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SIALOGLYCOPEPTIDES FROM BOVINE MILK FAT GLOBULE MEMBRANE

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SUMMARY

Milk fat globule membrane was shown to contain sialic acid, all of which could be released without disruption of the fat globule. Sialoglycopeptides were cleaved from the surface of intact fat globules by Pronase and fractionated on Sephadex G-50. Further fractionation of the major sialoglycopeptide peak on DEAE-Sephadex gave two groups of sialoglycopeptides eluted with 0.1 M NaCl (Group A) and 0.5 M NaCl (Group B), respectively. Refractionation gave a major sialoglycopeptide from each of the two groups together with a total of three minor sialoglycopeptides. All five sialoglycopeptides eluted as single peaks using shallow salt gradients on DEAE-Sephadex and contained a hydrophilic peptide chain together with galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid. Glycopeptides of Group A but not Group B contained fucose.

The major sialoglycopeptide of Group B released 35 % of its hexose and hexosamine on treatment with alkaline borohydride leaving a sialoglycopeptide which had reduced serine and threonine and elevated alanine levels and in addition contained 2-aminobutyric acid. An oligosaccharide fraction containing *N*-acetylgalactosaminitol, galactose and sialic acid in a molar ratio of 1 : 1 : 2 was partially characterised from the cleavage mixture.

The major sialoglycopeptide of Group A had a more complex carbohydrate structure and showed no released carbohydrate on treatment with alkaline borohydride.

The sialoglycopeptides of milk fat globule membrane show many similarities with those of erythrocyte membrane and have a potential use in comparative and structural studies.

INTRODUCTION

Available evidence indicates that during milk secretion the milk fat globule gains a membrane which is derived directly from the plasma membrane of the mammary

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cell [1–3] and possibly also from Golgi vesicle membrane [4, 5]. Recent electron-microscope studies [6, 7] suggest that despite gradual loss after leaving the secretory cell a considerable percentage of the original membrane remains on the surface of globules in freshly expressed cream. There is some evidence, therefore, that milk fat globule membrane can represent a convenient source of mammalian membrane material for structural studies.

The protein content of milk fat globule membrane has been examined by several workers [1, 8, 9] and fractions containing hexose, hexosamine and sialic acid have been described [10, 11]. However, little has been reported concerning the detailed composition and structure of purified glycoproteins. Recent microelectrophoretic studies [12] have shown that sialic acid, together with protein amine and carboxyl groups, contributes to the surface charge of milk fat globules, suggesting the presence of sialoglycoproteins on the membrane surface. We now report the isolation and partial characterisation of sialoglycopeptides enzymically cleaved from the surface of the milk fat globule membrane. A preliminary account of some of this work has been presented [13].

MATERIALS AND METHODS

Isolation of milk fat globules

Uncooled milk from 12 Friesian cows in mid-lactation was pooled in an insulated container. Within 20 min of milking, the milk was warmed to 40 °C and separated in a bench cream separator (Alfa-Laval AE Farm Separator) calibrated to give 40 % w/v butter fat. The cream was diluted with three times its volume of double-distilled water at 40 °C and reseparated. The dilution-separation process was repeated a further three times to give washed cream.

Preparation of milk fat globule membrane suspension

Washed cream (100 ml) was cooled to 4 °C and shaken until butter formed. The buttermilk was separated from the butter, which was melted at 40 °C giving butter oil and butter serum. Combination of butter serum with buttermilk gave total milk fat globule membrane suspension (40 ml).

Analytical methods

Evaporations were carried out in vacuo at 30–35 °C. Membrane filtration was performed using Oxoid Membrane filters (pore size 0.45 μm, Oxoid Ltd, Southwark Bridge Rd, London S.E.I.).

Hexose and hexosamine. Total hexose was determined colorimetrically by a modification of the cysteine/H_2SO_4 assay [14].

For gas-liquid chromatographic analysis of individual hexoses and hexosamines in soluble carbohydrate-containing fractions, aliquots (0.1 ml) of solution (containing approx. 0.5 mg carbohydrate/ml) were diluted with an equal volume of 6 M HCl, saturated with nitrogen, sealed and heated at 100 °C for 4 h. The hydrolysates were cooled and an internal standard of mannitol (25 μg) was added to each sample. The solutions were then freed from excess HCl by azeotropic distillation with ethanol/benzene (4 : 1, v/v). The residue in water (0.5 ml) was applied to a column (5 cm x 0.5 cm) of Bio-Rad AG-50W (×8, H^+ form) resin. Neutral and basic components of

the hydrolysate were eluted with water (5 ml) and 2 M HCl (5 ml), respectively. The eluates were separately evaporated, freed from water and HCl by azeotropic distillation with ethanol/benzene (4 : 1, v/v) and converted into *O*-trimethylsilyl derivatives by incubation with Trisil (50 μl) (see Enzymes and chemicals) at 37 °C for 15 min. Excess reagents were evaporated and the dry residue was reconstituted in *n*-hexane (25–200 μl). Aliquots (1–2 μl) were chromatographed on a column (6 ft x 0.25 inch) of coiled glass packed with 3 % silicone gum rubber SE-301, on High Performance (H.P.) Chromasorb W, 80–100 mesh. Samples were chromatographed isothermally at 175 °C with a nitrogen flow of 30.8 ml/min in a Perkin-Elmer F11 gas chromatograph equipped with a dual flame ionisation detector. Standard carbohydrate solutions were chromatographed immediately before and after each batch of samples to check detector response. Hexosamines were calculated as their *N*-acetyl derivatives.

Fucose. Aliquots (0.1 ml) of carbohydrate-containing solution (approx. 0.2 mg carbohydrate/ml) were diluted with 0.025 M H_2SO_4 (0.4 ml) containing an internal standard of mannitol (100 μg) and heated at 100 °C for 30 min. The hydrolysate was cooled to 0 °C and neutralised with saturated $Ba(OH)_2$ solution. $BaSO_4$ was removed by centrifugation at 3000 x *g* for 3 min and the supernatant applied to a column (5 cm x 0.5 cm) of Bio-Rad AG-50W (X 8, H^+ form) resin. The aqueous eluate (5 ml) was evaporated, *O*-trimethylsilylated and analysed by gas-liquid chromatography as described for hexose and hexosamine but at 165 °C. 90 % recovery of fucose was obtained when a control sample was subjected to the above procedure.

Sialic acid. For the determination of sialic acid in soluble carbohydrate-containing fractions, aliquots (0.1 ml) of solution (containing approx. 50 μg carbohydrate/ml) were diluted with 0.1 M H_2SO_4 (0.1 ml) and heated in a sealed tube at 80 °C for 1 h. The cooled hydrolysate containing free sialic acid was applied to a column (5 cm x 0.5 cm) of Bio-Rad AG-1 (X 8, formate form) anion-exchange resin as described by Svennerholm [15]. The resin was eluted successively with water (10 ml) and 0.3 M formic acid (10 ml). Formic acid was removed from the second eluate by azeotropic distillation with ethanol/benzene (4 : 1, v/v) and the resulting residue was finally reconstituted in water (0.2 ml) and assayed for sialic acid using the Aminoff [16] modification of Warren's [17] thiobarbituric acid method. 97 % recovery of *N*-acetylneuraminic acid was obtained when a control sample was subjected to the above procedure. Sialic acid was routinely calculated as *N*-acetylneuraminic acid which was the only sialic acid chromatographically detected after neuraminidase treatment of milk fat globules [12].

Sialic acid was released from the milk fat globule membrane either by dilute acid or neuraminidase treatment. In the former procedure washed cream (2 ml) was diluted with water (1 ml) and 0.2 M H_2SO_4 (5 ml) and heated at 80 °C for 100 min. These conditions gave maximal release of sialic acid without rupture of the milk fat globules. The cream was removed by centrifugation at 3000 x *g* for 30 min and the subnatant membrane-filtered before assay for free sialic acid as described above. Neuraminidase release of sialic acid was effected by incubation of washed cream (2 ml) with enzyme (1 mg) in 0.05 M barbiturate buffer, pH 5.5 [12] (10 ml) at 37 °C for 3 h. Cream was removed by centrifugation and the subnatant membrane-filtered and assayed for free sialic acid as above. Direct microscopic observation following both acid and neuraminidase treatment confirmed that the fat globules were intact.

Total sialic acid content of disrupted membranes was determined by a combi-

nation of acid and extensive Pronase treatment of a membrane suspension. Milk fat globule membrane suspension (5 ml) was diluted with 0.2 M H_2SO_4 (10 ml) and heated at 80 °C for 100 min. The cooled hydrolysate was centrifuged at 3000 x *g* for 15 min and the supernatant dialysed (3 x 100 ml water, 2 h each at 4 °C). The diffusate was concentrated, membrane-filtered and assayed for free sialic acid as described above. The non-diffusible material was concentrated and incubated with 2 vol. of Ca^{2+} buffer (4 mM $CaCl_2$ in 0.05 M Tris·HCl buffer, pH 7.8) and 1 vol. of buffered pronase (0.05 M Tris–HCl buffer, pH 7.8, containing 150 μg pronase/ml) at 37 °C for 7 h. The cooled mixture was centrifuged at 3000 x *g* for 15 min, membrane-filtered and assayed for sialic acid as described for soluble carbohydrate-containing fractions. The total sialic acid content of the membrane was taken as the sum of assays for the diffusate and non-diffusible material.

Amino acids. Sialoglycopeptide solutions (50 μl) were saturated with nitrogen and hydrolysed in 6 M HCl in a sealed tube at 100 °C for 24 h. Samples were membrane-filtered and acid was removed by azeotropic distillation with ethanol/benzene (4 : 1, v/v). An internal standard of DL-norvaline was added to the residue and analysis was carried out using a Technicon TSM auto analyser.

Protein. Total protein content of fractions obtained from alkaline BH_4^- cleavage experiments was assayed by differential absorption at 215 and 225 nm as described by Waddell [18].

Enzymes and chemicals

Neuraminidase (EC 3.2.1.18) from *Clostridium perfringens*, trypsin (EC 3.4.21.4) from bovine pancreas and *N*-acetylneuraminic acid were from Sigma London Ltd, Kingston-upon-Thames, Surrey. Pronase (B. grade, *Streptomyces griseus* protease) was from Calbiochem. Ltd, 10, Wyndham Place, London W.1. Trisil (hexamethyldisilazane and trimethylchlorosilane in dry pyridine) is a product of the Pierce Chemical Co. Ltd and was purchased from Pierce and Warriner Ltd, Chester, Cheshire. Unless otherwise stated all other laboratory reagents were from BDH Ltd, Poole, Dorset.

EXPERIMENTAL AND RESULTS

Sialic acid content of milk fat globule membrane

The total sialic acid content of milk fat globule membranes was released by subjecting a suspension of membrane fragments to a combination of extensive Pronase and mild acid treatment (Materials and Methods) when 110 ± 10 μg sialic acid were obtained from the membranes corresponding to 1 ml washed cream. Similar amounts of sialic acid (115 ± 4 μg/ml washed cream) were also obtained by treatment of milk fat globules with either mild acid or neuraminidase apparently without disruption of the globules. This suggests that the sialic acid is all present on the outer surface of the membrane although penetration of the membrane by neuraminidase or acid cannot be discounted.

Effect of washing milk fat globule membrane

Washed cream was gently stirred for 30 min with media of low and of high ionic strength (distilled water and 1.0 M NaCl) and also of varying pH (2–11). The result-

ing suspensions were centrifuged at 3000 x *g* for 3 min and the subnatants analysed for sialic acid-containing material. No significant quantities of sialic acid were released by any of the washing media. Extrinsic or absorbed proteins are normally removed by such washing techniques [19] and it appears therefore that the sialic acid of the milk fat globule membrane is not derived from adsorbed serum glycoproteins.

Proteolytic release of sialoglycopeptides from milk fat globule membrane

Trypsin. Washed cream was suspended in 4 vol. of buffered trypsin (0.01 M NaH_2PO_4 adjusted to pH 7.8 with 0.1 M NaOH, containing 50 μg trypsin/ml) and incubated at 37 °C. Release of sialoglycopeptides was followed by periodic removal of aliquots, centrifugation at 3000 x *g* for 3 min, membrane-filtration of the subnatant and assay for bound sialic acid (Materials and Methods). After 30 and 60 min incubation 23 % and 40 %, respectively, of the total membrane sialic acid had been released as sialoglycopeptides. Microscopic observation indicated extensive disruption of milk fat globules after 60 min incubation and this was confirmed by the appearance of butter oil.

Pronase. Washed cream was suspended in 2 vol. of Ca^{2+} buffer (4 mM $CaCl_2$ in 0.05 M Tris · HCl buffer, pH 7.8) and incubated at 37 °C with 1 vol. of buffered pronase (0.05 M Tris · HCl buffer, pH 7.8, containing 150 μg pronase/ml.) Release of sialoglycopeptides was determined as above for trypsin incubations. Approx. 40 % of the total membrane content of sialic acid was released after 30 min without visible disruption of the milk fat globules which became evident only after 60 min incubation. After 4 h incubation 100 % of the total sialic acid content of the milk fat globule membrane had been released and this was accompanied by total disruption of the fat globules and extensive butter oil formation.

Preparative-scale release of sialoglycopeptides from milk fat globule membrane

Conditions were chosen so as to obtain the maximum yield of sialoglycopeptides with the minium of fat globule disruption and in view of the above experiments Pronase was preferred to trypsin for this purpose. Washed cream (500 ml), Ca^{2+} buffer (1000 ml) and buffered pronase (500 ml) were separately warmed to 37 °C, mixed and incubated at 37 °C for 30 min with gentle stirring. Microscopic observation of the suspension after this treatment gave no evidence of milk fat globule disruption. The digest was cooled in ice and centrifuged at 3000 x *g* for 30 min to give a subnatant which was concentrated (500 ml) and extensively dialysed against distilled water at 4 °C. The non-diffusible material was concentrated (10 ml) and the resulting cream-coloured suspension centrifuged at 125 000 x *g* for 2 h. A pale yellow solution resulted which contained 18 mg hexose and 21 mg sialic acid. The solution was further concentrated to 2 ml.

Fractionation of Pronase-cleaved fragments on Sephadex G-50

The above concentrate (2 ml) was applied to a column (40 cm x 2.5 cm) of Sephadex G-50 (fine) (Pharmacia (OB) Ltd, Uxbridge Rd, London W.5), previously equilibrated with water at 4 °C. The column was eluted with water at 4 °C with a flow rate of 10 ml/h. Fractions (3.5 ml) were automatically collected and samples analysed for hexose and sialic acid.

$A_{280\ nm}$ was continuously monitored using an LKB Uvicord 2. The elution patterns

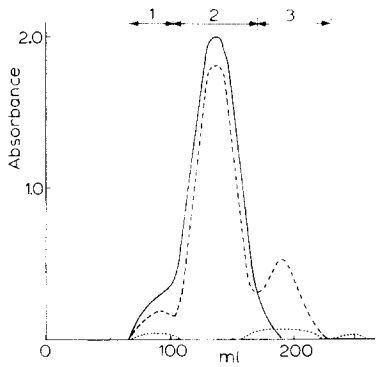


Fig. 1. Gel filtration of pronase -cleaved glycopeptides on Sephadex G-50. The dialysed, centrifuged pronase digest (Experimental and Results) was concentrated and applied to a column (40 cm × 2.5 cm) of Sephadex G-50 equilibrated with water at 4 °C. Elution was with water at 4 °C with a flow rate of 10 ml/h. Fractions (3.5 ml) were collected and analysed for hexose ($A_{420\ nm}$, - - -) and sialic acid ($A_{550\ nm}$, —). $A_{280\ nm}$ (. . .) was monitored using an LKB Uvicord 2.

are shown in Fig. 1. A major peak (Peak 2) contained approx. 90 % of the sialic acid applied to the column and about 70 % of the hexose. Calibration of the column using proteins of known molecular weight (bovine serum albumin, α-chymotrypsin, myoglobin and insulin) gave a mean molecular weight for this partially included peak of approx. 8000 with a spread from 7000–9000. Only very low $A_{280\ nm}$ was associated with this fraction, indicating a low content of aromatic amino acids.

A minor peak (Peak 1), eluted in the void volume, contained the remaining sialic acid and some hexose, while a small hexose-containing peak (Peak 3), free from sialic acid, followed the major peak.

Fractionation on DEAE-Sephadex A-25 of Peak 2 glycopeptides

Fractions corresponding to the major peak (Peak 2, Fig. 1) from Sephadex G-50 fractionation of Pronase-cleaved glycopeptide fragments from two batches (2 x 500 ml) of washed cream were combined, concentrated (150 ml), dialysed extensively against water at 4 °C and further concentrated (2 ml). The concentrate was applied to a column (40 cm x 2.5 cm) of DEAE-Sephadex A-25, previously equilibrated with 0.05 M Tris·HCl buffer (pH 7.8) at 4 °C. The column was successively eluted at 4 °C with buffer (85 ml), 0.1 M NaCl in buffer (85 ml) and 0.5 M NaCl in buffer (150 ml). Fractions (3 ml) were automatically collected and samples analysed for hexose and sialic acid. The elution patterns are shown in Fig. 2. Two major peaks, 2A and 2B, eluted with 0.1 M and 0.5 M NaCl, respectively, contained all the sialic acid and most of the hexose applied to the column. The hexose was distributed roughly equally between the two peaks while the sialic acid was divided in the ratio of approx.2 : 1 between Peaks 2B and 2A, respectively. The ratio varied slightly with different batches of cream. In addition to the two major fractions some sialic acid-free, hexose-containing material was eluted with buffer alone.

Fractions corresponding to Peak 2A (Fig. 2) were collected, extensively dialysed against water at 4 °C, concentrated and refractionated on a column (40 cm x 1 cm) of DEAE-Sephadex A-25 as before except that a linear salt gradient (0–0.1 M

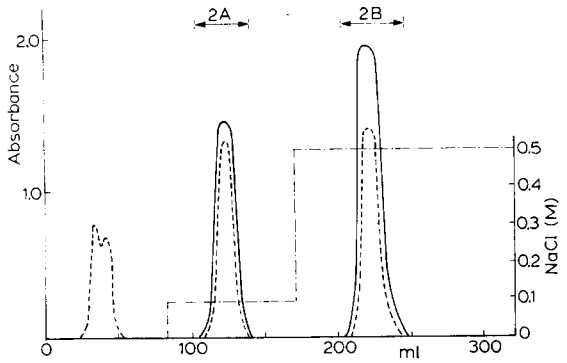
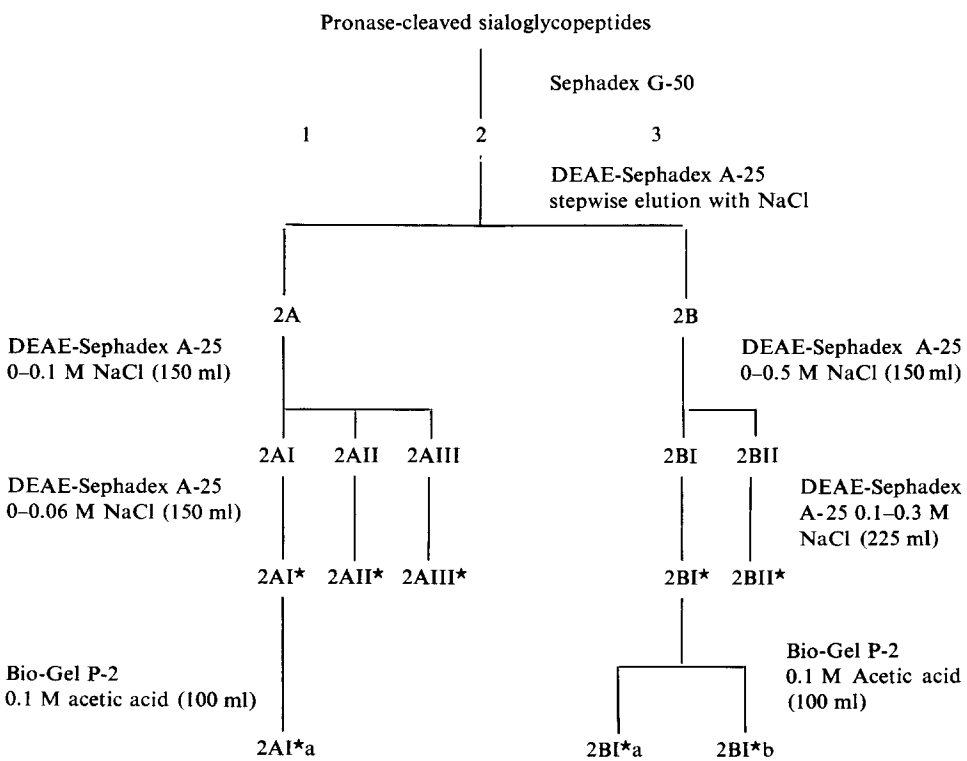


Fig. 2. Fractionation on DEAE-Sephadex of the major hexose-sialic acid peak (Fig. 1) from Sephadex G-50. Fractions corresponding to Peak 2 from Sephadex G-50 were combined, dialysed, membrane-filtered, concentrated and applied to a column (40 cm × 2.5 cm) of DEAE-Sephadex A-25 equilibrated at 4 °C with 0.05 M Tris·HCl buffer (pH 7.8). The column was eluted successively with 0.05 M Tris · HCl buffer, pH 7.8 (85 ml), 0.1 M NaCl in buffer (85 ml) and 0.5 M NaCl in buffer (150 ml) with a flow rate of 10 ml/h. Samples of fractions (3 ml) were analysed for hexose ($A_{420\ nm}$, - - -) and sialic acid ($A_{550\ nm}$, —). NaCl content of the eluant is shown (–·–·–·).

Scheme 1. Fractionation of pronase-cleaved sialoglycopeptides.



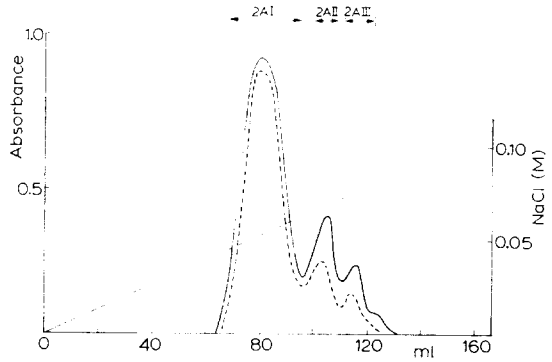


Fig. 3. Fractionation on DEAE-Sephadex of Peak 2A (Fig. 2) from DEAE-Sephadex. Fractions corresponding to Peak 2A were combined, dialysed, concentrated and applied to a column (40 cm × 1 cm) of DEAE-Sephadex A-25 equilibrated with 0.05 M Tris · HCl buffer (pH 7.8) at 4 °C. The column was eluted with a linear gradient of 0–0.1 M NaCl (–·–·–·) in buffer (150 ml) with a flow rate of 10 ml/h. Samples of fractions (3 ml) were analysed for hexose ($A_{420\ nm}$, - - -) and sialic acid ($A_{550\ nm}$, —).

NaCl (150 ml)) was used (Scheme 1). The elution pattern (Fig. 3) showed a major hexose-sialic acid peak (2AI) followed by two minor peaks (2AII and 2AIII). Fractions corresponding to all three peaks 2AI, 2AII and 2AIII were separately collected, treated and fractionated as above using shallower (0–0.06 MNaCl (150 ml)) gradients on DEAE-Sephadex A-25 (Scheme I) when single hexose-sialic acid Peaks 2AI*, 2AII* and 2AIII*, respectively, were obtained.

Fractions corresponding to Peak 2B (Fig. 2) were similarly refractionated on columns (40 cm x 1 cm) of DEAE-Sephadex A-25 using a linear salt gradient (0–0.5 M NaCl (150 ml)) when a major (2BI) and a minor (2BII) hexose-sialic acid peak were obtained (Fig. 4) together with two or more much smaller peaks, depending on the

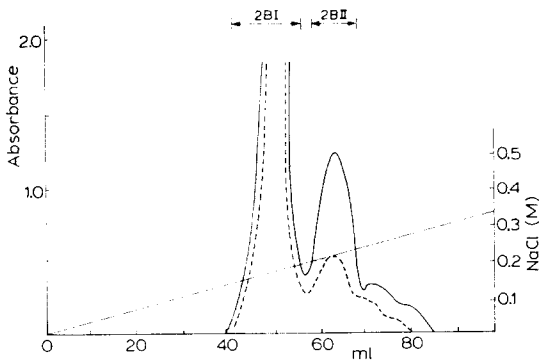


Fig. 4. Fractionation on DEAE-Sephadex of Peak 2B (Fig. 2) from DEAE-Sephadex. Fractions corresponding to Peak 2B were combined, dialysed, concentrated and applied to a column (40 cm × 1 cm) of DEAE-Sephadex A-25 equilibrated with 0.05 M Tris · HCl buffer (pH 7.8) at 4 °C. The column was eluted with a linear gradient of 0–0.5 M NaCl (–·–·–·) in buffer (150 ml) with a flow rate of 10 ml/h. Samples of fractions (3 ml) were analysed for hexose ($A_{420\ nm}$, - - -) and sialic acid ($A_{550\ nm}$, —).

batch of cream. Further fractionation of Peaks 2BI and 2BII (Scheme I) on shallow gradients (0.1–0.3 M NaCl (225 ml)) gave single hexose-sialic acid Peaks 2BI* and 2BII*. Solutions corresponding to the Peaks 2AI*, 2AII*, 2AIII*, 2BI* and 2BII* were separately collected, dialysed as previously and concentrated each to 2 ml.

Molecular-weight fractionation of major sialoglycopeptides 2AI and 2BI**

Concentrates (2 ml) of the major sialoglycopeptide Peaks 2AI* and 2BI* from DEAE-Sephadex fractionation were each applied to a column (40 cm x 1 cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Ltd, St. Albans, Herts.) previously equilibrated with 0.1 M acetic acid. The columns were eluted (10 ml/h) with 0.1 M acetic acid at 4 °C and fractions (1 ml) automatically collected. Samples of the fractions were assayed for hexose and sialic acid.

The concentrate of 2AI* eluted as a single hexose-sialic acid Peak 2AI*a while that of 2BI* gave rise to a major Peak 2BI*a, containing approx. 90 % of the hexose and sialic acid applied to the column, followed by a minor partially included Peak 2BI*b. Solutions of the sialoglycopeptide Fractions 2AI*a and 2BI*a were separately concentrated and freed from acetic acid by evaporation with ethanol/benzene (4 : 1, v/v). The residues were each made up to 2 ml with distilled water.

Analysis of purified sialoglycopeptides

The major (2AI*a and 2BI*a) and minor (2AII*, 2AIII* and 2BII*) purified fractions were analysed for individual sugars and amino acids (Tables I, II, III). All fractions were sialoglycopeptides containing galactose, mannose, *N*-acetylgalactosamine and *N*-acetylglucosamine as well as sialic acid (Table II). Similarities in relative carbohydrate content were apparent within each of the two groups of glycopeptides derived from Peaks 2A and 2B (Scheme I). Thus, Fractions 2AI*a, 2AII* and 2AIII* all contained relatively more *N*-acetylglucosamine and mannose than Fractions 2BI*a and 2BII* which had high percentages of galactose, *N*-acetylgalactosamine and sialic acid. Only fractions derived from the 2A-group contained fucose. The peptide portions of all fractions examined were chiefly composed of polar amino acids (Table III) and aromatic residues were completely absent, in accordance with the lack of A_{280nm} shown during initial fractionation (Fig. 1). Glycopeptides of the 2A-group contained a generally higher percentage of aspartate and a lower percentage of serine and threonine than those derived from Peak 2B. These differences were particularly marked in the case of the two major sialoglycopeptides 2AI*a and 2BI*a.

TABLE I

OVERALL COMPOSITION OF PURIFIED SIALOGLYCOPEPTIDES

Values are mg present in the total fraction and are averages from three batches. Variation was less than ±5 % of each value. Figures in parentheses are weight percent of total.

	Fraction				
	2AI*a	2AII*	2AIII*	2BI*a	2BII*
Total carbohydrate	5.70 (73)	2.00 (62)	2.87 (67)	2.45 (55)	0.80 (20)
Total protein	2.01 (27)	1.21 (38)	1.39 (33)	2.04 (45)	3.22 (80)
Total weight	7.71	3.21	4.26	4.49	4.02

TABLE II

CARBOHYDRATE COMPOSITION OF PURIFIED SIALOGLYCOPEPTIDES

Values are μmol/mg glycopeptide and are averages of five batches. Variation was less than ± 5 % of each value. Figures in parentheses are mol percent of total carbohydrate.

Carbohydrate	Fraction				
	2AI*a	2AII*	2AIII*	2BI*a	2BII*
Galactose	0.31 (9.4)	0.51 (18.8)	0.45 (15.6)	0.66 (29.0)	0.12 (15.2)
Mannose	0.35 (10.6)	0.34 (12.5)	0.32 (11.1)	0.13 (5.7)	0.004 (0.5)
Fucose	0.01 (0.3)	0.13 (4.8)	0.10 (3.5)	—	—
Glucose	—	—	—	—	—
N-Acetylgalactosamine	0.69 (20.9)	0.38 (14.0)	0.42 (14.5)	0.53 (23.2)	0.27 (34.2)
N-Acetylglucosamine	1.58 (47.9)	0.69 (25.4)	0.84 (29.1)	0.17 (7.5)	0.05 (6.3)
N-Acetylneuraminic acid (sialic acid)	0.36 (10.9)	0.67 (24.6)	0.76 (26.3)	0.79 (34.6)	0.35 (44.3)

The sialoglycopeptide 2AI*a contained approx. 70 % by weight of carbohydrate which, taken with the analytical figures (Table II) and a molecular weight of approx. 8000 would be in accord with a molar content of galactose (3 mol), mannose (3 mol), *N*-acetylgalactosamine (6 mol), *N*-acetylglucosamine (13 mol) and *N*-acetylneuraminic acid (3 mol) plus a small fucose content.

In the second major sialoglycopeptide 2BI*a the mole percentages of total

TABLE III

AMINO ACID COMPOSITION OF PURIFIED SIALOGLYCOPEPTIDES

Values are μmol/mg glycopeptide and are averages of two batches. Variation was less than ± 3 % of each value. Figures in parentheses are mol percent of total amino acid content.

Amino acid	Fraction				
	2AI*a	2AII*	2AIII*	2BI*a	2BII*
Lysine	0.40 (19.2)	0.70 (24.5)	0.33 (10.1)	0.32 (8.2)	0.42 (6.3)
Histidine	—	0.25 (8.7)	—	0.05 (1.3)	—
Arginine	—	0.08 (2.8)	0.59 (18.1)	0.02 (0.5)	0.05 (0.8)
Aspartic acid	0.39 (18.8)	0.28 (9.8)	0.28 (8.6)	0.17 (4.4)	0.62 (9.4)
Threonine	0.21 (10.1)	0.20 (7.0)	0.31 (9.5)	0.68 (17.4)	1.47 (22.1)
Serine	0.23 (11.1)	0.38 (13.1)	0.51 (15.6)	0.97 (24.8)	1.00 (15.1)
Glutamic acid	0.05 (2.4)	0.29 (10.1)	0.17 (5.2)	0.22 (5.6)	1.23 (18.6)
Proline	0.21 (10.1)	0.35 (12.2)	0.50 (15.3)	0.67 (17.1)	0.78 (11.8)
Glycine	0.04 (1.9)	0.12 (4.2)	0.13 (4.0)	0.24 (8.1)	0.46 (6.9)
Alanine	0.18 (8.7)	0.14 (4.9)	0.27 (8.3)	0.40 (10.2)	0.46 (6.9)
Cystine	—	—	—	—	—
Valine	0.35 (16.8)	0.03 (1.1)	0.08 (2.5)	0.10 (2.6)	0.07 (1.1)
Methionine	—	—	—	—	—
Isoleucine	0.01 (0.5)	—	0.05 (1.5)	0.04 (1.0)	—
Leucine	0.01 (0.5)	0.04 (1.4)	0.04 (1.2)	0.03 (0.8)	0.07 (1.1)
Tyrosine	—	—	—	—	—
Phenylalanine	—	—	—	—	—

carbohydrate of galactose (29.0), mannose (5.7), *N*-acetylgalactosamine (23.2) *N*-acetylglucosamine (7.5) and sialic acid (34.6) (Table II) correspond to an approximate ratio of 5 : 1 : 4 : 1 : 6. The presence of galactose (5 units), mannose (1 unit), *N*-acetylgalactosamine (4 units), *N*-acetylglucosamine (1 unit) and sialic acid (6 units) in one molecule of the sialoglycopeptide would fit well with the observed total carbohydrate content of 55 % by weight and an approximate molecular weight of 7500.

*Alkaline borohydride treatment of purified sialoglycopeptides 2AI*a and 2BI*a*

A sample (1.0 ml) of the solution of sialoglycopeptide 2BI*a was incubated for 24 h at 22 °C in the dark with a solution of 0.8 M $NaBH_4$ in 0.1 M NaOH (0.5 ml). Cleavage of alkali-labile glycopeptide linkages was followed by monitoring A_{241nm}, corresponding to appearance of olefinic amino acids. After 6 h no further increase in A_{241nm} was apparent and after 24 h excess borohydride was removed by acidifying the solution (to pH 6.5) with 2 M acetic acid. The neutralised solution was lyophilised and H_3BO_3 was removed from the residue by repeated evaporation with methanol/HCl (1000 : 1, v/v). The residue was made up to approx. 0.5 ml and applied to a column (100 cm x 1 cm) of Sephadex G-50 (fine). The column was eluted with 0.1 M acetic acid at 10 ml/h and fractions (1 ml) were automatically collected and analysed for hexose, sialic acid and A_{241nm}. The elution pattern is shown in Fig. 5. A large peak (F1) containing hexose and sialic acid and showing A_{241nm} was eluted near the void volume, and a smaller peak (F2) containing hexose and sialic acid, but no protein or A_{241nm}, was partially included. A third totally included peak (F3) contained only sialic acid. Fractions corresponding to Peaks F1, F2 and F3 were separately collected concentrated and freed of acetic acid by evaporation with ethanol/benzene (4 : 1, v/v). The residues of Peaks F1, and F2 and F3 were made up with distilled water to 2, 1 and 1 ml, respectively, and analysed for individual sugars and amino acids (Table IV).

Treatment of sialoglycopeptide 2AI*a with alkaline borohydride followed by gel filtration on Sephadex G-50, as described above for 2BI*a gave rise to a single sialo-

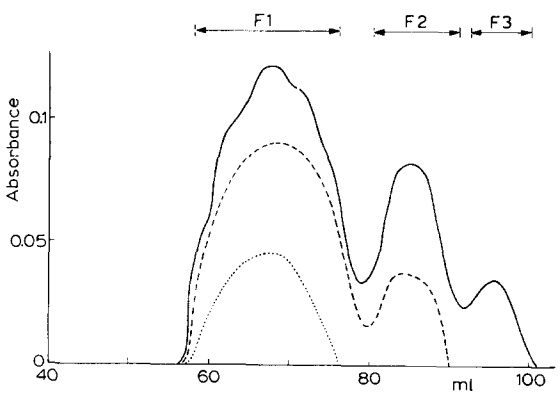


Fig. 5. Fractionation on Sephadex G-50 of products from alkaline borohydride treatment of sialoglycopeptide 2BI*a. The lyophilised reaction product was freed from H_3BO_3 (Experimental and Results) and applied to a column (100 cm × 1 cm) of Sephadex G-50 (fine). The column was eluted at 4 °C with 0.1 M acetic acid with a flow rate of 10 ml/h. Samples of fractions (1 ml) were assayed for hexose ($A_{420\ nm}$, - - -), sialic acid ($A_{550\ nm}$, —) and $A_{241\ nm}$ (. . .).

TABLE IV

AMINO ACID AND CARBOHYDRATE COMPOSITION OF SIALOGLYCOPEPTIDE 2BI*a AND OF ITS ALKALINE BOROHYDRIDE DEGRADATION PRODUCTS

Values are μmol present in the total fraction and are averages of two batches. Variation was less than ±5 % of each value.

	Fraction			
	2BI*a	F1	F2	F3
Lysine	0.73	0.66		
Histidine	0.13	0.13		
Arginine	0.06	trace		
Aspartic acid	0.38	0.38		
Threonine	1.55	0.96		
Serine	2.19	1.30		
Glutamic acid	0.50	0.42		
Proline	1.51	1.43		
Glycine	0.55	0.59		
Alanine	0.90	1.03		
Valine	0.22	0.19		
Isoleucine	0.09			
Leucine	0.08	0.08		
2-Aminobutyric acid		trace		
Galactose	1.50	0.71	0.08	
Mannose	0.29	0.29		
N-Acetylgalactosamine	1.20	1.00		
N-Acetylglucosamine	0.39	0.23		
N-Acetylgalactosaminitol			0.08	
N-Acetylneuraminic acid (sialic acid)	1.78	0.46	0.16	0.24

glycopeptide peak in the void volume with no evidence of further carbohydrate-containing fractions.

Comparison of the carbohydrate contents of Fraction 2BI*a and FI (Table IV) shows that alkaline borohydride treatment of Fraction 2BI*a resulted in loss of approx. 35 % by weight of the total hexose and hexosamine, mainly as galactose and *N*-acetylgalactosamine, together with most of the sialic acid. The amino acid compositions of Fractions 2BI*a and F1 were very similar except that F1 showed decreases in serine and threonine and a smaller, but significant, increase in alanine content together with the appearance of trace amounts of 2-aminobutyric acid (Table IV). These results are consistent with the activity of alkaline borohydride in releasing carbohydrate from Fraction 2BI*a by β-elimination from glycopeptide linkages to serine or threonine. Alkaline cleavage of such bonds is known [20] to result in formation of 2-aminoacrylic and 2-aminocrotonic acids, respectively, which can be reduced by borohydride to alanine and 2-aminobutyric acid.

Fraction F2, released by alkaline borohydride treatment of the glycopeptide 2BI*a, contained only galactose, *N*-acetylgalactosaminitol and sialic acid in the molar ratio 1 : 1 : 2 (Table IV). The position of elution of Fraction F2 from the Sephadex column is consistent with a molecular weight of approx. 1000, suggesting that the major component of this fraction is a tetrasaccharide containing two molecules of sialic acid

and one each of galactose and *N*-acetylgalactosaminitol. It is probable that *N*-acetylgalactosaminitol arises from borohydride reduction of an *N*-acetylgalactosamine unit following its release by the alkaline conditions from its involvement in a glycopeptide bond.

The totally included Peak F3 contained only sialic acid.

DISCUSSION

The present evidence that the sialic acid content of four times washed milk fat globules is contained in intrinsic membrane components and not in adsorbed serum glycoproteins is supported by an earlier report [12] that the electrophoretic mobility of such globules is not affected by five further washings with distilled water. Further support is afforded by the work of Jackson et al. [11] who showed that milk fat globule membrane glycoproteins are immunologically distinct from those found in milk serum.

The most detailed structural studies on mammalian membrane glycoproteins have been carried out on erythrocyte membrane components. In particular, considerable data have been derived from glycopeptides enzymically cleaved from erythrocytes and many parallels exist between such glycopeptides and those cleaved by Pronase from milk fat globule membrane in the present study. Sialoglycopeptides from the two sources fractionate on DEAE-Sephadex in a remarkably similar manner [21] and many common points of structure emerge. In both cases peptide chains composed of hydrophilic amino acids carry the sugars galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose and sialic acid. Alkaline borohydride treatment of erythrocyte-derived sialoglycopeptides releases a tetrasaccharide fragment containing galactose (1 mol), *N*-acetylgalactosaminitol (1 mol) and sialic acid (2 mol) which has been shown to result from cleavage of an *O*-glycosidic linkage between *N*-acetylgalactosamine and a serine or threonine residue in the polypeptide chain [22]. Treatment of the purified sialoglycopeptide Fraction 2BI*a with alkaline borohydride also release carbohydrate leaving a glycopeptide which shows little change in its amino acid composition apart from decreased serine and threonine levels and increased alanine and 2-aminobutyric acid content. This indicates that here too carbohydrate is released by β-elimination from serine and threonine residues. Not all the serine and threonine lost from the peptide chain is, in fact, accounted for as alanine and 2-aminobutyric acid suggesting that borohydride reduction of the double-bonded intermediate acids is incomplete. Such an explanation is supported by the presence of $A_{241\,nm}$ in the F1-fraction (Fig. 5).

The released carbohydrate in Fraction F2 contains only galactose, *N*-acetylgalactosaminitol and sialic acid and their molar ratios together with their elution position suggest that they are present as a tetrasaccharide containing two units of sialic acid. This again reflects results in the erythrocyte system. Galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid remain in the glycopeptide Fraction F1 following alkaline borohydride treatment and it is possible that they are attached to the peptide chain via an alkali-stable *N*-glycosidic linkage between *N*-acetylglucosamine and asparagine such as been shown to occur in erythrocyte glycopeptides. Indeed the molar ratios of *N*-acetylglucosamine and aspartic acid in Fraction F1 are roughly equivalent. Fraction F1 has a relatively low sialic acid content com-

pared with that of its precursor 2BI*a possibly as a result of loss during the recovery procedure following alkaline borohydride treatment. Some of the sialic acid is recovered as monosaccharide units in Fraction F3 which contains only sialic acid.

Alkaline borohydride treatment of the second major sialoglycopeptide 2AI*a results in no detectable released carbohydrate and only a single sialoglycopeptide peak is eluted from Sephadex G-50 following treatment. This indicates that most of the carbohydrate of Fraction 2AI*a is attached to the peptide chain by alkali-stable linkages which, as suggested for Fraction 2BI*a, probably involve *N*-acetylglucosamine and asparagine; a suggestion which is supported by the relatively high content of both *N*-acetylglucosamine and aspartic acid in this fraction. The structures of the oligosaccharides in Fraction 2AI*a are clearly complex and while little detail is available in this study it is clear that 2AI*a and the alkali-stable glycopeptides [22] from erythrocyte membrane contain largely the same monosaccharide units in similarly complex oligosaccharide structures.

Many of the structural features common to glycopeptides derived from milk fat globule membrane and from erythrocyte membrane have been demonstrated in other mamalian membranes [22, 23] and their occurrence together in the milk fat globule membrane glycopeptides suggests that the latter are derived from a true mammalian membrane. In view of its availability in relatively large quantities, milk fat globule membrane accordingly consitutes a potentially useful source of mammalian membrane glycoprotein for structural studies. In particular, membrane-derived glycopeptides of defined composition could be extremely useful in helping to elucidate the structures of less available cell surface receptors using comparative techniques such as hapten inhibition studies, etc.

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