Isolation and Structural Characterization of Alkali-Labile Oligosaccharides from Bovine Milk-Fat-Globule Membrane

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Treatment of sialoglycopeptides derived from bovine milk-fat-globule membrane with alkaline borohydride released a reduced oligosaccharide fraction from which a tetrasaccharide and two trisaccharides were isolated. Periodate-oxidation studies coupled with methylation analysis and g.l.c.–mass spectrometry established their structures as: *N*-acetylneuraminyl-(2→3)- β -D-galactopyranosyl-(1→3)-[*N*-acetylneuraminyl-(2→6)]-*N*-acetyl-D-galactosaminitol, *N*-acetylneuraminyl-(2→3)- β -D-galactopyranosyl-(1→3)-*N*-acetyl-D-galactosaminitol and β -D-galactopyranosyl-(1→3)-[*N*-acetylneuraminyl-(2→6)]-*N*-acetyl-D-galactosaminitol respectively.

Glycoproteins may be involved in cell recognition (Woodruff & Gesner, 1969) and adhesion (Roseman, 1970), antigenicity (Hughes, 1976) and in receptor sites for viruses (Gottschalk *et al.*, 1972) and hormones (Cuatrecasas, 1973). Despite these suggestions, detailed structural studies on the carbohydrate fraction of membrane glycoprotein have largely been confined to that of the erythrocyte membrane.

The milk-fat-globule membrane is surrounded by a membrane that is derived from the apical cell membrane, and probably also from the Golgi vesicle membrane, of the secretory cell (Patton & Keenan, 1975; Powell *et al.*, 1977; Wooding, 1977). In view of its ready availability on a large scale the bovine milk-fat-globule membrane constitutes a convenient source of mammalian membrane material for structural studies. We have previously reported (Harrison *et al.*, 1975; Harrison, 1977) that treatment of intact bovine milk-fat globules with proteolytic enzymes caused release from the membrane surface of sialoglycopeptides containing, in addition to sialic acid, glucosamine, galactosamine, galactose, mannose and fucose. Fractionation of the proteolysis fragments gave a major sialoglycopeptide that, on treatment with alkaline borohydride, released an oligosaccharide fraction containing *N*-acetylgalactosaminitol, galactose and sialic acid in the molar ratio 1:1:2. It was subsequently reported (Newman *et al.*, 1976) that alkaline borohydride treatment of a crude glycoprotein preparation from bovine milk-fat-globule membrane gave a fraction from gel filtration that contained the reduced disaccharide β -D-galactosyl-(1→3)-*N*-acetyl-D-galactosaminitol together with two molecules of sialic acid. Periodate-oxidation and alkaline-degradation studies on the glycoprotein mixture suggested that the major alkali-labile oligosaccharide component was a tetrasaccharide containing β -D-galactopyranosyl-(1→3)-*N*-acetyl-D-

galactosamine substituted by sialic acid at position C-3 of the galactose and position C-6 of the *N*-acetyl-D-galactosamine residues. In the present paper we report the isolation and complete structural determination of a tetrasaccharide and two trisaccharides cleaved by alkaline borohydride from sialoglycopeptides derived from the outer surface of bovine milk-fat-globule membrane.

Materials and Methods

Materials

N-Acetyl-D-galactosaminitol and β -D-galactopyranosyl-(1→3)-*N*-acetyl-D-galactosaminitol were prepared by reduction with NaBH₄ of *N*-acetyl-D-galactosamine and β -D-galactopyranosyl-(1→3)-*N*-acetyl-D-galactosamine respectively. The latter, obtained from human brain gangliosides (Klenk *et al.*, 1962), was a gift from Dr. R. A. Newman, Medical University Clinic, Köln, Germany. *N*-Acetylneuraminyl-lactose (bovine colostrum, type II), *N*-acetylneuraminic acid, methyl β -D-galactopyranoside and neuraminidase (*Clostridium perfringens* type VI, EC 3.2.1.18) were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Trisil Z was obtained from Pierce Chemical Co., Rockford, IL, U.S.A., and Pronase (grade B, *Streptomyces griseus* proteinase) was from Calbiochem, London W.1, U.K. Sephadex was from Pharmacia (G.B.), London W.5, U.K. Ion-exchange resins were from Bio-Rad Laboratories, St. Albans, Herts., U.K., and g.l.c.-column packings were from Phase Separations, Queensferry, Flintshire, Wales, U.K. NaBH₄, dimethyl sulphoxide (gold label grade), methyl iodide and sodium hydride were purchased from Aldrich Chemical Co., Gillingham, Kent, U.K., and all other reagents were from BDH Chemicals, Poole, Dorset, U.K., unless otherwise stated.

Methods

Preparation of sialoglycopeptides from milk-fat globules. Sialoglycopeptides were released from washed intact milk-fat globules (500ml) by incubation with Pronase and then dialysed, concentrated and centrifuged as described previously (Harrison *et al.* 1975) to give a solution (10ml) that contained approx. 20mg of hexose and 20mg of sialic acid. This solution (5ml) was applied to a column (90cm $\times$ 2.5cm) of Sephadex G-50 (medium grade) previously equilibrated with water at 22°C. The column was eluted with water at 22°C with a flow rate of 60ml/h. Fractions (20ml) were automatically collected and samples analysed for hexose and sialic acid. A major, partially included peak contained approx. 90% of the sialic acid applied to the column and approx. 60% of the hexose. Control experiments in which Pronase and milk-fat globules were each incubated in the absence of the other showed no significant hexose or sialic acid in the region of the major peak containing hexose and sialic acid obtained by incubation of fat globules and Pronase together.

Fractions corresponding to the major peak containing hexose and sialic acid from Sephadex G-50 were combined and freeze-dried to give a residue (approx. 140mg) that was dissolved in 0.1M-acetic acid (5ml). A sample (1ml) of this solution

was applied to a column of Sephadex G-25 and eluted with 0.1M-acetic acid to give a single major peak containing both hexose and sialic acid (Fig. 1). Fractions were combined so as to exclude the 'tail' of sialoglycopeptides with lower molecular weight (Fig. 1) and freeze-dried.

Cleavage by alkaline NaBH_4 of oligosaccharides from sialoglycopeptides. The total freeze-dried residue (approx. 20mg) containing the bulk of the sialoglycopeptides from the Sephadex G-25 column was dissolved in 1.0M- NaBH_4 in 0.05M- NaOH (20ml). A stream of N_2 was passed through the solution, which was then incubated in a stoppered flask at 50°C for 24h. The mixture was cooled, adjusted to pH 6.0 by addition of cation-exchange resin (Bio-Rad AG50W;X8; H^+ form), passed down a column (8cm $\times$ 1.4cm) of the same resin and eluted with water (20ml). The eluate from the ion-exchange column was freeze-dried and the residue was freed from boric acid by repeated addition and removal by distillation of methanol. This residue was dissolved in 0.1M-acetic acid (2ml), applied to a column of Sephadex G-25 and eluted with 0.1M-acetic acid. Fractions were collected and assayed for hexose and sialic acid giving the elution pattern shown in Fig. 2. Fractions corresponding to peaks B, C and D, containing the cleaved oligosaccharides, were combined in a single pool and freeze-dried.

Fig. 1. Chromatography on Sephadex G-25 of sialoglycopeptides from the major peak from Sephadex G-50. Fractions corresponding to the major peak from Sephadex G-50 (see the text) were combined, freeze-dried and dissolved in 0.1M-acetic acid (5ml). A sample of this solution was applied to a column (150cm $\times$ 1.0cm) of Sephadex G-25 equilibrated at 22°C with 0.1M-acetic acid. The column was eluted with 0.1M-acetic acid with a flow rate of 40ml/h. Samples of fractions (3ml) were analysed for hexose (A_{420} , $\circ$) and sialic acid (A_{550} , $\bullet$). Fractions collected before fraction 60 (vertical line) were collected and treated with alkaline borohydride as described in the Results section.

Fig. 2. Fractionation of carbohydrate-containing products from the alkaline borohydride treatment of sialoglycopeptides contained in fractions 30-60 from Sephadex G-25 chromatography (Fig. 1).

Fractions 30-60 from Sephadex G-25 chromatography (Fig. 1) were combined, freeze-dried and treated with alkaline borohydride as described in the Results section. The product, freed from borate, was dissolved in 0.1M-acetic acid (2ml) and applied to a column (150cm $\times$ 1.0cm) of Sephadex G-25 equilibrated at 22°C with 0.1M-acetic acid. The column was eluted with 0.1M-acetic acid with a flow rate of 40ml/h. Samples of fractions (3ml) were analysed for hexose (A_{420} , $\circ$) and sialic acid (A_{550} , $\bullet$).

Fig. 3. Fractionation on DEAE-Sephadex A-25 of fractions corresponding to peaks B, C and D from Sephadex G-25 chromatography (Fig. 2)

Fractions corresponding to peaks B, C and D from Sephadex G-25 chromatography (Fig. 2) were combined, freeze-dried, dissolved in 0.05M-pyridine acetate buffer (2ml), pH 5.0, and applied to a column (16cm $\times$ 1.5cm) of DEAE-Sephadex A-25 equilibrated at 22°C with 0.05M-pyridine acetate buffer, pH 5.0. The column was eluted with 0.05M-pyridine acetate buffer (70ml), pH 5.0, followed by a linear gradient of 0.05–0.65M-pyridine acetate buffer (200ml, ----), pH 5.0, with a flow rate of 40ml/h. Samples of fractions (7ml) were analysed for hexose (A_{420} , $\circ$) and sialic acid (A_{550} , $\bullet$).

Separation of oligosaccharides. Freeze-dried, pooled oligosaccharide fractions (peaks B, C and D) from the Sephadex G-25 column (Fig. 2) were dissolved in 0.05M-pyridine acetate buffer, pH 5.0, and applied to a column of DEAE-Sephadex A-25. The column was eluted with 0.05M-pyridine acetate buffer followed by a linear gradient of 0.05–0.65M buffer. Fractions were collected and assayed for hexose and sialic acid; three distinct peaks (Fig. 3) were obtained. Fractions corresponding to each of the three peaks I, II and III were separately combined and re-chromatographed on Sephadex G-25 (Fig. 4). Material from peaks I and III each gave single peaks (A' and B' respectively) containing both hexose and sialic acid, corresponding to peaks A and B respectively of Fig. 2. Material from peak II gave a major peak (C') containing hexose and sialic acid corresponding to peak C of Fig. 2, together with two shoulders, one of which (D') contained sialic acid but no hexose and chromatographed in the same position as peak D of Fig. 2. Fractions corresponding to peaks B' and C' were separately pooled and freeze-dried to give residues weighing approx. 1mg and 0.5mg respectively.

Separation of trisaccharides. A sample (1mg) of the trisaccharide mixture obtained from fraction C' (Fig. 4b) was chromatographed as a band on silica-gel G plates (20cm $\times$ 20cm $\times$ 0.5mm thick) and developed with propan-2-ol/butan-1-ol/water (5:3:2, by vol.). Positions of bands were determined from patterns of spots on a control plate that was sprayed with 3% (w/v) phenol in ethanol/conc. H_2SO_4 (19:1, v/v) and heated at 110°C for 10min. A major and a minor band (R_F 0.23 and 0.14 respectively) were scraped from the preparative plate and each was shaken with water (5ml). In each case the gel

was removed by centrifugation (3000g for 10min), resuspended in water (5ml) and centrifuged as before. The washing procedure was repeated and the supernatants were combined and freeze-dried. The residues were separately chromatographed on columns of Sephadex G-25 under the conditions described in the legend to Fig. 4 and in each case a sharp peak was obtained in the position of C' (Fig. 4). Fractions corresponding to the peaks were combined and freeze-dried. Samples of the residues were chromatographed on t.l.c. plates under the conditions described above, when each gave a single spot with R_F values 0.23 and 0.14 respectively.

Hexose and hexosaminitol determinations. Total hexose was determined colorimetrically by a modification of the cysteine/ H_2SO_4 assay (Dische & Danilchenko, 1967). Galactose and *N*-acetylgalactosaminitol in purified oligosaccharide fractions were determined by g.l.c. Oligosaccharides were hydrolysed with acid (see under 'Acid hydrolyses') and the released monosaccharides were identified as their *O*-trimethylsilyl derivatives and as their alditol acetates by g.l.c. (see under 'Gas-liquid chromatography') by using authentic standards. Alditol acetates were determined by comparing peak areas with those from identically treated standards. Addition of *N*-acetylneuraminic acid to the standards before hydrolysis was not shown to interfere with the assay.

Sialic acid determinations. Sialic acid in column eluates was determined after hydrolysing a sample (0.1ml) with 0.05M- H_2SO_4 (0.1ml) at 80°C for 1h, by the method of Warren (1959) as modified by Aminoff (1961).

Sialic acid in purified oligosaccharide fractions was determined by the above method except that the

Fig. 4. Chromatography on Sephadex G-25 of fractions I, II and III from DEAE-Sephadex A-25 chromatography (Fig. 3)

Fractions corresponding to peaks I, II and III (Fig. 3) were combined into three separate pools, each of which was freeze-dried, dissolved in 0.1 M-acetic acid (2 ml) and applied to a column (150 cm $\times$ 1.0 cm) of Sephadex G-25 equilibrated at 22°C with 0.1 M-acetic acid. The columns were eluted with 0.1 M-acetic acid with a flow rate of 40 ml/h. Samples of fractions (3 ml) were analysed for hexose (A_{420} , $\circ$) and sialic acid (A_{550} , $\bullet$). (a) Peak I from DEAE-Sephadex A-25 chromatography; (b) peak II from DEAE-Sephadex A-25 chromatography; (c) peak III from DEAE-Sephadex A-25 chromatography.

cooled hydrolysate containing free sialic acid was applied to a column (5 cm $\times$ 0.5 cm) of anion-exchange resin (Bio-Rad AG 1;X8; formate form) as described by Svennerholm (1958). The column was eluted with water (10 ml) to remove neutral saccharides and then with 0.3 M-formic acid (10 ml). The latter eluate was freeze-dried before assay as described above. Authentic *N*-acetylneuraminic acid treated identically was used as a standard.

Alternatively sialic acid was released from purified oligosaccharide fractions by incubation with neuraminidase (0.2 mg) in 0.05 M-sodium barbiturate buffer (2.5 ml) pH 5.5, at 37°C for 3 h. The incubation mixture was freed from salts and protein by passage down a column (100 cm $\times$ 1 cm) of Sephadex G-15 and elution with 100 mM-acetic acid before anion-exchange chromatography as described above.

Sialic acid in purified oligosaccharide fractions was identified as *N*-acetylneuraminic acid by a g.l.c. procedure based on that described by Yu & Ledeen (1970). The dried oligosaccharide (approx. 0.5 mg) in methanolic 0.05 M-HCl (2 ml) was heated at 80°C for 2 h under an atmosphere of N_2 in a sealed tube. Solvent was removed in a stream of N_2 at 22°C and the residue was freed from HCl by repeated distillation and evaporation of methanol under the above conditions and dried over P_2O_5 . Methyl neuraminic acid methyl ester in the residue was compared with an authentic sample of the *N*-acetylneuraminic acid derivative (Yu & Ledeen, 1969) by conversion into the *O*-trimethylsilyl ethers and g.l.c. (see under 'Gas-liquid chromatography').

Methylation. Saccharide samples (0.5–1.0 mg) were methylated by using methyl iodide and methylsulphinyln sodium in dimethyl sulphoxide as originally reported by Hakomori (1964) and modified as described by Lindberg (1972), except that the methylation step was extended from 30 min to 16 h.

Periodate oxidation. Oligosaccharide samples (0.5–1.0 mg) in 0.2 M-sodium acetate buffer (2 ml), pH 4.5, were freed from O_2 by passing a stream of N_2 through the solution. Sodium metaperiodate (0.1 M; 1 ml) was added and the mixture was stored at 0°C under an atmosphere of N_2 in the dark for 20 h. Ethylene glycol (20 mM; 1 ml) was added to destroy excess periodate and the mixture was treated with $NaBH_4$ as described below.

$NaBH_4$ reduction of reducing saccharides. $NaBH_4$ (0.2 M) in 0.05 M-NaOH (1 ml) was added to a reducing saccharide sample (0.5–1.0 mg) and the solution was stored at 22°C for 20 h. Excess $NaBH_4$ was removed by addition of 3 M-acetic acid and the mixture was freed from boric acid by repeated addition and removal by distillation of methanol. The residue was directly converted into a derivative or hydrolysed (in periodate-oxidation experiments) unless otherwise stated. In the preparation of β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol for g.l.c. as its *O*-trimethylsilyl ether, the reduced product in water (1.0 ml) was desalted by passage down a column (100 cm $\times$ 1.0 cm) of Sephadex G-15 before freeze-drying and derivative formation.

Acid hydrolyses. Methylated saccharides (0.5–1.0 mg) in 0.1 M- H_2SO_4 were heated at 100°C for 14 h under an atmosphere of N_2 in a sealed tube. Initial short periods of immersion in an ultrasonic cleaning bath (Dawe Sonicleaner, supplied by Dawe Instruments, London W.3, U.K.) were used to aid emulsification of the mixture. The cooled hydrolysate was neutralized with saturated aqueous $Ba(OH)_2$ solution and centrifuged at 4000g for 15 min to remove $BaSO_4$. The precipitate was suspended in water and re-centrifuged. The process was repeated twice and the combined supernatants were acidified with

dilute acetic acid and concentrated, under reduced pressure, to dryness.

Unmethylated oligosaccharides (0.5–1.0mg) were completely hydrolysed by heating in 0.6M-HCl at 100°C for 14h under an atmosphere of N₂ in a sealed vial. Acid was removed by repeated freeze-drying before derivative formation for g.l.c.

The disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol was released from oligosaccharide samples by selective acid hydrolysis of sialic acid linkages. The oligosaccharide sample (0.5–1.0mg) in 4M-formic acid (2ml) was heated at 70°C for 2h in a sealed tube under an atmosphere of N₂. The hydrolysate was freeze-dried, re-dissolved in water and again freeze-dried to remove acid. Trehalose (30 μ g) was added as a standard before preparation of the *O*-trimethylsilyl derivative of the remaining disaccharide for g.l.c.

Gas-liquid chromatography. Alditol acetate derivatives of reducing saccharides (0.5–1.0mg) were prepared by reduction with NaBH₄ as described above, followed by addition of dry pyridine (0.25ml) and acetic anhydride (0.25ml). A stream of N₂ was passed through the mixture, which was then heated at 100°C for 1h in a stoppered tube. The mixture was concentrated to dryness in a stream of N₂ at 80°C and the residue was dissolved in water (0.5ml) and extracted with chloroform (0.5ml). Samples (0.1ml) of the chloroform extract were concentrated under reduced pressure to dryness in a stream of N₂ and dissolved in dichloromethane (50 μ l) before g.l.c. on OV-17 (unmethylated derivatives) or OV-225 methylated derivatives).

O-Trimethylsilyl derivatives of simple monosaccharides were prepared by dissolving the dry sample (0.2–1.0mg) in Trisil Z (0.1–0.2ml) and incubating at 37°C for 30min as described by Sweeley *et al.* (1963). Excess reagents were evaporated under reduced pressure and the residue was reconstituted in dry *n*-hexane (50–100 μ l) before chromatography on SE-30. Methyl neuraminic acid methyl ester was treated as described above except that incubation conditions were extended to 60°C for 1h and g.l.c. was carried out on OV-225 at 190°C. The disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol was incubated with Trisil Z at 70°C for 2h and chromatographed on SE-30 (250°C) and on OV-17 (230°C).

Samples (0.5–1.0 μ l) of solutions of saccharide derivatives were chromatographed on columns (2m $\times$ 6.4mm) of coiled glass containing 3% OV-17 or OV-225 on High Performance (H.P.) Gas-Chrom Q or 3% silicone gum rubber SE-30 on H.P. Chromasorb W. Chromatography was carried out isothermally at 180°C (OV-225), 190°C (SE-30) or 210°C (OV-17) unless otherwise stated, with a flow rate of 45ml/min in a Perkin-Elmer F11 gas chromatograph equipped with a dual-flame ionization detector.

Unless otherwise stated retention times are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (prepared from methyl α -D-glucopyranoside by methylation, acid hydrolysis, reduction with NaBH₄ and acetylation as described above).

Gas-liquid chromatography-mass spectrometry. Samples were chromatographed with the column packings and conditions described above in a Pye 104 gas chromatograph coupled to a 12F mass spectrometer supplied by VG Micromass, Altrincham, Lancs., U.K., operating at 70eV.

Amino acid analyses. The absence of amino acids from purified oligosaccharide fractions was checked by using an amino acid analyser. Samples (100–300 μ g) in 6M-HCl (1ml), containing 20nm-norleucine as internal standard, were purged with N₂ and heated at 103°C in sealed tubes under an atmosphere of N₂ for 12, 18 and 24h. HCl was removed under vacuum and the residue was analysed in a Rank Hilger Chromaspek Auto high-pressure amino acid analyser.

Results and Discussion

Sialoglycopeptides were released by Pronase from intact milk-fat globules and purified by gel filtration on columns of Sephadex G-50 and Sephadex G-25 (Fig. 1) as described in the Materials and Methods section. Oligosaccharides were cleaved by alkaline NaBH₄ from the purified sialoglycopeptides, separated by gel filtration on a column of Sephadex G-25 (Fig. 2) and fractionated on columns of DEAE-Sephadex A-25 (Fig. 3) and Sephadex G-25 (Fig. 4) to give freeze-dried samples B' and C' (see the Materials and Methods section).

Structural characterization of fraction B' from Sephadex G-25

Amino acids were not detected in fraction B', which contained galactose, *N*-acetylgalactosaminitol and sialic acid in the molar proportions 1:1:2. The g.l.c. procedures used (see the Materials and Methods section) gave no evidence for the presence of other monosaccharide components and showed that *N*-acetylneuraminic acid was the only sialic acid present. Mild acid hydrolysis of the tetrasaccharide released the disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol, which was identified as its *O*-trimethylsilyl derivative by g.l.c. on two column packings by comparison with the derivative of an authentic sample (Newman *et al.*, 1976) (see the Materials and Methods section).

The tetrasaccharide was treated with sodium metaperiodate, reduced and acid hydrolysed, and alditol acetate derivatives were prepared as described in the Materials and Methods section. Parallel experiments were performed in which only the periodate step was omitted. Comparison by g.l.c.

indicated that periodate oxidation destroyed all the *N*-acetylgalactosaminitol moiety, whereas the galactose component was more than 95% preserved. Concomitant with the disappearance of the peak corresponding to *N*-acetylgalactosaminitol, only one significant new peak was observed (retention time 0.34 relative to that of 1,2,3,4,5,6-hexa-*O*-acetyl-galactitol), which had retention time and mass spectrum (major peaks, *m/e* 43, 60, 72, 84, 96, 102, 114, 144, 156) identical with those of 1,3,4-tri-*O*-acetyl-*N*-acetyl-D-threosaminitol, which was the only significant component of the gas chromatogram obtained when the disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol was oxidized with periodate and treated as described for the tetrasaccharide.

The above evidence indicates that the tetrasaccharide, fraction B', contains the core disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol, in which the galactopyranosyl, but not the *N*-acetylgalactosaminitol, moiety is protected from periodate oxidation by *N*-acetylneuraminic acid substitution. This, together with the production of threosaminitol after periodate oxidation, limits the possible tetrasaccharide structures to those in which the galactopyranosyl moiety is suitably disubstituted by *N*-acetylneuraminic acid or to that shown in Fig. 6(a) in which the galactopyranosyl moiety is mono-substituted at position C-3 and the *N*-acetyl-galactosaminitol unit is also substituted at position C-6.

Evidence that the galactopyranosyl moiety is not disubstituted by sialic acid was provided by further periodate-oxidation experiments. The tetrasaccharide was treated with sodium metaperiodate, reduced with NaBH₄, dissolved in 0.05M-pyridine acetate buffer, pH 5.0 (2.5ml), and applied to a column (16cm $\times$ 1.5cm) of DEAE-Sephadex A-25. Elution with 0.05M-pyridine acetate buffer followed by a linear gradient of 0.05–0.65M-buffer, as described in the legend to Fig. 3, gave a single broad hexose-containing peak in the position (peak II, Fig. 3) characteristic of saccharides carrying a single carboxylic acid group. This is consistent with an original tetrasaccharide structure in which the *N*-acetylgalactosamine moiety is substituted by sialic acid at position C-6. No hexose-containing peak was observed in the region characteristic of saccharides carrying two carboxylic acid groups (peak III, Fig. 3) such as would arise from periodate oxidation of a tetrasaccharide in which the galactopyranosyl moiety was disubstituted.

The hexose-containing fractions obtained after periodate oxidation, as described above, were combined, freeze-dried and analysed by g.l.c. for sialic acid (see the Materials and Methods section). Sialic acid could not be detected. This provides further evidence that the tetrasaccharide does not

contain a galactopyranosyl moiety carrying both sialic acid moieties (joined together by a 2 $\rightarrow$ 8 linkage) at the C-3 position. The non-terminal sialic acid in such a structure would be expected to be protected against periodate oxidation.

The structure of the tetrasaccharide was confirmed as that shown in Fig. 6(a) by methylation analysis. The tetrasaccharide was methylated and hydrolysed as described in the Materials and Methods section. G.l.c. of the alditol acetate derivatives of the resulting methylated monosaccharides showed only two significant peaks, I and II, with retention times 1.94 and 9.17 respectively (Fig. 5a). Peak I was identified as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol by mass-spectrometric comparison with a standard

Fig. 5. Gas-liquid chromatography of the alditol acetate derivatives of the hydrolysis products from methylated oligosaccharides

Purified oligosaccharides were methylated, hydrolysed and the products were converted into their alditol acetate derivatives as described in the Materials and Methods section. Samples (0.5–1.0 μ l) of the resulting dichloromethane solutions were chromatographed on columns (2m $\times$ 6.4mm) of coiled glass containing 3% OV-225 on high performance Gas-Chrom Q at 180°C with a flow rate of 45 ml/min in a Perkin-Elmer F11 gas-liquid chromatograph equipped with a dual-flame ionization detector. (a) Tetrasaccharide fraction B'; (b) trisaccharide fraction C'; (c) disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol; (d) major separated trisaccharide fraction C_I; (e) minor separated trisaccharide fraction C_{II}.

(major peaks: m/e 43, 45, 87, 101, 117, 129, 161, 233) derived from *N*-acetylneuraminyllactose. *N*-Acetylneuraminyllactose (Sigma Chemical Co.) is a mixture of *N*-acetylneuraminyllactose (β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucose (85%) and *N*-acetylneuraminyllactose (β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucose (15%) and, after borohydride reduction, methylation, hydrolysis and derivative formation, peaks corresponding to 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol (retention time 2.74) were obtained in the expected proportions. This identification of peak I from methylation of the tetrasaccharide as a trimethylalditol acetate and the absence of any peaks in the region expected for dimethylated galactitol derivatives (retention times 3.1–5.5; Jansson *et al.*, 1976) limit the structure of the tetrasaccharide to that of Fig. 6(a). The retention time and mass spectrum (major peaks: m/e 43, 88, 101, 117, 130, 142, 161, 243) of peak II from the methylated tetrasaccharide are consistent with its identification as 3,6-di-*O*-acetyl-1,4,5-tri-*O*-methyl-*N*-acetyl-*N*-methyl-D-galactosaminitol, which is expected on the basis of the proposed tetrasaccharide structure (Fig. 6a).

Structural characterization of fraction C' from Sephadex G-25

As with fraction B', fraction C' was found to be free of amino acids and to contain only galactose, *N*-acetylgalactosaminitol and *N*-acetylneuraminic acid, in this case in the molar proportions 1:1:1. Mild acid hydrolysis of this trisaccharide fraction released the disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol, which was identified as its *O*-trimethylsilyl derivative by g.l.c. as described above.

The trisaccharide fraction was treated with sodium metaperiodate, reduced, acid hydrolysed and alditol acetate derivatives were prepared as described in the

Materials and Methods section. Control experiments were carried out in which only the periodate-oxidation step was omitted. Analysis by g.l.c. indicated that the effect of periodate oxidation was to destroy completely the *N*-acetylgalactosaminitol moiety and approx. 18–20% of the galactose. As with the tetrasaccharide, the disappearance of the peak corresponding to *N*-acetylgalactosaminitol was accompanied by the appearance of a peak attributable to 1,3,4-tri-*O*-acetyl-*N*-threosaminitol.

The trisaccharide fraction was methylated, hydrolysed, reduced and acetylated as described for the tetrasaccharide. G.l.c. of the resulting mixture gave the pattern shown in Fig. 5(b), in which four major peaks include peaks I and II, obtained from the methylated tetrasaccharide together with two further peaks III (retention time 1.17) and IV (retention time 3.54).

Methylation and derivative formation of the disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosaminitol as described for the trisaccharide fraction gave a gas-liquid chromatogram (Fig. 5c) showing two peaks, which were identified by mass spectrometry as peaks III and IV derived from the trisaccharide fraction. The expected products from the methylated disaccharide are 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol and 3-*O*-acetyl-1,4,5,6-tetra-*O*-methyl-*N*-acetyl-*N*-methyl-D-galactosaminitol. Peak III was identified as the galactitol derivative by mass-spectrometric comparison (major peaks: m/e 43, 45, 71, 87, 101, 117, 129, 145, 161, 205) with an authentic standard prepared by methylation, acid hydrolysis, borohydride reduction and acetylation of methyl β -D-galactopyranoside. Peak IV must then correspond to the galactosaminitol derivative, which is consistent with its mass spectrum (major peaks: m/e 43, 45, 88, 101, 130, 133, 142, 216, 243).

The data obtained for the trisaccharide fraction C' can be fully explained if the fraction is a mixture of the two trisaccharides C'_I (Fig. 6b) and C'_{II} (Fig. 6c). In the gas-liquid chromatogram of methylated derivatives (Fig. 5b) peaks I and IV would then arise from trisaccharide C'_I and peaks II and III from trisaccharide C'_{II}. The observed ratio of peak I : peak II is approx. 4:1, indicating a similar C'_I:C'_{II} ratio. This ratio is further supported by the periodate-oxidation results reported above.

The trisaccharides C'_I and C'_{II} were separated by preparative t.l.c. as described in the Materials and Methods section and each trisaccharide was methylated, hydrolysed, reduced and acetylated as previously described for the mixture. Gas-liquid chromatograms for the major and minor components are shown in Figs. 5(d) and 5(e) respectively, which show that the original four-peak pattern of Fig. 5(b) has been completely resolved into two two-peak patterns [peaks I and IV (Fig. 5d) and peaks II and

Fig. 6. Structures of alkali-labile oligosaccharides (a) Tetrasaccharide fraction B': R = R' = α -*N*-acetylneuraminyllactose. (b) Trisaccharide fraction C'_I: R = α -*N*-acetylneuraminyllactose, R' = H. (c) Trisaccharide fraction C'_{II}: R = H, R' = α -*N*-acetylneuraminyllactose.

III (Fig. 5e)], consistent with the assignment of structures C'_I and C'_{II} (Figs. 6b and 6c) to the major and minor trisaccharide components respectively.

The experimental data obtained for the tetrasaccharide B' and for the trisaccharides C'_I and C'_{II} fully support their chemical structures as shown in Fig. 6. The finding that *N*-acetylneuraminic acid is the only sialic acid present in the oligosaccharides is consistent with previous failure to detect other sialic acids on the surface of the milk-fat-globule membrane (Newman & Harrison, 1973). The *N*-acetylneuraminyl linkages are designated in the α -anomeric form, in view of the known specificity of neuraminidase (Gottschalk & Drzeniek, 1972), which was found to be as effective as acid hydrolysis in releasing sialic acid in quantitative assays.

It has been shown previously (Harrison *et al.*, 1975) that release of oligosaccharide fragments by alkaline borohydride treatment of bovine milk-fat-globule-membrane glycopeptides is accompanied by a decrease in the serine and threonine content of the glycopeptide and by an increase in alanine and 2-aminobutyric acid contents. This evidence, together with the presence of *N*-acetylgalactosaminitol in all three oligosaccharides now characterized, indicates that the oligosaccharides arise from β -elimination of *O*-glycosidic linkages between *N*-acetylgalactosamine and serine or threonine in the membrane-derived glycopeptides. Each of the two trisaccharides, C'_I and C'_{II} , differs from the tetrasaccharide B' in lacking an *N*-acetylneuraminyl residue linked to either *N*-acetylgalactosaminitol (C'_I) or galactose (C'_{II}). Whereas it is possible that the trisaccharides, linked to protein, are present in the glycoprotein of the membrane, their production from the parent tetrasaccharide in the course of the isolation procedure cannot be ruled out.

Structures similar to that shown in Fig. 6 have been assigned, largely on the basis of periodate-oxidation data, to tetrasaccharides released from the erythrocyte membranes of humans (Thomas & Winzler, 1969), horses and sheep (Glöckner *et al.*, 1976) and from fetuin, the major glycoprotein of calf serum (Spiro & Bhoyroo, 1974). Finne (1975) has used methylation analysis to derive unambiguously the same structure for a tetrasaccharide obtained from glycopeptides resulting from papain digestion of lipid-free rat brain. From human erythrocyte membrane, fetuin and rat brain, a trisaccharide having the structure shown in Fig. 6(b) was isolated and characterized by the methods used for the parent tetrasaccharide.

Our isolation of the tetrasaccharide (Fig. 6a) from the surface of a mammalian membrane other than that of the erythrocyte poses questions about its location and role in cell membranes generally. The presence of the core disaccharide, β -D-galactopyrano-

syl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine, in sialic acid-substituted form, has been demonstrated on human and bovine lymphocyte plasma membranes (Newman & Uhlenbruck, 1977), and it is possible that the above tetrasaccharide structure is also present on these and other mammalian membranes. Structures of this type have been implicated in MN-blood-group specificity (Thomas & Winzler, 1971; Springer & Desai, 1975; Lisowska & Duk, 1975; Dahr *et al.*, 1975) and the availability, from bovine milk, of the tetrasaccharide, together with two related trisaccharides, in milligram quantities offers the possibility of using these oligosaccharides to define the structural specificity of similar membrane-surface antigens.

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