thiogalactoside at 0 °C at pH 7.3 (○), 8.0 (●), 8.9 (▼), or 9.6 (■). The number of galactose residues incorporated into α-amylase was determined as described in Materials and Methods.

Anal. Calcd for $C_{12}H_{22}N_2O_7S$ (338.4 daltons): C, 42.59; H, 6.55; N, 8.28; S, 9.48. Found: C, 42.23; H, 6.56; N, 8.16; S, 9.15.

Attachment of IME-Thioglycosides to Proteins. The efficiency and rate of coupling of IME-thiogalactoside (**11**) to α-amylase were compared at pH 7.3 (0.2 M sodium phosphate), 8.0, 8.9, and 9.6 (0.2 M sodium borate) at 0 °C. As shown in Figure 6, all the reactions reached their respective maximal levels after 25–30 h. Both the rate and extent of coupling increased with increasing pH. At room temperature, the same level of coupling was achieved as at 0 °C (pH 8.5), but the reaction was complete in less than 6 h. CNM-Thiogalactoside incubated under the same conditions did not react with α-amylase.

The effect of increasing the imidate/protein ratio on the level of coupling to α-amylase and lysozyme is shown in Figure 7. A maximum of about 12 mol of thiogalactoside was coupled to amylase, and about 4 mol to lysozyme. Similar levels of incorporation were obtained with **10**, **12**, and **13**. The enzymatic activities of 24 preparations with varying amounts of sugar

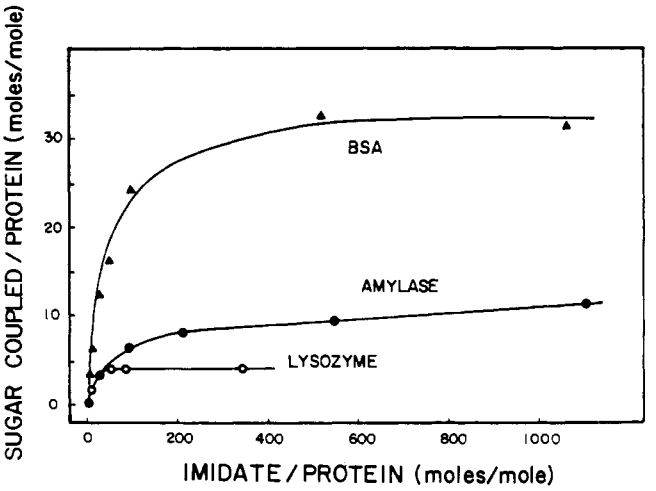


FIGURE 7: Effect of imidate concentration on the extent of amidination of proteins by IME-thiogalactoside. α-Amylase (●), lysozyme (○), and bovine serum albumin (▲) at 10 mg/ml in 0.25 M sodium borate, pH 8.5, were reacted with various molar excesses of IME-galactoside at room temperature for 24 h. The number of sugars incorporated into the proteins was determined as described in Materials and Methods.

incorporated are summarized in Table I. Some loss of enzymatic activity was observed in the most highly modified derivatives, but low levels of modification appeared to have no effect or even slightly increased enzyme activity.

The rate of the reaction of the IME-thioglycosides with bovine serum albumin was more rapid than with either of the two enzymes. At room temperature and pH 8.5, the reaction of **10** or **11** with bovine serum albumin leveled off in less than 0.5 h. The effect of increasing the ratio of imidate to protein on the number of sugars incorporated into bovine serum albumin is shown in Figure 7. Up to 28–41 sugars could be attached to bovine serum albumin with a 30-fold excess of the four IME-thioglycosides to amino groups.

The amidinated proteins were stored in 0.1 M NaCl or 0.1 M sodium acetate (pH 5.5) at −20 °C for at least 6 months with no change in sugar content. Even the most highly modified derivatives showed no changes in solubility during this time.

Discussion

CNM-Thioglycosides of four monosaccharides were synthesized and used to generate 2-imino-2-methoxyethyl thioglycosides. Since the imidates (IME-thioglycosides) could not be isolated in crystalline form, it was necessary to use imidate generated in the reaction mixture in situ. Two methods of determining imidate content, based on either their acid lability or their ability to react with primary amines, were developed and used in this study. Since estimates of imidate in a particular sample using both methods agreed closely, the more convenient hydrolysis–TNBS assay was used routinely.

The behavior of the putative imidate in acidic and basic aqueous solutions is consistent with the known properties of imidate as is its ability to react with amines. Further evidence is provided by the isolation and purification of the product of the reaction between imidate and GABA at pH 10. Elemental analysis of this product is consistent with the proposed structure of **15**, in agreement with the results of Browne and Kent (1975a). At lower pHs they found a mixture of amidine and nonamidine products.

Imidates have been made from nitriles in alcohol using either acid or base catalysts. The acid-catalyzed reaction is efficient with many unsubstituted aromatic and aliphatic nitriles,

TABLE I: Effect of Thioglycoside[a] Attachment on Enzyme Activity.

Protein	Moles Coupled/ Mole of Protein	% Act.
α-Amylase	1.2–2.0 (4)[b]	92–154
	3.8–5.2 (5)	85–128
	8.3–11.5 (4)	84–110
Lysozyme	0.7–2.2 (5)	72–112
	3.2–4.4 (6)	62–96

[a] 2-Imino-2-methoxyethyl 1-thio-β-D-galactopyranoside or -β-D-glucopyranoside was used. [b] The numbers in parentheses indicate the number of preparations examined.

whereas the base-catalyzed reaction is efficient with electronegatively substituted aromatic and aliphatic nitriles (Schaefer and Peters, 1961). Therefore the two methods are complementary and can be used to make a wide variety of imidates.

In our studies, both methods were used to generate imidate from the CNM-thioglycosides. Using HCl as the catalyst, however, the yields were poor, perhaps due to the instability of the product. The low yield and long reaction time made this reaction impractical.

Useful yields of about 60% could be obtained in a reasonable amount of time when sodium methoxide was used as the catalyst. Base-catalyzed imidate formation from nitriles is known to be reversible and commonly does not go to completion, especially when the nitrile is unsubstituted or substituted with a weak electrophile (Bayliss et al., 1956; Schaefer and Peters, 1961). Since the extent of this reaction at a given temperature is not affected by changing the concentration of nitrile or base or their ratio, it seems likely that an equilibrium is established between nitrile and imidate, slightly favoring the imidate.

The yield of imidate from CNM-thioglycoside was, however, sensitive to temperature. The yields were lower at temperatures greater than 37 °C and unchanged at temperatures below 20 °C. This observation is consistent with the suggestion that imidate formation is an exothermic process and would be inhibited by higher temperatures (Schaefer and Peters, 1961).

The rate of imidate formation was also sensitive to temperature and reactant concentrations. At 20 °C the reaction leveled off between 24 and 48 h, whereas at 37 and 55 °C the maximum yield was obtained within several hours. The rate was increased when the concentrations of CNM-thioglycoside or sodium methoxide were increased. The ratio of concentrations is also critical. The rate of reaction was more rapid when the ratio of sodium methoxide to nitrile was 0.1 even though the concentration of the reactants was lower than in a reaction where the ratio was 0.04.

The reaction mixture with methanolic sodium methoxide containing the IME-thioglycosides and the CNM-thioglycosides at equilibrium is apparently very stable under anhydrous conditions. Thus it is practical to treat a large quantity of CNM-thioglycoside in methanol with sodium methoxide and store the reaction mixture as a stock solution of IME-thioglycoside. If necessary, imidate concentration can be measured with one of the methods described above. Based on the above results, IME-thioglycosides were routinely generated by treating 0.1 M CNM-thioglycoside in dry methanol with 0.01 M sodium methoxide at room temperature for 1–2 days. This method has been giving satisfactory results reproducibly.

The IME-thioglycosides coupled readily to GABA, Jeffamine, α-amylase, lysozyme, and bovine serum albumin. With large molar excesses of GABA or Jeffamine the reactions were rapid and quantitative. On the other hand, not all of the amino groups of any of the proteins could be modified even using a 50-fold excess of imidate to amino group. After exhaustive amidination (room temperature, pH 10, 48 h), 12 of the 22 amino groups of α-amylase and 4 of 7 of lysozyme could be modified. Using a 30-fold excess of imidate up to 41 of the 59 amino groups of bovine serum albumin could be modified after 2 h at room temperature at pH 8.5.

The number of amino groups in these proteins reactive to the IME-thioglycosides is similar to the numbers reactive to other reagents. Using dinitrobenzenesulfonic acid, up to 14 of the 21 ε-amino groups of α-amylase could be dinitrophenylated (Ikenaka, 1959) whereas 12 were reactive to the IME-thioglycosides. All 7 amino groups of lysozyme are available for modification by acetic anhydride or *O*-methylisourea in accordance with the observation that all 6 lysines are on the surface (Imoto et al., 1972). However, the reaction of lysozyme with several imidates does not go to completion (Davies and Neuberger, 1969; McCoubrey and Smith, 1966). McCoubrey and Smith estimated that up to 5 of the ε-amino groups can be amidinated by small imidates but that this number is decreased as the size of the imidate increases. Using the IME-thioglycosides only 4 amino groups could be modified. Lemieux et al. (1975) have reported the incorporation of 30–38 mol of carboxyalkyl glycosides of oligosaccharides into bovine serum albumin by the acyl azide method, whereas in this study 28–41 mol of monosaccharide could be incorporated by amidination.

Since amidination proceeds readily within these limits, it was possible to carry out the reactions under conditions mild enough to preserve the structure and activity of these two enzymes. Even at the highest levels of coupling, α-amylase retained virtually full activity while the activity of lysozyme was only slightly impaired. Apparently neither the reaction conditions nor the introduction of the monosaccharides caused gross changes in conformation necessary for enzymatic activities. While it has been shown that amino groups are not involved in the catalytic process of either enzyme, dinitrophenylation of α-amylase (Ikenaka, 1959) or acetylation of lysozyme (Davies and Neuberger, 1969) greatly reduces their activities, probably because of the loss of the ionizable amino groups and subsequent changes in conformation. On the other hand, when the amino groups of lysozyme are converted to the ionizable guanidino or amidino-derivatives, full activity is retained (Imoto et al., 1972). At low levels of coupling, a slight activation of the enzymes was observed. Amidination has been observed to slightly activate lysozyme (Davies and Neuberger, 1969) and strongly activate human and horse liver alcohol dehydrogenases (Fries et al., 1975; Browne and Kent, 1975a,b). The glycoside-amidinated derivatives of proteins made in this study were found to be stable in neutral and acidic buffers as expected from the known behavior of amidino compounds (Roger and Neilson, 1961) and can tolerate prolonged storage.

The techniques described in this report for coupling thioglycosides to proteins offer a number of advantages that will permit efficient and versatile use. Thioglycosides are more resistant to glycosidases and acid than the corresponding *O*-glycosides but can be hydrolyzed readily and specifically by mercuric salts in aqueous solution (Krantz and Lee, 1976). The susceptibility of the thio linkage to mercury salts made it possible to analyze for thioglycosides in the presence of *O*-glycosides. The IME-thioglycosides can be generated easily and in good yield from crystalline CNM-thioglycoside acetates, which can be readily prepared. These imidates can be stored in the reaction mixture for extended periods. Amidination is specific for amino groups and can be accomplished rapidly under conditions mild enough to preserve protein structure. The reaction conditions and the conversion of an amino to an amidino group did not impair the activities of the enzymes studied here, and are probably safe for many other enzymes.

The techniques described here can be used to investigate the contribution of sugars to the structure and activity of biologically active proteins such as hormones and enzymes. The effect of adding specific sugars on the binding of proteins to liver plasma membranes has been investigated using these reagents (Krantz et al., 1976). These reagents may also be useful for modifying whole cells in order to investigate the roles of sugars in such cell surface properties as antigenicity, lectin binding, and adhesion. The versatility of this reagent will make it a useful tool for understanding the role of sugars in complex carbohydrates.

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References

Ashwell, G. (1972), *Methods Enzymol. 28,* 219.
Bayliss, N. S., Heppolette, R. L., Little, L. H., and Miller, J. (1956), *J. Am. Chem. Soc. 78,* 1978.
Bernfeld, P. (1951), *Adv. Enzymol. 12,* 379.
Browne, D. T., and Kent, S. B. H. (1975a), *Biochem. Biophys. Res. Commun. 67,* 126.
Browne, D. T., and Kent, S. B. H. (1975b), *Biochem. Biophys. Res. Commun. 67,* 133.
Chipowsky, S., and Lee, Y. C. (1973), *Carbohydr. Res. 31,* 339.
Davies, R. C., and Neuberger, A. (1969), *Biochim. Biophys. Acta 178,* 306.
Eyler, E. H. (1965), *J. Theor. Biol. 10,* 89.
Fries, R. W., Bohlken, D. P., Blakely, R. T., and Plapps, B. U. (1975), *Biochemistry 14,* 5233.
Goebel, W. F., and Avery, O. T. (1929), *J. Exp. Med. 50,* 521.
Gray, G. (1974), *Arch. Biochem. Biophys. 163,* 426.
Hunter, M. J., and Ludwig, M. L. (1972), *Methods Enzymol. 25,* 585.
Ikenaka, T. (1959), *J. Biochem. (Tokyo) 46,* 177.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1972), *Enzymes 3rd Ed. 7,* 780.
Jeffrey, A. M., Zopf, D. A., and Ginsburg, V. (1975), *Biochem. Biophys. Res. Commun. 62,* 608.
Krantz, M. J., Holtzman, N. A., Stowell, C. P., and Lee, Y. C. (1976), *Biochemistry,* following paper in this issue.
Krantz, M. J., and Lee, Y. C. (1976), *Anal. Biochem. 71,* 318.
Lee, Y. C. (1972), *Methods Enzymol. 28,* 63.
Lemieux, R. U., Bundle, D. R., and Baker, D. A. (1975), *J. Am. Chem. Soc. 97,* 4076.
Maekawa, K., and Liener, I. E. (1960a), *Arch. Biochem. Biophys. 91,* 101.

Maekawa, K., and Liener, I. E. (1960b), *Arch. Biochem. Biophys. 91,* 108.
McBroom, C. R., Samanen, C. H., and Goldstein, I. J. (1972), *Methods Enzymol. 28,* 212.
McCoubrey, A., and Smith, M. H. (1966), *Biochem. Pharmacol. 15,* 1623.
McKelvy, J. F., and Lee, Y. C. (1969), *Arch. Biochem. Biophys. 132,* 99.
Moczar, E. (1973), *Experientia 29,* 1576.
Moczar, E., and Le Boul, J. (1975), *FEBS Lett. 50,* 300.
Okuyama, T., and Satake, K. (1960), *J. Biochem. (Tokyo) 47,* 454.
Roger, R., and Neilson, D. G. (1961), *Chem. Rev. 61,* 179.
Roseman, S. (1970), *Chem. Phys. Lipids 5,* 270.
Roth, S. (1973), *Q. Rev. Biol. 48,* 541.
Schaefer, F. C., and Peters, G. A. (1961), *J. Org. Chem. 26,* 412.
Shugar, D. (1952), *Biochim. Biophys. Acta 8,* 302.
Winterburn, P. J., and Phelps, C. F. (1972), *Nature (London) 236,* 147.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2,* 104.
Zamenof, S. (1957), *Methods Enzymol. 3,* 702.
Zull, J. E., and Chuang, J. (1975), *J. Biol. Chem. 250,* 1668.

Attachment of Thioglycosides to Proteins: Enhancement of Liver Membrane Binding†

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With Technical Assistance of Jerry W. Weiner and Hans H. Liu

ABSTRACT: Thioglycosides of D-galactose, D-glucose, *N*-acetyl-D-glucosamine, and D-mannose were covalently attached to *Aspergillus oryzae* α-amylase, hen's egg lysozyme, and bovine serum albumin by amidination, diazo coupling, and amide formation. The binding of the newly formed glycoproteins (neoglycoproteins) to rabbit liver membranes was measured, using asialoorosomucoid as a reference. Attachment of D-galactosides by any of the three methods enhanced binding by several orders of magnitude. Coupling of a comparable number of D-mannosides or *N*-acetyl-D-glucosaminides had little or no effect. Attachment of D-glucosides also enhanced binding but to a variable extent depending on the method of attachment. Thus, the behavior of neoglycoproteins toward rabbit liver membranes closely paralleled that of serum glycoproteins (Ashwell and Morell, 1974) with respect to sugar specificity.

Numerous hypotheses about the biological function of the carbohydrate groups in glycoconjugates have been advanced (Eyler, 1966; Roseman, 1970; Winterburn and Phelps, 1972; Roth, 1973). Although the relationship between carbohydrate structure and the biological behavior of glycoconjugates is not fully understood, there have been many examples in which subtle modifications of the carbohydrate structure of glycoconjugates result in drastic changes in their biological behavior (for examples, see Watkins, 1972; Ashwell and Morell, 1974; Moyle et al., 1975).

Since their initial observation that desialylated glycoproteins were rapidly removed from the circulation of the rabbit by the liver, Ashwell, Morell, and co-workers (see Ashwell and Morell, 1974) have extensively studied the characteristics of the clearance mechanism. They have demonstrated that the asialoglycoproteins are taken up and catabolized by liver parenchymal cells. Uptake of a glycoprotein is dependent upon the exposure of galactosyl residues by desialylation. Subsequent removal of the penultimate galactosyl residue thereby exposing *N*-acetylglucosaminide prevents its clearance. The same sugar specificity was observed for the clearance of exoglycosidase-treated antibodies (Winkelhake and Nicolson, 1976) and bovine serum albumin and lysozyme to which desialylated fetuin glycopeptides had been covalently attached (Rogers and Kornfeld, 1971). Asialoglycoproteins are bound to liver membranes in vitro by a saturable, calcium-dependent process which is sensitive to phospholipases A and C (Lunney and Ashwell, 1974) and neuraminidase. A glycoprotein which is responsible for the binding of desialylated serum glycoproteins has been isolated from liver membranes (Hudgin et al., 1974) and extensively characterized (Kawasaki and Ashwell, 1976).

We have approached the problem of the relationship between carbohydrate structure and biological behavior by attaching monosaccharides to proteins under controlled conditions. In this paper we report the results of attaching thioglycosides to three proteins by three methods including by amidination with 2-imino-2-methoxyethyl 1-thioglycosides, new reagents which we have described in the preceding paper (Lee et al., 1976). The abilities of the neoglycoproteins to bind to liver membranes were quantitatively evaluated using the inhibition binding assay of Van Lenten and Ashwell (1972), thereby enabling a comparison of the effects of incorporating varying amounts of four monosaccharides by different techniques.

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