Types of oligosaccharide sulphation, depending on mucus glycoprotein source, corpus or antral, in rat stomach

Yukinobu GOSO* and Kyoko HOTTA

Department of Biochemistry, School of Medicine, Kitasato University, Sagamihara 228, Japan

Radiolabelled mucus glycoprotein was obtained from tissue and a culture medium each of the corpus and antrum of rat stomach incubated with [^{35}S]sulphate *in vitro*. Gel-filtration analysis of oligosaccharides liberated by alkaline-borohydride treatment from glycoproteins indicated that ^{35}S -labelled oligosaccharides from the corpus vary considerably with respect to chain length whereas those from antral mucus glycoprotein are composed of small oligosaccharides. Examination of the reduced radiolabelled products obtained by HNO_2 cleavage of the hydrazine-treated oligosaccharides indicated sulphate esters of *N*-acetylglucosamine to be present at three locations on a carbohydrate unit: [^{35}S]sulphated monosaccharide (2,5-anhydromannitol 6-sulphate), [^{35}S]sulphated disaccharide [galactosyl(β 1-4)-2,5-anhydromannitol 6-sulphate] and [^{35}S]sulphated trisaccharide [fucosyl(α 1-2)-galactosyl(β 1-4)-2,5-anhydromannitol 6-sulphate]. Sulphated disaccharide and trisaccharide, possibly originating from the *N*-acetyl-lactosamine and fucosyl-*N*-acetyl-lactosamine sequences respectively, were detected in the corpus, especially as large oligosaccharides, but were present in the antrum in only very small amounts. The sulphated monosaccharide, however, most probably originating from 6-sulphated *N*-acetylglucosamine residues at non-reducing termini, was present in all oligosaccharide fractions in both the corpus and antrum.

INTRODUCTION

Glycoprotein in the gastric mucosa is considered to protect the mucosa from acid, pepsin and other destructive reagents. The sulphation of oligosaccharide chains in glycoprotein may be important to this function, since this process imparts a strongly negative charge to glycoprotein molecules, thus influencing rheological properties. The role of sulphated glycoprotein *in vivo* is still unclear, but it has been found to provide protection from peptic attack by covering the protein substrate (Mikuni-Takagaki & Hotta, 1979). Change in the degree of sulphation in human gastric glycoproteins as a result of gastric disorder may be an indication that sulphation performs an important role (Kakei *et al.*, 1984; Goso *et al.*, 1985).

Slomiany & Meyer (1972) have proposed a structure of sulphated oligosaccharide bearing an internal sulphated *NN'*-diacetylchitobiose sequence in pig gastric glycoprotein. The presence of mono- and bis-sulphated *N*-acetylglucosamine and galactose residues in rat gastric glycoprotein has been demonstrated with glycoprotein synthesized *in vitro* (Liau & Horowitz, 1982). Subsequently Ohara *et al.* (1983) found evidence for possible regional differences in the biosynthesis of gastric sulphated glycoprotein in the rat. Sulphotransferase has quite recently been shown to be present in a Golgi-rich membrane fraction from rat stomach (Slomiany *et al.*, 1987; Carter *et al.*, 1988; Murty *et al.*, 1988). Nevertheless additional studies need to be conducted to clarify structural features and biosynthetic mechanisms of sulphated mucus glycoprotein.

In the present study, differences in the sulphation of mucus glycoproteins in the corpus and antrum were

investigated by examining ^{35}S -labelled sulphated glycoproteins newly synthesized *in vitro*.

MATERIALS AND METHODS

Incubation of stomach pieces and isolation of mucus glycoproteins

White male Wistar rats (150–170 g) were killed by decapitation and their stomachs rapidly excised and gently washed with ice-cold Dulbecco's phosphate-buffered saline (Bashor, 1979) without Ca^{2+} or Mg^{2+} . Incubation of stomach pieces (antrum or corpus, 2 mm $\times$ 2 mm, ten pieces/dish) was carried out at 37 °C for 5 h with $\text{Na}_2^{35}\text{SO}_4$ (carrier-free; New England Nuclear) (150 μCi /dish) in a manner previously described (Ohara *et al.*, 1983). After being rinsed with ice-cold phosphate-buffered saline, the tissue pieces were boiled for 3 min in 50 mM-Tris/acetate buffer, pH 7.2, containing 2% (v/v) Triton X-100 so as to prevent any degradation by enzymes. The tissue pieces were homogenized with a Physcotron tissue homogenizer (dial setting 6, 30 s twice) and centrifuged at 8000 *g* for 30 min. Homogenization and centrifugation were repeated for re-extraction. The combined supernatant was applied onto a column (1.5 cm $\times$ 45 cm) of Bio-Gel A-1.5 m (100–200 mesh; Bio-Rad Laboratories) previously equilibrated with 50 mM-Tris/acetate buffer, pH 7.2, containing 2% Triton X-100. The excluded fraction containing mucus glycoprotein (Azumi *et al.*, 1980) was collected and subjected to CsCl equilibrium centrifugation for further purification. Centrifugation was performed at 152000 *g* for 120 h at 10 °C with a starting density of 1.45 g/ml. The purified mucus glycoproteins were desalted with Toyoparl HW50S (Tosoh) gel-filtration chromatography

Abbreviations used: AnManH₂, 2,5-anhydro-D-mannitol; AnTalH₂, 2,5-anhydro-D-talitol.

* To whom correspondence should be addressed.

by elution with distilled water. Mucus glycoprotein in the culture medium was similarly purified except that no extraction was carried out.

Unlabelled mucus glycoproteins were isolated directly from the corpus and antrum and purified as above, but with hexose as an indicator.

Preparation of oligosaccharides from mucus glycoproteins

The ^{35}S -labelled mucus glycoproteins (1×10^6 – 6×10^6 d.p.m.) were incubated with 1 M- NaBH_4 in 50 mM- NaOH for 15 h at 45°C to prepare oligosaccharides (Carlson, 1968). Unlabelled mucus glycoproteins were treated in the same manner but with NaB^3H_4 (New England Nuclear) (adjusted to 100 mCi/mmol, 0.1 mCi/nmol of oligosaccharides). After acidification with 1 M-acetic acid, the samples were passed through a Dowex 50 X2 (H^+ form) column and boric acid was removed by repeated evaporation with methanol. The oligosaccharides were purified by preparative t.l.c. on a cellulose plate with butan-1-ol/ethanol/water (4:1:1, by vol.) as solvent. The cellulose layer between the origin and elution position of GalNAcH_2 was scraped off and extracted with water.

Treatment with hydrazine, HNO_2 and borohydride

Treatment with hydrazine and HNO_2 was carried out by a previously described procedure (Edge & Spiro, 1984) on samples thoroughly dried *in vacuo* over P_2O_5 overnight. After the addition of 0.5 ml of freshly distilled hydrazine, each sample was heated at 100°C for 15 h under N_2 in a sealed tube. The reagent was removed by evaporation *in vacuo* over a trap of conc. H_2SO_4 , during which toluene was added several times. The deacetylated samples were then treated with 0.5 ml of 0.2 M- NaNO_2 in 0.5 M-acetic acid for 2 h at room temperature. After having been passed through a Dowex 50 X4 (H^+ form) column, the samples were incubated with 0.1 M- NaBH_4 in 50 mM- NaOH for 3 h at room temperature. All products thus produced were recovered by acidification, passage through a Dowex 50 X4 column and removal of boric acid as described above.

Smith periodate oxidation

The radiolabelled saccharides were oxidized with 50 mM-sodium metaperiodate in 50 mM-sodium acetate buffer, pH 4.5, for 24 h at 4°C . The reactions were terminated by the addition of glycerol and, after 1 h at room temperature, the samples were reduced with 50 mM- NaBH_4 in 0.1 M-sodium borate buffer, pH 10.0, at room temperature for 4 h. After the addition of acetic acid, the samples were applied on to a Darco G60/Celite 535 column (1:1, by wt.) for removal of salts. The oxidized saccharides were recovered in the eluent with 30% (v/v) ethanol, and then treated with 0.1 M-HCl at 80°C for 1 h. After dilution with water, the acid was removed by repeated freeze-drying with additions of water.

Enzymic treatments

Bovine kidney α -L-fucosidase and coffee-bean α -D-galactosidase (Boehringer) digestion of ^{35}S -labelled oligosaccharides (1000 d.p.m.) were carried out in 50 μl of 50 mM-sodium acetate buffer, pH 4.5, with 6 munits of each of the enzymes. *Charonia lampas* β -D-galactosidase (Seikagaku Kogyo) digestion was conducted in 0.1 M-

sodium citrate/phosphate buffer, pH 4.0, containing 0.5 M- NaCl with 6 munits of the enzyme. Almond emulsion fucosidases I and II were prepared by the procedure of Ogata-Arakawa *et al.* (1977) except that Sephacryl S-200 (Pharmacia) was used instead of Sephadex G-200. Digestion was performed at 37°C for 16 h in the presence of toluene in 50 mM-sodium citrate/phosphate buffer, pH 5.0, containing 5 mg of galactono-1,4-lactone/ml.

For digestions with glycosaminoglycan-degrading enzymes, the ^{35}S -labelled macromolecules from the CsCl centrifugation were precipitated by adding 3 vol. of ice-cold 95% (v/v) ethanol containing 13 g of potassium acetate/l so as to remove salts and then redissolved in 0.2 ml of 0.1 M-Tris/acetate buffer, pH 7.3, containing 30 mM-sodium acetate or 5 mM-calcium acetate (in the case of heparitinase) and the proteinase inhibitors 10 mM-EDTA (sodium salt), 10 mM-N-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-benzamidine hydrochloride and 0.1 M-6-amino-hexanoic acid. This was followed by incubation of the samples for 1 h at 37°C with one of the following enzymes (Seikagaku Kogyo): 0.5 unit of *Proteus vulgaris* chondroitin ABC lyase, 0.05 unit of *Flavobacterium heparinum* heparitinase or 0.5 unit of *Pseudomonas* sp. keratanase. About 200 μg of an appropriate glycosaminoglycan standard (Seikagaku Kogyo), shark cartilage chondroitin sulphate, bovine kidney heparan sulphate or bovine cornea keratan sulphate, was mixed with the sample as an internal control to monitor the enzyme reactions.

Gel-filtration chromatography

The oligosaccharide samples were applied on to a Toyopearl HW50S column (1.6 cm $\times$ 80 cm) previously equilibrated with 0.1 M-pyridine/acetate buffer, pH 5.0, and eluted with the same solution at a flow rate of 12 ml/h. Fractions (1 ml) were collected and each was assayed for radioactivity. The column was calibrated by eluting Dextran T-500 (Pharmacia), glucose oligomers, GalNAcH_2 , $\text{Gal}\beta 3\text{GalNAcH}_2$ prepared from $\text{Gal}\beta 3\text{-GalNAc}$ (Bio-Carb) by reduction and $\text{GalNAcH}_2(6\text{-SO}_3)$ prepared from $^4\Delta\text{GlcA}\beta 3\text{GalNAc}(6\text{-SO}_3)$ (Seikagaku Kogyo) by Smith periodate degradation.

The glycoprotein samples that had been treated with one of the glycosaminoglycan-degrading enzymes were applied on to a Bio-Gel A-1.5m column (1.5 cm $\times$ 28 cm) equilibrated with 50 mM-Tris/acetate buffer, pH 7.2, containing 4 M-guanidinium chloride, 0.2% Triton X-100 and the proteinase inhibitors specified above. Each fraction eluted from the column was assayed for radioactivity and for hexuronic acid or hexose in the case of keratanase digests. Hexuronic acid was determined after the samples had been precipitated by adding 3 vol. of 95% ethanol containing 13 g of potassium acetate/l to remove salts.

High-performance anion-exchange chromatography

High-performance anion-exchange chromatography was carried out on a TSK-gel DEAE-5PW column (acetate form; Tosoh) (0.75 cm $\times$ 7.5 cm). The samples were applied on to the column and, after being washed with water, were eluted with a linear gradient of 0–2 M-pyridine/acetate buffer, pH 5.0, at a flow rate of 0.5 ml/min for 60 min. Fractions (1 ml) were collected and assayed for radioactivity.

T.l.c.

The saccharides were resolved by t.l.c. on plastic sheets precoated with cellulose (0.1 mm thickness; E. Merck) in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.) (solvent A) or in butan-1-ol/acetic acid/1 M-NH₃ (3:2:1, by vol.) (solvent B). A wick of Whatman 3MM paper was clamped to the top of the thin-layer plate and chromatography proceeded for 6 h in solvent A and for 24 h in solvent B. Components were located after the plate had been sprayed with 4-methylnaphthalene-based enhancer (Spiro & Spiro, 1985) by fluorography at -80 °C with XAR-5 film (Eastman-Kodak). The following ³H-labelled sulphated saccharides were used as markers: AnManH₂(3-SO₄) and AnManH₂(6-SO₄) obtained from glucosamine 3-sulphate and glucosamine 6-sulphate (Sigma Chemical Co.) respectively by HNO₂ deamination followed by NaB³H₄ reduction as described by Edge & Spiro (1985), Galβ4AnManH₂(6-SO₄) and Gal(6-SO₄)β4AnManH₂(6-SO₄) prepared from bovine cornea keratan sulphate (Seikagaku Kogyo) by treatment with hydrazine and HNO₂ followed by NaB³H₄ reduction, and AnTalH₂(4-SO₄) and AnTalH₂(6-SO₄) prepared from chondroitin sulphate as described by Edge & Spiro (1984).

Chemical analysis

Hexose was measured by a phenol/H₂SO₄ method (Dubois *et al.*, 1956) with galactose as the standard. Protein was determined by the BCA method (Smith *et al.*, 1985) with the Pierce protein assay kit and bovine serum albumin as the standard. Hexuronic acid was measured by the method of Galambos (1967).

RESULTS

Preparation of ³⁵S-labelled mucus glycoprotein

Radiolabelled macromolecules were extracted with Triton X-100 from tissue pieces of corpus or antrum of rat stomach previously incubated with [³⁵S]sulphate. They were subsequently obtained from a Bio-Gel A-1.5m column as the excluded fraction, as previously described (Ohara *et al.*, 1983). ³⁵S-labelled macromolecules were also obtained from the culture medium by Bio-Gel A-1.5m chromatography. These macromolecules were further purified by CsCl centrifugation to remove nucleic acids and proteins (Spee-Brand *et al.*, 1980). Fig. 1 shows that ³⁵S-labelled macromolecules were recovered from the centrifuge tube as a single peak at a density of about 1.55 g/ml (corpus tissue) or 1.50 g/ml (antrum tissue). Similar elution profiles were also obtained for the ³⁵S-labelled macromolecules in the medium, though the peaks were somewhat broader.

Since unlabelled mucus glycoprotein had a lower density (about 1.50 g/ml) (Fig. 1a), there is the question as to whether ³⁵S-labelled macromolecules contain proteoglycans whose density is higher. For clarification of this point, the ³⁵S-labelled macromolecules in Fig. 1(b) were pooled and, after being desalted, were digested with glycosaminoglycan-degrading enzymes. Fig. 2 shows that no ³⁵S radioactivity was included in the column after digestion with chondroitin ABC lyase when carrier chondroitin sulphate was completely digested. It is thus evident that ³⁵S-labelled macromolecules do not contain chondroitin sulphate. Neither did heparitinase digest any ³⁵S-labelled macromolecules. While some ³⁵S radio-

Fig. 1. CsCl equilibrium centrifugation of rat gastric macromolecules

Unlabelled macromolecules obtained from the corpus tissue (a) by extraction with 2% Triton X-100 followed by Bio-Gel A-1.5m chromatography, and ³⁵S-labelled macromolecules from the corpus tissue (b), antral tissue (c), corpus medium (d) and antral medium (e), were subjected to CsCl centrifugation as described in the text. The designated areas were pooled for further study.

activity was detected in the included fraction after keratanase digestion, more than 95% of the ³⁵S radioactivity was excluded from the column. The same digestion studies on antral ³⁵S-labelled macromolecules showed nearly all ³⁵S radioactivity to be excluded from the column after digestion with each of the above enzymes. It is evident from these results that nearly the ³⁵S-labelled macromolecules in Fig. 1 are not proteoglycans but sulphated glycoproteins.

The amount of ³⁵S radioactivity in corpus mucus glycoproteins was about 3 times that in the antral mucus glycoproteins (Table 1), this being consistent with our previous observations (Ohara *et al.*, 1983). About 90% and 60% of ³⁵S-labelled mucus glycoproteins were recovered from the tissue pieces of corpus and antrum

Fig. 2. Digestion of ^{35}S -labelled macromolecules with glycosaminoglycan-degrading enzymes

A portion of ^{35}S -labelled macromolecules from Fig. 1(b), after the addition of appropriate glycosaminoglycan standard, was incubated in the absence of any enzyme (a), and with *Proteus vulgaris* chondroitin ABC lyase (b), *F. heparinum* heparitinase (c) or *Pseudomonas* sp. keratanase (d), followed by analysis by Bio-Gel A-1.5m chromatography. Each fraction eluted from the column was assayed for radioactivity and hexuronic acid content (indicated by the broken line). Hexuronic acid was measured for the sample precipitated with ethanol to remove guanidinium chloride. The arrow indicates the column volume calibrated by eluting galactose.

respectively, and the remainder from the medium in each case. Subsequent to acid hydrolysis of the ^{35}S -labelled mucus glycoproteins, more than 95% of the radiolabel was bound to the Dowex 1 column and, after elution, could be almost entirely precipitated as BaSO_4 in the presence of a carrier.

Gel filtration of β -eliminated oligosaccharides from mucus glycoprotein

Purified mucus glycoprotein was treated with alkaline borohydride and, after being purified by preparative t.l.c., the released oligosaccharides were analysed by Toyopearl HW50S gel-filtration chromatography. The ^{35}S -labelled oligosaccharides released from mucus glycoprotein of corpus tissue showed considerable variation in size (Fig. 3a). The elution pattern was essentially the same when oligosaccharides from the culture medium were used (Fig. 3c).

The chemically radiolabelled oligosaccharides obtained from unlabelled mucus glycoprotein by alkaline-borotritide treatment differed somewhat from metabolically labelled oligosaccharides. Thus large oligosaccharides were observed in small amounts in ^3H -labelled oligosaccharides (Fig. 3a, broken line). This may indicate that large oligosaccharides are extensively sulphated in corpus mucus glycoproteins.

Though a calibrated column with glucose oligomers was used, ^{35}S -labelled oligosaccharide size could not be determined by gel-filtration chromatography because of the abnormal elution of the sulphated saccharide. Consequently, standard *N*-acetylgalactosaminitol 6-sulphate was eluted behind *N*-acetylgalactosaminitol. This abnormality may have been due to interactions between the sulphate residues on the oligosaccharides and resin.

When the ^{35}S -labelled oligosaccharides of mucus glycoprotein in antrum tissue were subjected to gel-filtration chromatography, the results differed from those for the corpus. Small oligosaccharides were detected as major components but large oligosaccharides were present in only small amounts (Fig. 3b). In contrast, ^3H -labelled oligosaccharides gave elution patterns similar to those of corpus mucus glycoprotein. Also, no significant differences in elution patterns of oligosaccharides were observed for the tissue and medium in the antrum (Fig. 3d).

^{35}S -labelled oligosaccharide fractions, designated C1, C2, C3, A1, A2, A3 and A4, were pooled separately and used for further characterization. Because they were available in only limited amounts, ^{35}S -labelled oligosaccharides of mucus glycoproteins in the medium were not used for further characterization.

Table 1. Incorporation of [^{35}S]sulphate into mucus glycoprotein from rat stomach incubated *in vitro*

For experimental details see the text. Values for protein content and ^{35}S incorporation are expressed for 50 tissue pieces.

	Protein (mg)	$10^{-5} \times ^{35}\text{S}$ incorporation (d.p.m.)			$10^{-4} \times$ Specific radioactivity (d.p.m./mg of protein)
		Tissue	Medium	Total	
Corpus	11.7	6.1	0.9	7.0	6.0
Antrum	9.9	1.0	0.7	1.7	1.7

Fig. 3. Toyopearl HW50S chromatography of ^{35}S -labelled oligosaccharides liberated by alkaline-borohydride treatment from mucus glycoproteins of corpus tissue (a), antral tissue (b), corpus medium (c), and antral medium (d)

The oligosaccharides were applied on to a column (1.6 cm $\times$ 80 cm) of the gel equilibrated with 0.1 M-pyridine/acetate buffer, pH 5.0. Elution was achieved at a flow rate of 12 ml/h. The designated areas were pooled for further study. Elution of the ^{35}S -labelled oligosaccharide was compared with that of separately chromatographed ^3H -labelled oligosaccharides (designated by the broken line) liberated by alkaline-borotritide treatment from unlabelled mucus glycoprotein. The column was calibrated with the following saccharides: 1, Gal β 3GalNAcH $_2$; 2, GalNAcH $_2$; 3, GalNAcH $_2$ (6-SO $_4$); G1-G16, glucose oligomers prepared by acid hydrolysis of dextran. The excluded volume was determined by eluting Dextran T-500 with the sample.

Position of [^{35}S]sulphate on oligosaccharides

For clarification of sulphate group location on oligosaccharides, the oligosaccharides, after deacetylation by hydrazinolysis, were cleaved by HNO $_2$ deamination and

Fig. 4. T.l.c. of HNO $_2$ deamination products from ^{35}S -labelled oligosaccharides

Oligosaccharide fractions separated by gel filtration, C1, C2 and C3 from Fig. 3(a) and A1, A2, A3 and A4 from Fig. 3(b), were subjected to deacetylation with hydrazine followed by HNO $_2$ deamination. After reduction, the products were subjected to t.l.c. Migration of ^3H -labelled standards is shown by arrows: aM(3S), AnManH $_2$ (3-SO $_4$); aM(6S), AnManH $_2$ (6-SO $_4$); aT(4S), AnTalH $_2$ (4-SO $_4$); aT(6S), AnTalH $_2$ (6-SO $_4$); Gal-aM(6S), Gal β 4AnManH $_2$ (6-SO $_4$); Gal(6S)-aM(6S), Gal(6-SO $_4$) β 4AnManH $_2$ (6-SO $_4$).

Fig. 5. Toyopearl HW50S chromatography of saccharide III

This saccharide (2000 d.p.m.) was applied on to a column (1.6 cm $\times$ 80 cm) of the gel. Arrows indicate the positions of elution from the column of the ^3H -labelled standards applied to a column with the sample: Gal(6S)-aM(6S), Gal(6-SO $_4$) β 4AnManH $_2$ (6-SO $_4$); Gal-aM(6S), Gal β 4AnManH $_2$ (6-SO $_4$); aM(6S), AnManH $_2$ (6-SO $_4$).

Fig. 6. High-performance anion-exchange chromatography of saccharide III on DEAE-5PW

Saccharide III (500 d.p.m.) was applied on to a DEAE-5PW column (0.75 cm $\times$ 7.5 cm) and eluted with a linear gradient of 0–2 M-pyridine/acetate buffer, pH 5.0, for 60 min at a flow rate of 0.5 ml/min. O, ^{35}S radioactivity; ●, ^3H radioactivity of ^3H -labelled standards were eluted with the sample: 1, GalNAcH_2 ; 2, $\text{Gal}\beta 4\text{AnManH}_2(6\text{-SO}_4)$; 3, $\text{Gal}(6\text{-SO}_4)\beta 4\text{AnManH}_2(6\text{-SO}_4)$.

Fig. 7. Smith periodate degradation of ^{35}S -labelled saccharide III and ^3H -labelled $\text{Gal}\beta 4\text{AnManH}_2(6\text{-SO}_4)$

The samples (1000 d.p.m. and 15000 d.p.m. respectively) were subjected to Smith periodate degradation as described in the Materials and Methods section. The products thus obtained as well as the starting material were analysed by t.l.c. The same markers as in Fig. 2 were used.

Fig. 8. Glycosidase digestion of saccharide III

The samples (1000 d.p.m. each) were incubated without (lanes 1 and 4) or with bovine kidney α -L-fucosidase (lane 2), *C. lampas* β -D-galactosidase (lane 3), almond emulsin α -L-fucosidase I (lane 5) and almond emulsin α -L-fucosidase II (lane 6), and then subjected to t.l.c.

the reduced products were identified by t.l.c. ^{35}S -labelled products from the oligosaccharides gave three major spots, I, II and III, on t.l.c. (Fig. 4). A time-course study of hydrazinolysis indicated the formation of their maximum yield at approx. 15 h. It was also apparent that none of them consisted of incompletely degraded oligosaccharides, since none of them showed significant decrease with prolonged treatment. The radiolabelled materials remaining near the origin, possibly representing incompletely degraded oligosaccharides or larger sulphated products, were not characterized.

Saccharides I and II were identified as $\text{AnManH}_2(6\text{-SO}_4)$ and $\text{Gal}\beta 4\text{AnManH}_2(6\text{-SO}_4)$ respectively from their chromatographic migration in solvent systems A (Fig. 4) and B (not shown). In contrast, saccharide III

did not co-migrate with any sulphated saccharide standard used in the t.l.c. Further characterization of saccharide III was made for determination of its structure. First, following its purification by preparative t.l.c., saccharide III was subjected to gel filtration on a Toyoparl HW50S column. That this saccharide was eluted at a position between $\text{Gal}(6\text{-SO}_4)\beta 4\text{AnManH}_2(6\text{-SO}_4)$ and $\text{Gal}\beta 4\text{AnManH}_2(6\text{-SO}_4)$ (Fig. 5) indicates that it possibly could be a disaccharide mono- or bis-sulphate or trisaccharide mono-sulphate. Secondly, saccharide III was applied on to an anion-exchange column of DEAE-5PW to study its electrical properties. It was eluted somewhat faster than standard $\text{Gal}\beta 4\text{AnManH}_2(6\text{-SO}_4)$ (Fig. 6), thus indicating it to have not two but only one sulphate residue. Further characterization was made by periodate

Table 2. Quantification of sulphated saccharides obtained by the HNO₂ deamination of hydrazine-treated oligosaccharides of mucus glycoprotein

Sulphated saccharides obtained from the oligosaccharides C1, C2, C3, A1, A2, A3 and A4 indicated in Fig. 3 were separated by t.l.c., and the spot corresponding to each product was scraped off and its radioactivity counted. Each value is radioactivity per mg of protein of corpus or antral tissue.

	10 ⁻³ × ³⁵ S radioactivity (d.p.m./mg of protein)						
	Corpus			Antrum			
	C1	C2	C3	A1	A2	A3	A4
AnManH ₂ (6-SO ₄)	4.6	0.85	2.3	0.76	0.38	1.1	0.68
Galβ4AnManH ₂ (6-SO ₄)	3.4	0.86	0.39	0.50	0.15	0.14	0.10
Fuca2Galβ4AnManH ₂ (6-SO ₄)	4.0	1.5	0.32	0.15	0.09	0.01	0.05

degradation and exoglycosidase digestion. Fig. 7 shows this saccharide as well as standard Galβ4AnManH₂(6-SO₄) used as control to be sensitive to Smith periodate oxidation and to produce AnManH₂(6-SO₄). Fig. 8 demonstrates its conversion into Galβ4AnManH₂(6-SO₄) by digestion of bovine kidney α-L-fucosidase; *C. lampas* β-D-galactosidase, however, failed to change its migration on t.l.c., as was also noted for coffee-bean α-D-galactosidase. The same results were observed with t.l.c. with the solvent system B. Saccharide III thus appears to be fucosyl-Galβ4AnManH₂(6-SO₄). For determination of the location of the fructose residue, saccharide III was digested separately with almond emulsin α-L-fucosidases I and II. The former enzyme has been reported to digest α1,3- or α1,4-linked fucose and the latter to be specific to α1,2-linked fucose residues (Ogata-Arakawa *et al.*, 1977). Fig. 8 (lanes 4, 5 and 6) shows saccharide III to undergo conversion into Galβ4AnManH₂(6-SO₄) only by fucosidase II. From the experimental results presented above, saccharide III is concluded to be Fuca2Galβ4AnManH₂(6-SO₄).

Distribution of sulphated saccharides in the oligosaccharides of mucus glycoproteins

Fig. 4 and Table 2 show that the radiolabelled AnManH₂(6-SO₄) is present in all fractions of oligosaccharides prepared from both corpus and antral mucus glycoproteins, whereas Galβ4AnManH₂(6-SO₄) and Fuca2Galβ4AnManH₂(6-SO₄) that are obtained from oligosaccharides of corpus mucus glycoprotein occur in only small amounts in antral mucus glycoprotein. Galβ4AnManH₂(6-SO₄) was specific to large oligosaccharides, and Fuca2Galβ4AnManH₂(6-SO₄) was found on large and medium-size oligosaccharides, but on small ones their amounts were only slight.

DISCUSSION

³⁵S-labelled mucus glycoproteins were extracted with Triton X-100 from rat stomach tissues incubated *in vitro* and purified by means of Bio-Gel A-1.5m chromatography followed by CsCl equilibrium centrifugation. A glycosaminoglycan-degradation study failed to show ³⁵S-labelled proteoglycan to be present in significant amounts in the purified fractions. It was thus concluded that almost all ³⁵S-labelled macromolecules are mucus glyco-

proteins. It is unlikely that proteoglycans would have been fully extracted with 2% Triton X-100, since guanidium chloride was usually required for extracting proteoglycans (Yanagishita *et al.*, 1987). That limited amounts of ³⁵S-labelled proteoglycans were synthesized under the conditions used is a possibility, however. The differences in the density of ³⁵S-labelled and non-labelled mucus glycoproteins (Fig. 1) may be due to differences in the extent of their sulphation (Van Beurden-Lamers *et al.*, 1989).

Sulphate group locations on oligosaccharides of gastric mucus glycoprotein were determined by methods that included treatment with hydrazine and HNO₂. These methods have been shown to be quite useful for characterizing keratan sulphate (Hopwood & Elliott, 1983) and sulphated saccharides of thyroid plasma-membrane glycoprotein (Edge & Spiro, 1984) and thyroglobulin (Spiro & Bhoyroo, 1988). The detection of Galβ4AnManH₂(6-SO₄) indicates that sulphated *N*-acetyl-lactosamine sequences are present in mucus glycoprotein, and that of Fuca2Galβ4AnManH₂(6-SO₄) demonstrates that some of the galactose residues in the sulphated *N*-acetyl-lactosamine sequences undergo a fucosylation. Although the locations of sulphated *N*-acetyl-lactosamine sequences on oligosaccharides could not be determined by the present methods, it is quite likely that they are located at internal sites, since the sulphated *N*-acetyl-lactosamine sequence was specifically observed in large oligosaccharides, whose number of *N*-acetyl-lactosamine sequences should be sufficient for this to be so. The sulphated fucosyl-*N*-acetyl-lactosamine sequence, however, may be present at non-reducing termini on oligosaccharides. The Fuca2Galβ4GlcNAc sequence carries the blood-group-O antigen and is generally located at non-reducing termini.

The formation of AnManH₂(6-SO₄) by HNO₂ deamination indicates that 6-*O*-sulphated *N*-acetylglucosamine residues are possibly at non-reducing termini on oligosaccharide chains. The fact that rat stomach has been found to contain a sulphotransferase that produces sulphated oligosaccharides bearing terminal 6-*O*-sulphated *N*-acetylglucosamine (Carter *et al.*, 1988) supports this hypothesis. The possibility that AnManH₂(6-SO₄) was formed from the GlcNAcβ4GlcNAc(6-SO₄) sequence, however, cannot be ruled out, since such a sequence has been reported to occupy an internal position in the

oligosaccharide chain of pig gastric mucus glycoprotein (Slomiany & Meyer, 1972). Liao & Horowitz (1982) reported that 6-*O*-sulphated *N*-acetylglucosamine is liberated as a major product by mild acid hydrolysis from rat gastric mucus glycoprotein labelled metabolically with [³H]glucosamine and [³⁵S]sulphate. The present data are consistent with this finding, and thus sulphate residues may be concluded to be located at the 6-position of *N*-acetylglucosamine. They, however, reported that hexosamine bis-sulphate and galactose monosulphate are also present in glycoprotein. The reason for this inconsistency is not apparent at the present, but an HNO₂ deamination product containing such sulphated sugars may be too little in amount to permit detection as a clear spot on t.l.c. or may be located near the origin.

Sulphated saccharide sequences have been shown in the present study to differ according to site, whether in the corpus or the antrum. Sulphated *N*-acetyl-lactosamine and sulphated fucosyl-*N*-acetyl-lactosamine were found to be present as minor sequences in the antrum, and both could be detected in the corpus. Decrease in the degree of sulphation on the *N*-acetyl-lactosamine sequence in antrum remains to be explained, since large oligosaccharides, which should have *N*-acetyl-lactosamine sequences, are present in the antrum. Their presence was confirmed by gel-filtration patterns of ³H-labelled oligosaccharides prepared directly from unlabelled mucus glycoprotein from the antral glycoprotein (Fig. 3). Chromatographic patterns by hexose content and sugar composition analysis of unlabelled oligosaccharides also support their presence (Ohara *et al.*, 1986, 1988). Decrease in sulphation at *N*-acetyl-lactosamine sequences of oligosaccharides in antral mucus glycoprotein may be explained as resulting from a decrease in the amount of sulphotransferase(s) that produces sulphated lactosamine and/or sulphated fucosyl-*N*-acetyl-lactosamine sequences. Should this actually be the case, more than one enzyme would be involved in the sulphation of mucus glycoproteins. Enzymes in the antrum would not be capable of transferring sulphate to internal *N*-acetyl-lactosamine sequences of oligosaccharides. The possibility of the involvement of more than one enzyme is considerable, since two enzymes that transfer sulphate to the 6-position of terminal and internal *N*-acetylglactosamine of chondroitin sulphate have been reported (Nakanishi *et al.*, 1981). Decrease in the sulphotransferase that acts on *N*-acetyl-lactosamine would also partially explain the decrease in the sulphation of antral mucus glycoprotein (Table 1). The lesser extent of sulphation of large oligosaccharides at *N*-acetyl-lactosamine sequences in antral mucus glycoprotein may possibly account for differences in the elution profiles of ³⁵S-labelled oligosaccharides in the corpus and antrum.

That the transfer of *N*-acetylglactosamine to fucosyl-*N*-acetyl-lactosamine sequences in non-reducing termini inhibits subsequent sulphation may also adequately serve to explain the lesser sulphation of fucosyl-*N*-acetyl-lactosamine sequences in antral glycoprotein oligosaccharides. The presence of *N*-acetylglactosamine residues in addition to those located at reducing termini on the oligosaccharides of antral mucus glycoproteins (Ohara *et al.*, 1986) and that of *N*-acetylglactos-

aminyltransferase, a blood-group-A-producing enzyme, in antral microsomal fractions (Iwase *et al.*, 1988) appear consistent with such a possibility.

We are grateful to Dr Hitoo Iwase and Dr Susumu Ohara for their valuable comments. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Azuumi, Y., Ohara, S., Ishihara, K., Okabe, H. & Hotta, K. (1980) *Gut* **21**, 533–536
- Bashor, M. M. (1979) *Methods Enzymol.* **58**, 119–131
- Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626
- Carter, S. R., Slomiany, A., Gwozdziński, K., Liao, Y. H. & Slomiany, B. L. (1988) *J. Biol. Chem.* **263**, 11977–11984
- Dubois, M., Gilles, K. A., Hamilton, J. K., Roebbers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Edge, A. S. B. & Spiro, R. G. (1984) *J. Biol. Chem.* **259**, 4710–4713
- Edge, A. S. B. & Spiro, R. G. (1985) *Arch. Biochem. Biophys.* **240**, 560–572
- Galambos, J. T. (1967) *Anal. Biochem.* **19**, 119–132
- Goso, K., Ishihara, K., Ohara, S., Kakei, M. & Hotta, K. (1985) *Digestion* **31**, 205–212
- Hopwood, J. J. & Elliott, H. (1983) *Carbohydr. Res.* **117**, 263–274
- Iwase, H., Ishii, I., Ohara, S. & Hotta, K. (1988) *Anal. Biochem.* **173**, 317–320
- Kakei, M., Ohara, S., Ishihara, K., Goso, K., Okabe, H. & Hotta, K. (1984) *Digestion* **30**, 59–64
- Liao, Y. H. & Horowitz, M. I. (1982) *J. Biol. Chem.* **257**, 4709–4718
- Mikuni-Takagaki, Y. & Hotta, K. (1979) *Biochim. Biophys. Acta* **584**, 288–297
- Murty, V. L. N., Slomiany, A., Zalesna, G., Mizuta, K. & Slomiany, B. L. (1988) *Biochem. Pharmacol.* **37**, 3311–3316
- Nakanishi, Y., Shimizu, M., Otsu, K., Kato, S., Tsuji, M. & Suzuki, S. (1981) *J. Biol. Chem.* **256**, 5443–5449
- Ogata-Arakawa, M., Muramatsu, T. & Kobata, A. (1977) *Arch. Biochem. Biophys.* **181**, 353–358
- Ohara, S., Ishihara, K., Goso, K. & Hotta, K. (1983) *Comp. Biochem. Physiol. B* **76**, 5–8
- Ohara, S., Ishihara, K. & Hotta, K. (1986) *Comp. Biochem. Physiol. B* **83**, 273–275
- Ohara, S., Ishihara, K. & Hotta, K. (1988) *J. Biochem. (Tokyo)* **103**, 1050–1053
- Slomiany, B. L. & Meyer, K. (1972) *J. Biol. Chem.* **247**, 5062–5070
- Slomiany, B. L., Liao, Y. H., Sarosiek, C. J., Tsukada, H. & Slomiany, A. (1987) *Digestion* **38**, 178–186
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Spee-Brand, R., Strous, G. J. A. M. & Kramer, M. F. (1980) *Biochim. Biophys. Acta* **621**, 104–116
- Spiro, M. J. & Spiro, R. G. (1985) *J. Biol. Chem.* **260**, 5808–5815
- Spiro, R. G. & Bhoyroo, V. D. (1988) *J. Biol. Chem.* **263**, 14351–14358
- Van Beurden-Lamers, W. M. O., Spee-Brand, R., Dekker, J. & Strous, G. J. (1989) *Biochim. Biophys. Acta* **990**, 232–239
- Yanagishita, M., Midura, R. J. & Hascall, V. C. (1987) *Methods Enzymol.* **138**, 279–289