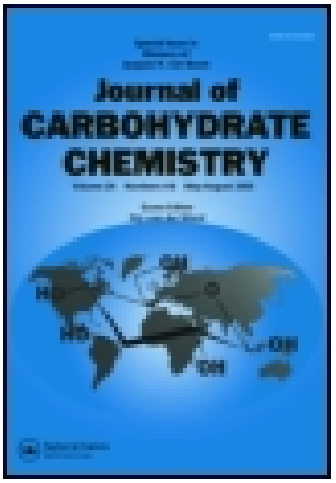

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IS SULFATE LOST DURING THE CHEMICAL RELEASE OF OLIGOSACCHARIDES FROM GLYCOPROTEINS?

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ABSTRACT

Model experiments using methyl α-D-galactopyranoside 6-sulfate have implied that the Carlson conditions (1 M sodium borohydride and 0.05 M sodium hydroxide at 45 °C) for the release of *O*-linked oligosaccharides from glycoproteins do not cause significant loss of sulfate ester groups. However the more vigorous conditions used to release *N*-linked oligosaccharides are more likely to cause considerable sulfate loss from a galactoside 6-sulfate as a result of 3,6-anhydride formation. Experiments with dextran sulfate suggested that sulfate loss due to oxirane formation was also not significant under the Carlson conditions. These results were supported by experiments with [^{35}S]sulfate-labelled human rectal mucus glycoprotein.

INTRODUCTION

The characterisation of the oligosaccharide moieties of glycoproteins requires their release from the protein by chemical or enzymatic means. Chemical methods, in particular the alkaline borohydride conditions of Carlson,[1] are the key methods for the release of *O*-linked oligosaccharides in view of the limitations of enzymic methods, especially for mucus glycoproteins. Sulfate groups have been recognised as important constituents of certain

glycoproteins in recent years[2-13] and it is clearly important that methods used to release and isolate oligosaccharides do not cause any loss of sulfate. Since the widely used alkaline borohydride method can, in principle, cause sulfate loss when a hydroxyl group is favourably located to form a cyclic anhydride by nucleophilic displacement of sulfate, it was considered desirable to ensure that no sulfate loss occurred during the use of this method.

Sulfate loss with concomitant anhydride formation has been shown[14,15] to occur under alkaline conditions for hexopyranose 6-sulfates, which form 3,6-anhydrides, and for pyranose 2-sulfates (or 3-sulfates or 4-sulfates) with neighbouring *trans*-related hydroxyl groups, which form oxiranes. In most of the structural studies of glycoprotein oligosaccharides sulfate groups were found at C-6 of D-galactose and/or 2-acetamido-2-deoxy-D-glucose units;[4,5,12,13] there are a few reports of sulfate groups at C-3 of D-galactose, and C-3 and C-4 of 2-acetamido-2-deoxy-D-glucose units.[6,8] 3,6-Anhydride formation under alkaline conditions has been used to establish the position of sulfate groups at C-6 of galactose residues in mucus glycoproteins.[16] Methyl α-D-galactopyranoside 6-sulfate was chosen as a model compound with which to study the extent of sulfate loss under the alkaline conditions used to isolate *O*-linked and *N*-linked oligosaccharide. Dextran sulfate was used to study the extent of loss of sulfate resulting from oxirane formation, and finally a [^{35}S]sulfate-labelled glycoprotein was used in a control experiment that more closely represented a glycoprotein digestion.[17]

RESULTS

Methyl α-D-galactopyranoside 6-sulfate, was synthesised from methyl 2,3,4-tri-*O*-benzyl-6-*O*-trityl-α-D-galactopyranoside by sequential detritylation, sulfation and debenzylation. The proton 250 MHz NMR spectrum of a D_2O solution of the galactoside sulfate was highly second order, and this spectrum was identical to the simulated spectrum obtained using data taken from the 500 MHz spectrum.[18] A spectrum amenable to first order analysis was obtained with a d_5-pyridine solution containing 5% D_2O. The standard conditions used to release *O*-linked oligosaccharides are 0.05 M sodium hydroxide and 1 M sodium borohydride at 45 °C for 15 hours.[1] A recent study showed that increasing the reaction time to 30 hours improved the recovery of released oligosaccharides from gastric mucus glycoproteins.[19] Therefore the galactoside 6-sulfate was exposed to the latter conditions except that the sodium borohydride was omitted to simplify the analysis. The 3,4-borate complex of the galactoside that could form (reversibly) in the presence of

borohydride would not be expected to interfere with 3,6-anhydride formation, which is an irreversible reaction. Aliquots of the reaction mixture were neutralised with hydrochloric acid and analysed by HPLC for the presence of methyl 3,6-anhydro-α-D-galactopyranoside using 1-propanol as an internal standard. The amount of anhydride detected was <2% and there was a trace of methyl α-D-galactopyranoside.

The more vigorous conditions that have been used[20] to release *N*-linked oligosaccharides involve a higher temperature (100 °C) and more concentrated (1 M) NaOH. Under these conditions methyl 3,6-anhydro-α-D-galactopyranoside was formed in 86% yield and a trace of methyl α-D-galactopyranoside was also detected.

Dextran sulfate (average MW 5000) was used to check for sulfate loss resulting from oxirane formation. In this case the sulfate content was determined before and after exposure to the alkaline conditions. Any released sulfate was to be separated from the dextran sulfate by gel permeation chromatography, so the dextran sulfate was first purified by this method (Fig.). Exposure of the purified dextran sulfate (with sulfate content of 7.6%) to 0.05 M sodium hydroxide and 1 M sodium borohydride at 45 °C for 30 hours resulted in the loss of 15% of the sulfate. A control experiment however established that sulfate loss occurred during the work-up used to remove borate. Modification of the work-up to avoid exposure to acid conditions reduced the sulfate loss to less than 3%, and a separate experiment demonstrated that this small loss resulted from the work-up and not during the alkaline digestion.

A further check on sulfate loss was made using [^{35}S]sulfate labelled human rectal mucus glycoprotein prepared by incubating biopsy samples with sodium [^{35}S]sulfate and 2-amino-2-deoxy-D-[6-^{3}H]glucose. The labelled glycoprotein was isolated, exposed to Carlson conditions, and the released oligosaccharides were isolated by gel-permeation chromatography. In six similar experiments less than 1% of the original mucus bound [^{35}S]-label eluted as free sulfate.

DISCUSSION

3,6-Anhydride formation from a glucopyranose or galactopyranose derivative involves the formation of the unfavourable $^{1}C_{4}$ chair (or a skew boat) conformation and this is less demanding for a galactoside (one fewer axial hydroxyl group) than a glucoside. Thus a galactoside 6-sulfate was adjudged to be a more sensitive probe for 3,6-anhydride formation in the glycoprotein context.

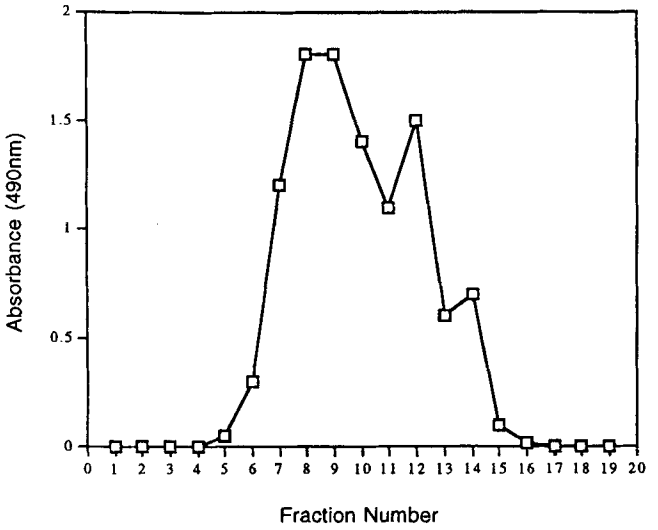

Figure Chromatography of dextran sulfate on BioGel P2; fractions 5-7 were pooled. Sodium sulfate eluted in fractions 10-14 in a separate experiment.

The results of the experiments with methyl α-D-galactopyranoside 6-sulfate show that the strongly alkaline conditions (1 M NaOH at 100 ^{0}C), which resemble those used to release *N*-linked oligosaccharides, would cause substantial sulfate loss from galactopyranose units with sulfate groups at C-6 and a free hydroxyl group at C-3. Thus, provided the sodium borohydride, which is an additional constituent of the digestion mixture, does not have a stabilising effect on sulfated monosaccharide units, such vigorous conditions should not be used to release *N*-linked oligosaccharides that contain sulfate. It is possible that internal and external galactose units would react at different rates since the conformational change required for cyclisation would require more extensive reorientation of the oligosaccharide chain for an internal unit than would be the case for a terminal unit.

The less vigorous conditions, which resembled those used for the release of *O*-linked oligosaccharides, caused very little loss of sulfate from methyl α-D-galactopyranoside 6-sulfate. This implies that 3,6-anhydride formation should not be a problem assuming that the sodium borohydride constituent of Carlson digests does not enhance anhydride formation. This leaves the possibility of sulfate loss via oxirane formation to consider. The dextran sulfate used for the model experiment had a low sulfate

content (7.6%) and the sulfate groups are likely to be present on either C-2 or C-3 or C-4 of one seventh of the glucose units. The ^{13}C NMR spectrum of the dextran sulfate indicated that most of the sulfate groups were on positions 2 (C-1 at d 96.9, C-2 at 79.8) and position 3 (C-3 at 82.3) in separate glucose residues. The ease of oxirane formation is likely to vary with the position of the sulfate group and a thorough study requires that all possible isomers of the monosulfates of the monosaccharide constituents of glycoproteins be examined. An alternative approach involves the study of the loss of sulfate from the glycoprotein of interest. This can be conveniently done when it is possible to isolate radiolabelled material.

In such an experiment the mucus glycoproteins in human rectal biopsies were labelled with [^{35}S]sulfate and this provided a simple method for the assay of loss of sulfate (as inorganic sulfate). The significance of the absence of sulfate loss must await the characterisation of the oligosaccharides. If the sulfate groups are found at C-3 of galactose units, as for meconium mucus glycoprotein[8] and human bronchial mucus glycoprotein,[2] and the hydroxyl group at C-2 is free this would indicate that oxirane formation in such a structure was slow under the Carlson conditions.

CONCLUSION

The mild Carlson-like alkaline borohydride conditions used to release *O*-linked oligosaccharides from GPs are not likely to cause serious loss of sulfate ester groups. However the more vigorous conditions (higher temperatures and more concentrated alkali) used to release *N*-linked oligosaccharide may cause substantial loss of sulfate.

EXPERIMENTAL

General methods. Melting points were uncorrected. NMR spectra were measured on Bruker WM 250 and WHP 400 spectrometers with sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate (TSP) as the reference for solutions in D_2O. Thin layer chromatography (TLC) was carried out with plastic plates coated with silica gel. HPLC was carried out on a reverse phase (ODS, 5 mm Spherisorb) column (0.49 x 25 cm) with a refractive index (RI) detector. Standard mixtures of 1-propanol (internal standard) and methyl 3,6-anhydro-α-D-galactopyranoside were used to calibrate the RI detector and water was used as eluent. Gel-permeation chromatography was carried out on a column (34

x 2.5 cm) of Bio-Gel P-2, calibrated with blue dextran and sodium sulfate, eluted with 0.1 M acetic acid. Carbohydrate was located by the phenol-sulfuric acid method.[21] Sulfate was assayed by the barium rhodizonate method[22] which gave a linear calibration for the range 0-100 nmoles of sulfate.

Methyl α-D-Galactopyranoside-6-(potassium sulfate). A solution of chlorosulfonic acid (0.574 g, 4.9 mmol) in alcohol-free chloroform (5 mL) was added dropwise to a stirred solution of methyl 2,3,6-tri-*O*-benzyl-α-D-galactopyranoside[23] (1.142 g, 2.5 mmol) in anhydrous pyridine (60 mL) at -15 to -18 °C. The reaction mixture was allowed to warm to room temperature and the addition of chlorosulfonic acid (with cooling) was repeated three times. Water was then added and the solution evaporated under reduced pressure, with periodic additions of water to aid the removal of pyridine. The residue was chromatographed on a silica gel column with 2:1 toluene:methanol as eluent. The appropriate fractions were pooled and concentrated to give the product (1.142 g, 74%), R_f (2:1 toluene:methanol) 0.31. A portion (0.22 g, 0.4 mmol) of the product was dissolved in methanol (10 mL) and hydrogenated over Pd/C catalyst (50 mg) for 48 h. A further 50 mg of catalyst was added and the reaction continued for 3 days when debenzylation was complete (TLC evidence). Filtration followed by concentration under reduced pressure gave the galactoside sulfate (0.135 g), Rf 0.15 (ethyl acetate:acetic acid:water 6:3:2), which was shown by ^{1}H NMR to be a piperidinium rather than a pyridinium salt (δ, 100 MHz, D_2O, 3.2, m, 4 H and 1.8, m, 6 H). An aqueous solution of this salt was passed through a small column of Amberlyte IR 120 (H^+ form) resin, which was washed with water. The effluent plus washings were neutralised with saturated $KHCO_3$ and concentrated to dryness under reduced pressure to give methyl α-D-galactopyranoside 6-(potassium sulfate) (0.12 g) which crystallised on trituration with methanol. The methanol washed crystals (45 mg, 38%) had mp 210 °C (dec.), [lit.[24] 210-215 °C (dec)], ^{1}H NMR (19:1 d_5-pyridine:D_2O) δ 5.14 (d, $J_{1,2}$ 3.8 Hz, H-1), 5.00 (dd, $J_{5,6}$ 6.1 Hz, H-6), 4.91 (dd, $J_{5,6'}$ 6.6, $J_{6,6'}$ 10.5 Hz, H-6'), 4.58 (dd, $J_{2,3}$ 10.1 Hz, H-2), 4.51 (d, $J_{3,4}$ 3.4 Hz, H-4), 4.43 (t, H-5), 4.34 (dd, H-3), 3.45 (s, OCH_3).

Methyl 3,6-Anhydro-α-D-galactopyranoside. An authentic sample was prepared by deacetylation of the diacetate derivative, which was isolated as a by-product in the preparation of methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-iodo-α-D-galactopyranoside;[25] mp 139-141 °C, lit.[26] 140 °C.

Model Experiments: exposure of methyl α-D-galactopyranoside-6-(potassium sulfate) to alkaline conditions. Mild conditions: methyl α-D-

galactopyranoside-6-(potassium sulfate) (5 mg) was dissolved in 0.05 M NaOH (0.5 mL) containing 1.6% 1-propanol as internal standard. Duplicate solutions in sealed ampoules were incubated at 45 ^{o}C for 30 h. Aliquots (2 x 0.2 mL) were cooled in ice, neutralised to pH 6-7 by addition of 0.05 M HCl, and analysed by HPLC (chromatogram illustrated in ref. 17). **Harsh conditions**: similar weights of methyl α-D-galactopyranoside-6-sulfate were incubated in 1 M NaOH (0.5 mL) containing 1.6% 1-propanol at 100 ^{o}C for 5 h. Cooled aliquots were neutralised with 1 M HCl and analysed by HPLC;[17] the galactoside 6-sulfate, methyl α-D-galactopyranoside, methyl 3,6-anhydro-α-D-galactopyranoside and propanol had retention times 1.3, 2.1, 5.2 and 5.9 min respectively (eluent: water, flow rate 2 mL/min).

Model Experiments With Dextran Sulfate. Dextran sulfate (average molecular weight 5,000 Da, Sigma) was purified on a Bio-Gel P-2 column (Fig) and material eluting in the void region was pooled and freeze dried; ^{13}C NMR (100.6 MHz, partial data, acetone reference) sulfated glucose units: δ 96.60 and 79.81 (C-1 and C-2 respectively of 2-sulfate), 83.11 (C-3 of 3-sulfate); non-sulfated glucose: 98.50 (C-1), 74.19 (C-3), 72.20 (C-2), 70.99 (C-4), 70.33 (C-5), and 66.31 (C-6). Preliminary experiments established that the work up procedure[19] (involving removal of sodium ions with a cation exchange resin followed by methanolysis to remove boric acid) for the isolation of the dextran sulfate after exposure to alkaline sodium borohydride caused the loss of 15.7% of the sulfate. A modified procedure was used. Dextran sulfate (sulfate content 7.6%) was exposed to 0.05 M NaOH, 1 M $NaBH_4$ at 45 ^{o}C for 30 h. The ice-cooled solution was neutralised to pH 6-7 with Amberlyte IR 120 resin (H^+), filtered with washing of the resin, and treated with cold methanol followed by acetic anhydride added in 5 portions over one h. After a further 75 min the solution was extracted with diethyl ether (3x) and the combined ether layers were washed with water. The combined aqueous layers were cooled in ice, adjusted to pH 5 with NaOH and freeze dried. Boric acid was removed from the residue as methyl borate by the addition of chilled methanol and glacial acetic acid followed by solvent evaporation under reduced pressure. Addition and evaporation of methanol was repeated four times. The residue was dissolved in ice-cold water, the pH adjusted to 6 with NaOH, and the resulting solution freeze dried. Any released sulfate was removed by chromatography on Bio-Gel P-2; the dextran sulfate eluted in the same void region had a sulfate content of 7.4%.

Alkaline digestion of ^{35}S-labelled mucus glycoprotein. Radio-labelled human colorectal mucus glycoproteins were prepared by incubating tissue with 925 kBq

sodium [35S]-sulfate and 370 kBq [3H]-2-amino-2-deoxy-D-glucose hydrochloride for 24 h.[27] The mucus glycoproteins were isolated by chromatography on Sepharose CL 4B and characterised by density gradient centrifugation, SDS-polyacrylamide gel electrophoresis, and enzyme digestion with chondroitinase ABC. Six separate samples of secreted mucus glycoproteins, containing 0.67-0.84 kBq [^{35}S] and 0.81-0.93 kBq [^{3}H], were subjected to alkaline borohydride and the released oligosaccharides, after removal of sodium ions by Dowex 50W (H^+) resin and boric acid as methyl borate, were isolated by chromatography on Sepharose CL 4B (in 0.01 M Tris/HCl pH 8.0 buffer) and then Bio-Gel P-6 (in 0.1 M pyridinium acetate buffer, pH 5.0). The alkaline treatment resulted in a shift in the elution profile on Sepharose of radioactive label to a position near the lower included limit, accounting for 96+/-5% (0.70-0.76 kBq) of [^{35}S] and 97.4+/-4% (0.84-0.90 kBq) of [^{3}H] for six samples. Chromatography of this material on Bio-Gel P-6 gave a major peak at the excluded volume with coincident [^{35}S] and [^{3}H] profiles. Less than 1% of the radioactivity due to [^{35}S] (0.006-0.007 kBq) was present in the included fraction where free sulfate eluted. The overall recovery of radioactivity in these experiments was 88+/-6% for 6 samples.

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