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Quantitation of Oligosaccharides Released by the β-Elimination Reaction[1]

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A quantitative micromethod has been described for monitoring the rate and extent of the β-elimination reaction as applied to *O*-glycosyl-glycoproteins utilizing alkaline tritiated borohydride. The procedure simultaneously labels the released oligosaccharides by their reduction to the corresponding tritiated alditols. The alkaline tritiated borohydride treatment also results in the labeling of the protein moiety of the glycoprotein and this can be quantitatively separated from the carbohydrate moiety on a cation exchange resin; the carbohydrate moiety is not adsorbed, while the protein moiety is adsorbed and then eluted with HCl. The radioactivity in the aqueous eluate of the resin is therefore a direct measure of the amount of oligosaccharides released by the β-elimination reaction. The sensitivity of the method is dependent on the specific activity of the tritiated sodium borohydride used. The stoichiometry of the reaction has been established by the use of *N*-acetylgalactosaminyl-*O*-glycoproteins, demonstrating that at the completion of the β-elimination reaction: (a) none of the radioactivity attributable to the protein moiety contaminates the carbohydrate moiety, (b) all the carbohydrate components of the glycoprotein are found in the aqueous eluate from the cationic exchange resin, (c) all the radioactivity in this aqueous eluate is associated with the sugar known to be at the reducing end of the oligosaccharide chain bound to serine or threonine of the glycoprotein (in the examples discussed, *N*-acetylgalactosamine), and (d) there is no additional hydrolysis of the oligosaccharide chains during the processing.

Glycoproteins containing oligosaccharides linked glycosidically to the hydroxyl groups of serine and threonine residues occur widely in nature and perform a variety of biological functions (1). The alkaline β-elimination reaction is commonly used for the excision of the carbohydrate moieties from the protein of such glycoproteins. Treatment of these glycoproteins under alkaline conditions results in the cleavage of the glyco → peptide bond. Oligosaccharides are thereby obtained in which the sugar that had been linked to serine or threonine is at the reducing end of the oligosaccharide, while the serine or threonine, still in the peptide chain, are in the form of 2-aminoacrylic acid (dehydroalanine) and 2-aminobutenoic acid, respectively. If the alkaline treatment is performed in the presence of borohydride (2), the carbohydrate residue at the reducing end of the oligosaccharide is converted to the corresponding sugar alcohol as it is released, and the "peeling reaction" normally accompanying alkaline degradation of carbohydrates is thereby also arrested.

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As a consequence of some of our recent investigations (3,4) on the structure of the carbohydrate moiety of *O*-glycosyl-glycoproteins it became apparent that there is a need for a convenient method by which it would be possible to: (a) monitor the rate and extent of β-elimination, and (b) simultaneously label the carbohydrate components so that they may be identified, separated, and quantitated. This we have accomplished by performing the alkaline reaction in the presence of tritiated borohydride, thereby incorporating tritium into the C-1 position of the sugar alditols (5).

MATERIALS AND METHODS

Chemicals. Tritium-labeled potassium borohydride, in lots of 100 mCi, was dissolved in 20 ml of a solution of 2 M KBH_4 in 0.1 N KOH to give a final specific activity of 2.5 mCi/mmol. The tritiated borohydride was obtained from New England Nuclear Corporation and had a low "acid-stable" blank. The problems associated with high "acid-stable" blank of tritiated borohydride have been discussed in a previous paper (5).

The following materials were obtained from commercial sources: *N*-acetyl-D-galactosamine (GalNAc)[5] D-galactosamine hydrochloride, ribonuclease A (Sigma Chemical Co.), D-galactose (Pfanstiehl Laboratories, Inc.), *N*-acetylneuraminic acid (Koch–Light Laboratories Ltd.), and Dextran blue 2000 (Pharmacia). Sugar alditols were prepared under the conditions of the assay procedure described below.

Glycoproteins. The glycoproteins used in these studies were obtained from a number of sources. Crude hog gastric mucin was purchased from Wilson Company (Chicago, Ill.). It consists of mucin derived from a pool of hog stomachs with blood group A and H specificities. Fresh ovine submaxillary glands were obtained commercially (Pelfreez). Fresh hog submaxillary glands were obtained from a local slaughterhouse and serologically typed A^+, H^+, or A^-H^- (inactive) (6). These mucins were purified as previously described (7). Ovine submaxillary asialo mucin (a-OSM) was prepared by controlled acid hydrolysis (0.1 N HCl at 80°C for 45 min) (8). Glycoproteins from edible bird's nest (*Collacalia*) as well as from colostrum were prepared as previously described (8).

Ion-exchange resins. The cation-exchange resin Bio-Rad AG 50W-X8 (200–400 mesh, H^+) and Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories) were used in these experiments. The cation-exchange resin was contained in a small plastic disposable tube sold under the name of Quik-Sep (IsoLab Inc.).

Scintillation mixture. The scintillation mixture was prepared by dissolving 18 g of 2,5-diphenyl oxazole (PPO) in a solution consisting of 2.0 liters of toluene and 1.0 liter of Triton X-100. Radioactivity was measured with a Packard Model C2425 liquid scintillation spectrometer.

General methods. The carbohydrate composition of the glycoproteins was determined as follows: Total hexose was determined by the anthrone method (9), total sialic acid by a modification of the Svennerholm procedure (10) with 1-butanol replacing isoamyl alcohol for the development of color (11). Free sialic acid was measured by the thiobarbituric acid method (8). In both the resorcinol and thiobarbituric acid assays, NeuNAc was used as the standard and the appropriate correction was applied to allow for the difference in the molecular extinction coefficients for NeuNAc and NeuNGc (8). Total hexosamine was

[5] Abbreviations used: GalNAc, *N*-acetyl-D-galactosamine; Gal, D-galactose; NeuNAc, *N*-acetylneuraminic acid; NeuNGc, *N*-glycolyl neuraminic acid; GalNActitol, *N*-acetylgalactosaminitol; hog submaxillary mucins with A, H or In (A^-H^-) reactivity are A^+PSM, H^+PSM, and A^-H^-PSM, respectively; ovine submaxillary glycoprotein and its desialated derivative are OSM and a-OSM, respectively; GM, hog gastric mucin; TBA, thiobarbituric acid assay (8); TPTZ, 2, 4, 6–Tripyridyl-*s*-triazine.

determined by a modification of the Elson–Morgan procedure after acid hydrolysis (12,13).

Chromatography was performed on Schleicher and Schuell No. 589 green ribbon paper using the solvent 1-butanol:pyridine:water (6:4:3, v/v/v). The papers were irrigated in descending fashion for 16 h. The sugars or oligosaccharides were detected with the $AgNO_3$–NaOH method following a periodate dip (14). Amino acids were detected by spraying the chromatogram with 0.5% ninhydrin in acetone (15). When tritium-labeled alditols were involved, the location of radioactivity on the chromatogram was determined by cutting the paper into 2 × 5-cm strips and counting each strip separately in 10 ml of the scintillation mixture. Sugars and oligosaccharides were also separated by passage through a column (1.5 × 90 cm) of Bio-Gel P-2, collecting fractions of 1.5 ml. The elution pattern of carbohydrate material was determined colorimetrically by the TPTZ procedure (16). An aliquot of each fraction, 100 μl, was added to a standard amount of periodate, and after a 1-h incubation at 37°C, the excess periodate was determined with TPTZ. The elution pattern of tritium-labeled sugar or oligosaccharide alditols was also determined by counting a 50-μl aliquot of each fraction in 10 ml of scintillation mixture.

Reductive equivalent for N-*acetylgalactosamine*. The reductive equivalent of tritiated borohydride with reference to reducing sugars, free or as in oligosaccharides, was determined as previously described (5), but at 45°C. Aliquots up to 1.0 μmol of GalNAc were incubated at 45°C for 24 h with 10 μl of the alkaline [^{3}H]-borohydride reagent (2.5 mCi/mmol) in a final volume of 100 μl. The mixtures were then cooled in an ice bath, and titrated to pH 4.5 with 4 M acetic acid. Each sample was taken to dryness and redissolved in water and this was repeated several times. The final dried product was dissolved in 200 μl of water, and a 50-μl aliquot was added to 10 ml of scintillation mixture and counted.

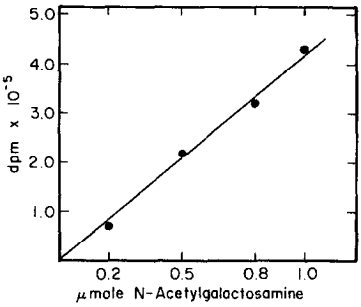

FIG. 1. Determination of the reductive equivalent of GalNAc. GalNAc (0–1 μmol), contained in a final volume of 100 μl, was incubated at 45°C for 24 h with 10 μl of the alkaline [^{3}H]borohydride solution. See text for experimental details. The reductive equivalent was calculated from the slope.

A typical calibration curve is shown in Fig. 1 from which the reductive equivalent of GalNAc can be readily calculated.

Assay procedure. A 0.25-ml aliquot of a 0.5% aqueous solution of the glycoprotein (e.g., OSM) was added to 0.25 ml of 2.0 M potassium [^{3}H]borohydride (specific activity 2.5 mCi/mmol) in 0.1 N potassium hydroxide. An appropriate reagent blank was prepared simultaneously with water replacing the solution of glycoprotein. After incubation for 24 h at 45°C, the reaction mixtures were cooled in an ice bath and titrated to pH 4.5 with 4.0 M acetic acid in order to decompose the excess [^{3}H]borohydride. The mixtures were dried and dissolved in 50% methanol and redried using a flash evaporator. This procedure was repeated several times to remove excess borate as its volatile methyl ester derivative (17). The final residues were dried overnight *in vacuo* in a desiccator over NaOH/H_2SO_4. They were then redissolved in 0.5 ml of distilled water and a 50-μl aliquot was used to determine the total radioactivity in the incubation mixtures. Another aliquot, 0.4 ml, was applied to a column containing 3 ml of the cation-exchange resin. Elution was with 20 ml of distilled water, followed by 20 ml of 2.0 N hydrochloric acid. Both the aqueous and acid eluates were evaporated to dryness and the residues were redissolved in water and redried using a flash

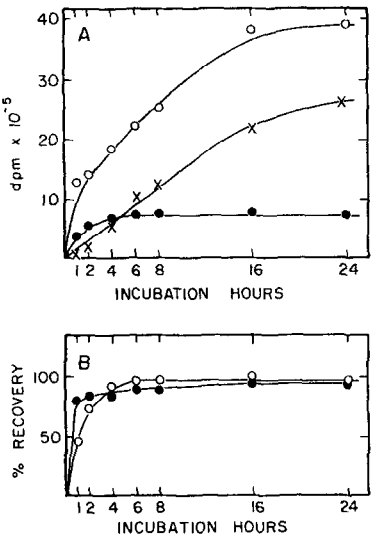

FIG. 2. Incorporation of tritium into the β-elimination products of OSM. Samples containing 0.25 ml of 0.5% OSM were incubated with 0.25 ml of the alkaline [^{3}H]borohydride solution at 45°C for various periods of time. (A) The total radioactivity of the incubation mixture (○) and the amount of radioactivity incorporated into the aqueous eluate (●) and the acid eluate (×) were monitored. (B) The percentage recovery of NeuNAc (●) and hexosamine as *N*-[^{3}H]acetylgalactosaminitol (○) was determined for the aqueous eluates.

evaporator. This was repeated several times to completely remove all volatile tritium. The final residues were each redissolved in 1 ml of distilled water. A 50-μl aliquot from each pool of the aqueous and acid eluates from the cation-exchange resin was used for quantitation of radioactivity. Furthermore, each was also analyzed for its carbohydrate content. Great care should be exercised at each of the flash evaporation steps to remove all of the volatile tritium present.

RESULTS

Incorporation of Tritium into the Products of the β-Elimination Reaction

The reduction of proteins by alkaline borohydride has been previously reported (18). Under the conditions specified above we were able to separate the radioactively labeled amino acids and peptides (18) from the labeled oligosaccharides with a cation exchange resin.

Figure 2A shows the rate and extent of the β-elimination reaction as applied to OSM, which consists predominantly of disaccharide chains of NeuNAc and GalNAc linked to the protein core (19). Inspection of this figure shows that while the total radioactivity and the amount of radioactivity in the acid eluate is continually rising throughout the 24-h period, radioactivity in the aqueous eluate reaches a maximum in approximately 6 to 8 h. At this point, the β-elimination reaction is considered to be complete, i.e., all of the *O*-glycosidically linked oligosaccharide chains have been cleaved from the glycoprotein and have been converted by the [^{3}H]borohydride to their corresponding tritium-labeled oligosaccharide alditols. The radioactivity incorporated into the acid eluate, and therefore the incubation mixture as a whole, continues to rise throughout the time interval, presumably due to the degradation and tritium labeling of the protein moiety of the glycoprotein. Similar results were obtained with A^+ porcine submaxillary mucin, which contains more complex oligosaccharide chains (4).

Effective Removal of Protein and Its Degradation Products by Cation-Exchange Resin

Figure 3 shows the effectiveness of separation of the β-elimination products of OSM into two distinct peaks when adsorbed at pH 4 and eluted from the cation-exchange resin with water and 2 N HCl. Upon chromatographic analysis of β-elimination products in the aqueous eluate, GalNActitol and oligosaccharide alditols were readily detected with the $AgNO_3$–NaOH staining technique, but no amino acids or peptides could be detected with ninhydrin, indicating the presence of carbohydrate material only. In turn, the acid eluate gave positive results for peptides and amino acids and negative results for carbohydrate material. Further-

more, when ribonuclease, a carbohydrate-free protein, was treated with alkaline [^{3}H]borohydride, the labeled degradation products were completely adsorbed onto the cation-exchange resin and all the radioactivity was recovered in the acid eluate, as would be expected. The sharp separation of carbohydrate and protein moieties was found to be effective for all *O*-glycosyl-glycoproteins examined. It was not, however, as effective with *N*-glycosyl-glycoproteins or with phosphoproteins. We are presently working on procedures to circumvent the problems posed by these classes of conjugated proteins.

Effect of Temperature

The extent of the incorporation of tritium into the "aqueous eluate" of OSM was investigated at 25, 35, and 45°C, results of which are presented in Fig. 4. Since the incorporation of tritium into the reducing end of the oligosaccharide or monosaccharide is not the limiting factor (5), it is apparent that the higher temperatures are necessary for the rapid and complete cleavage of the oligosaccharides from the glycoprotein.

Effect of Reagents Used to Arrest the β-Elimination Reaction

Lowering the pH by addition of acid results in the decomposition of the borohydride as well as arresting the β-elimination reaction. However, since many of the oligosaccharides that we are interested in contain sialic acid and fucose, care has to be exercised not to release these during the concentration and drying processes when dealing with solutions of low pH. The following reagents were therefore tested for their effectiveness: acetone, 2 N HCl, and 4 M acetic acid.

In the first test, in which the remaining

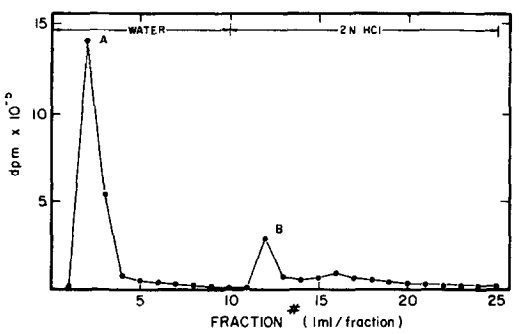

FIG. 3. Elution pattern of OSM β-elimination products adsorbed at pH 4. The β-elimination products of OSM, as determined by radioactivity, were eluted from the cation-exchange column with (A) water and (B) 2 N HCl.

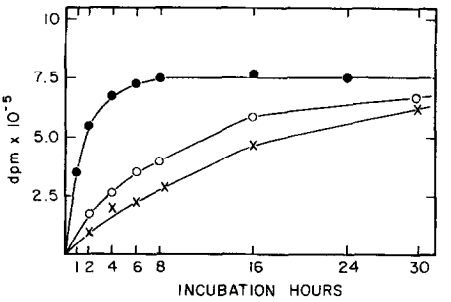

FIG. 4. The effect of temperature on the rate of incorporation of tritium into the aqueous eluate of OSM. Samples containing 0.25 ml of 0.5% solution of OSM were incubated with 0.25 ml of the alkaline [^{3}H]borohydride solution for various time intervals at 25°C (×), 35°C (○), and 45°C (●) and the respective aqueous eluates were counted.

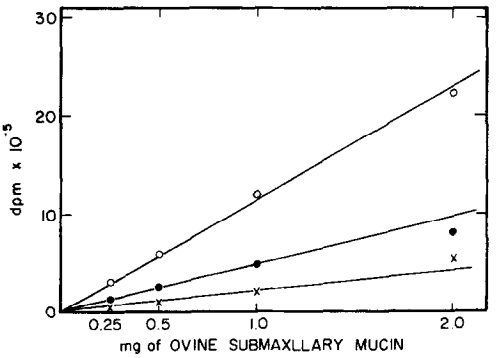

FIG. 5. The effect of concentration of OSM on the extent of incorporation of tritium. Samples containing 0 to 2.0 mg of OSM and 0.25 ml of the alkaline [^{3}H]borohydride solution in a final volume of 0.5 ml were incubated for 16 h at 45°C, and the total radioactivity of the incubation mixtures (○) and the amount of radioactivity incorporated into the aqueous (●) and acid (×) eluates of the cation-exchange column were measured.

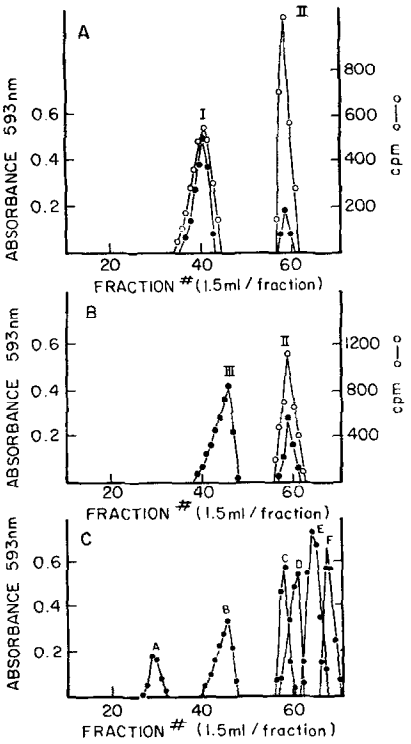

FIG. 6. Elution pattern of OSM oligosaccharide alditols from Bio-Gel P-2 column. (A) The elution of oligosaccharide alditols of OSM, obtained from the water eluate of the cation-exchange column and monitored by the periodate–TPTZ assay (●) and by radioactivity (○). (B) The elution pattern of tubes 35–45 from Fig. 6A after pooling and acid hydrolysis (0.1 N HCl at 80°C for 45 min) monitored by the periodate–TPTZ assay (●) and by radioactivity (○). (C) Elution pattern of standard sugars from Bio-Gel P-2 column monitored by the periodate–TPTZ assay; A = dextran blue, read at 280 nm, B = NeuNAc, C = GalNActitol, D = GalNAc, E = Galactitol, and F = Gal.

radioactivity was measured after the addition of each of the possible arresting reagents in excess, it was found that acetone left a substantially higher blank than hydrochloric acid or acetic acid. Acetone was thus eliminated as a possible reagent to stop the reaction. In the second test, OSM was incubated for 16 h and the reaction was stopped by titrating the solutions to pH values from 3 to 6 using either HCl or acetic acid. The measurement of free sialic acid in each sample as determined by TBA (8) showed that all incubations stopped with HCl, and also those stopped with acetic acid at pH 4.0 or less, contained free sialic acid. Thus, to minimize the "acid-stable" blank and cleavage of sialyl residues, all β-elimination reactions were titrated to pH 4.5 with 4 M acetic acid.

Effect of Concentration of Glycoprotein on the Incorporation of Tritium

Various amounts of OSM, ranging from 0 to 2.0 mg, were subjected to the alkaline [^{3}H]borohydride treatment. After an incubation period of 16 h at 45°C, the total radioactivity incorporated into the aqueous and acid eluates were measured. As shown in Fig. 5, there is a linear relationship between the concentration of glycoprotein and the extent of tritium incorporation.

Identification of the Labeled Sugar as N-[^{3}H]Acetylgalactosaminitol

Oligosaccharide alditols, obtained from OSM by the assay procedure outlined, were applied to a Bio-Gel P-2 column and eluted with water, resulting in the elution pattern presented in Fig. 6A. Two sharp peaks were obtained, I and II, that were detectable by the periodate–TPTZ assay, as well as by their radioactivity. Peak I, moreover, gave the reactions for sialic acid, and upon mild acid hydrolysis (0.1 N HCl, 80°C, 45 min) and reapplication to the Bio-Gel P-2 column resulted in two peaks, II and III, as presented in Fig. 6B. Peak III, both by its color reaction and position in the elution profile, corresponds to NeuNAc (Fig. 6C). This peak was not radioactive. Peak II, Figs. 6A and B, on the other hand, corresponds in position to and reacts like *N*-acetylgalactosaminitol. Peak II was radioactive and, as would be expected, represents the reducing end of the disaccharide alditol, peak I (Fig. 6A), corresponding to NeuNAc → GalNActitol.

A second experiment was also performed on the same oligosaccharide alditols obtained from OSM. After acid hydrolysis

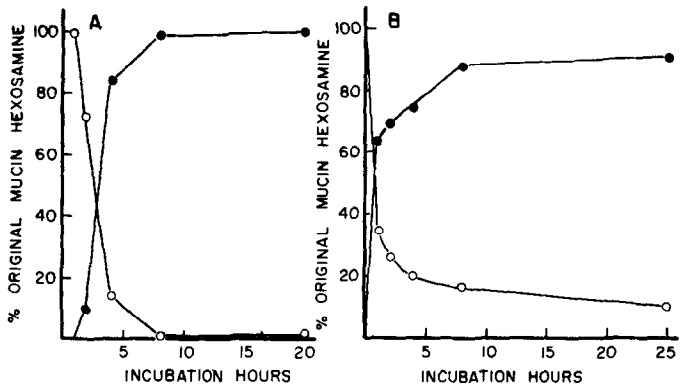

FIG. 7. Relationship between the loss of hexosamine and incorporation of tritium into the aqueous eluate. (A) Samples containing 0.5 ml of 0.5% a-OSM and 0.5 ml of the alkaline [^{3}H]borohydride solution were incubated at 45°C for various time intervals. The percentage of hexosamine (○) recovered in the incubation mixture was monitored by the Elson–Morgan procedure, and the percentage *N*-[^{3}H]acetylgalactosaminitol (●) recovered was calculated from the amount of radioactivity found in the aqueous eluate. See text for experimental details. (B) The identical procedure was performed on OSM.

(0.5 N HCl, 100°C, 4 h), the end products were separated by paper chromatography. Since GalNAc is deacetylated to galactosamine under the conditions of the hydrolysis, [^{3}H]-galactosaminitol was used as a standard for comparative purposes. The results indicate that only one component of the hydrolysate contained tritium, and it migrated exactly as authentic [^{3}H]galactosaminitol.

Stoichiometry of Loss of Hexosamine and Formation of Radioactive Alditol

The rate and extent of the β-elimination reaction was followed by correlating the loss of hexosamine with the appearance of radioactivity in the aqueous eluate. Moreover, calculating the amount of GalNActitol present from the reductive equivalent of GalNAc demonstrated the stoichiometry of the reaction. The results, presented in Fig. 7, show that the two parameters are equivalent, indicating that the rate of incorporation of radioactivity in the "aqueous eluate" is a reliable indicator of the rate of the β-elimination reactions and an accurate estimate of the number of *O*-glycosyl-oligosaccharides released.

Quantitative Recovery of Carbohydrate Components in Aqueous Eluates

Each of the aqueous eluates previously discussed in Fig. 2A were analyzed for their sugar content and also for the amount of *N*-[^{3}H]acetylgalactosaminitol as calculated from the reductive equivalent for GalNAc. The results in Fig. 2B indicate that nearly 100% of all the monosaccharides have been recovered in the aqueous eluate.

To demonstrate the usefulness of this assay, several *O*-glycosyl-glycoproteins were incubated with the alkaline [^{3}H]-borohydride for 6 and 24 h, and the amount of radioactivity present in the aqueous eluate, as well as the percentage recovery of the different carbohydrate components in the fraction, was calculated. The results, presented in Table 1, clearly indicate the quantitative recovery of the carbohydrate components present in the original glycoprotein and the versatility of the reaction. The low percentage conversion of hexosamine of A^+ PSM, GM, and *Collocalia* glycoproteins to the corresponding radioactive alditols in comparison to the generally good recovery of other carbohydrate components in the aqueous eluate is attributable

TABLE 1

QUANTITATIVE ANALYSIS OF AQUEOUS ELUATES OBTAINED FROM VARIOUS GLYCOPROTEINS AFTER β-ELIMINATION

Sample	Hours	dpm in aqueous eluate (10^{-5})	Aqueous eluate (%μmol)			Oligosaccharide released (μmol)
			GalNActitol[a]	Sialic acid	Hexose	
OSM	6	3.2	77	89	—	0.46
	24	3.9	93	100	—	0.56
a-OSM	6	0.9	45	—	—	0.12
	24	2.1	103	—	—	0.28
A^+PSM	6	6.0	79	94	94	0.87
	24	6.0	79	97	98	0.87
H^+PSM	6	2.5	75	71	85	0.36
	24	3.3	99	91	100	0.48
A^-H^-PSM	6	3.5	83	93	80	0.50
	24	4.1	98	100	89	0.59
GM	6	1.5	16	—	72	0.22
	24	2.7	29	—	85	0.39
Milk glycoprotein	6	1.4	72	84	85	0.20
	24	1.8	93	100	100	0.26
Collacalia glycoprotein	6	1.8	34	85	80	0.26
	24	2.8	53	94	95	0.40

[a] Calculated from the reductive equivalent for GalNAc and expressed as the percentage of total hexosamine.

to the presence of hexosamine residues in the middle of the chain and/or at the nonreducing end of the oligosaccharide chains (4,20,21).

Precision and Accuracy of Assay

Five identical aliquots of four different glycoproteins were analyzed following the standard assay procedure. The precision and accuracy are demonstrated by comparing the results obtained by this assay to the expected results based on the amount of GalNAc present in each glycoprotein (Table 2). The mono- and disaccharides of OSM were further separated from each other by descending paper chromatography, allowing for the quantitation of each individual oligosaccharide (Table 2).

DISCUSSION

We have used tritiated borohydride under alkaline conditions to cleave the oligosaccharides from the protein in several glycoproteins and have found that this procedure can simultaneously resolve many problems associated with the β-elimination reaction. Thus, this method can be applied: (a) to monitor the kinetics of β-elimination reactions, (b) to simultaneously label and quantitate the reduced oligosaccharides, and (c) to quantitate *O*-glycosyl-glycoproteins over a wide range, its applicability to a microscale depending on the specific activity of the tritiated borohydride used.

Sodium borohydride is a very versatile reducing agent, effective not only in the conversion of reducing sugars to alditols, but also by reducing sulfide linkage in proteins (18). We have shown that it is possible to effectively separate the radioactively labeled sugar alditols from the protein degradation products by the use of a cation exchange resin. The neutral and acidic carbohydrate residues are unadsorbed by the resin and appear in the aqueous eluate, while the amino acid and peptide residues are completely adsorbed by the resin and are readily eluted with 2 N HCl.

From the kinetics of the various reac-

tions, it is apparent that at 45°C the conversion of reducing sugars and oligosaccharides to their corresponding alditols is extremely rapid, a matter of a few minutes. The rate of β-elimination is much slower, a matter of hours, and is therefore the rate-limiting reaction in the appearance of radioactivity in the aqueous eluate. The completion of cleavage is indicated by the plateau attained in the rate curves. The continuous and extensive incorporation of radioactivity into the protein moiety is shown by the rate curves of the acid eluate and the results suggest that this reaction is the slowest of the three reactions at 45°C.

The rate of incorporation of tritium was found to vary from glycoprotein to glycoprotein. Thus, following the rate of incorporation of radioactivity into the aqueous eluate it is possible to determine the time actually required for completion of the β-elimination reaction. The observed difference in the rate of incorporation of tritium into the carbohydrate moiety of ovine (8 h) and porcine submaxillary (4 h) mucins can be attributed to a number of factors, such as: (a) the proportion of serine to threonine residues that are glycosylated, (6), (b) length of oligosaccharide chain, and (c) presence and proximity of sialyl residues to the carbohydrate–protein linkage area (cf. the analogous difference in stability of hexosamine oligosaccharides to acid hydrolysis depending on the state of acylation of the amino group of the hexosamine (22)). The data for OSM and a-OSM (Table 1) would appear to support this deduction.

The radioactivity in the aqueous eluate fraction derived from the β-elimination products of the glycoproteins (OSM, a-OSM, and A^+PSM) was found to be associated with the *N*-acetylgalactosaminitol moiety of the oligosaccharides. The loss of total hexosamine was found to be directly proportional to the amount of radioactivity in the aqueous eluate fraction. This radioactivity translated to *N*-acetylgalactosaminitol from the reductive equivalent of the tritiated borohydride was found to be stoichiometrically related to the hexosamine lost. Further confirmation of the stoichiometry was afforded by the observation that all the radioactivity was associated with only one sugar, *N*-acetylgalactosaminitol by gel filtration, using columns of Bio-Gel P-2, and to galactosaminitol by paper chromatographic analysis of the products of acid hydrolysis.

TABLE 2

PRECISION AND ACCURACY OF ASSAY

Glycoprotein	Expected amount of oligosaccharides[a] (μmol)	Experimental results[b] (μmol)
a-OSM	0.31	0.30 ± 0.04
OSM		
Monosaccharide	0.16	0.15 ± 0.02
Disaccharide	0.50	0.50 ± 0.03
Total	0.66	0.65 ± 0.035
Asialo-A^-H^-PSM	0.44	0.43 ± 0.02
A^-H^-PSM	0.64	0.66 ± 0.02

[a] Based on total hexosamine and sialic acid assays.
[b] Mean ± SD for five determinations.

The method described here satisfies the following criteria, desirable for an analytical procedure for the quantitation of *O*-glycosyl-glycoproteins in milli- and microgram amounts:

(i) The radioactivity in the aqueous fraction obtained from the cation-exchange resin is equal to the loss of hexosamine.

(ii) No radioactivity attributable to extraneous protein degradation products is present in the aqueous eluate fraction.

(iii) Quantitative recovery of the carbohydrate components of the *O*-glycosyl-glycoprotein in the aqueous eluate fraction.

(iv) There is no free sialic acid in the aqueous eluate, indicating that the oligosaccharides retain their native structure with no loss of labile sugars during the β-elimination reaction and subsequent processing of the material for analysis.

(v) All the radioactivity is identifiable with the one sugar, known from previous work to be at the reducing end of the

oligosaccharide chains. In the case of the *O*-glycosyl-glycoproteins it is *N*-acetyl-galactosamine.

(vi) The results indicate that the analytical procedure is reproducible at the level of total carbohydrate released as well as that of the individual oligosaccharides after separation.

This assay procedure should also be applicable to other *O*-glycosyl-glycoproteins involving galactosyl, xylosyl, and mannosyl residues linked to the hydroxyamino acids serine and threonine in the peptide linkage. The method could thus differentiate these types of *O*-glycosyl linkages from that of collagen and basement membranes which involve alkali-stable galactosyl residues linked to hydroxylysine residues of the proteins (23).

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