Biochimica et Biophysica Acta, 433 (1976) 344–356

BBA 77290

ALKALI-LABILE OLIGOSACCHARIDES FROM BOVINE MILK FAT GLOBULE MEMBRANE GLYCOPROTEIN

ROLAND A. NEWMAN[a], ROGER HARRISON[b] and GERHARD UHLENBRUCK[a]

[a] *Department of Immunobiology, Medical University Clinic, Cologne, Kerpener Strasse 15 (G.F.R.) and*
[b] *Department of Biochemistry, University of Bath, Claverton Down, Bath (U.K.)*

(Received September 16th, 1975)

SUMMARY

Phenol extraction of bovine milk fat globule membrane gave a glycoprotein fraction which, in sodium dodecyl sulphate electrophoresis, showed three major bands, all staining for both protein and carbohydrate. Alkaline borohydride treatment and desialylation of the glycoprotein fraction released the reduced disaccharide β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosamine (T-antigen), which was identified by gas chromatography using a standard. All of the disaccharide units in the native glycoprotein were shown to be substituted by sialic acid, and a tetrasaccharide containing the disaccharide plus two molecules of sialic acid was isolated following alkaline borohydride treatment of the glycoprotein and gel filtration. Periodate oxidation of native and desialylated glycoprotein, together with paper chromatography of alkali degraded oligosaccharide fragments, indicated that the major alkali-labile oligosaccharide of the glycoprotein fraction is a tetrasaccharide containing β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosamine substituted by sialic acid at position C3 of the galactosyl and position C6 of the *N*-acetyl-D-galactosamine residue. Evidence was also obtained for the presence of small amounts of unsubstituted alkali-labile *N*-acetyl-D-galactosamine linked directly to protein in the native glycoprotein.

Serological evidence using agglutinins from *Vicia graminea*, *Arachis hypogoea* and human anti-T serum confirmed the presence in the native glycoprotein of a sialic acid substituted T-antigen. Similar evidence using agglutinins from *Helix pomatia* and *Cepaea hortensis* also confirmed the presence of terminal alkali-labile *N*-acetyl-D-galactosamine in the native glycoprotein.

INTRODUCTION

Two types of carbohydrate-protein linkage are commonly found in membrane, serum and secreted glycoproteins: *N*-acetylgalactosaminyl-serine (threonine) and *N*-acetylglucosaminyl-asparagine [1]. Both types of linkage have been shown to occur in sialoglycopeptides isolated from the surface of the milk fat globule membrane by pronase digestion [2], the former being cleaved in alkali via a β-elimination reaction. Treatment of pronase-cleaved glycopeptides from the milk fat globule membrane

with $NaOH/NaBH_4$ leads to the destruction of the hydroxyamino acids serine and threonine with the concomitant release of a reduced oligosaccharide. The released oligosaccharide contains the sugars sialic acid, galactose and *N*-acetylgalactosaminitol in the molar ratio 2 : 1 : 1 [2].

The work reported in this paper investigates the structure of the alkali-labile oligosaccharide, its intersaccharide linkages and serological activity against several heterophile agglutinins and antibodies.

MATERIALS AND METHODS

Reagents

Neuraminidase (*Vibrio cholerae*: 500 units/ml) was obtained from Behringwerke (Marburg/Lahn). Monosaccharides for gas chromatographic standards, *N*-acetylneuraminic acid, Dowex 50W×8 (200–400 mesh) and Dowex 1×2 (200–400 mesh) were obtained from Serva Ltd. (Heidelberg). Crystalline β-D-galactosyl (1 → 3)-*N*-acetyl-D-galactosamine from human brain gangliosides [3] was a gift from Professor W. Gielen (Pharmakologisches Institut der Universität Köln).

Anti-A agglutinins from *Helix pomatia* and *Cepaea hortensis* and the lectin from *Arachis hypogoea* were prepared as described by Dahr et al. [4, 5]. Anti-T antibody was prepared from human serum (group O) after appropriate absorption [4, 5].

An extract of *Vicia graminea* was prepared by grinding approximately 30 seeds in a mortar and extracting the resulting powder with unbuffered physiological saline (2 ml) for 2 h with frequent shaking. The resulting suspension was centrifuged (4000× *g*, 15 min,) the supernatant filtered through a millipore filter (pore size 0.45 μm) and the extract used immediately. Overnight storage at 4 °C or freezing considerably weakened or totally abolished the agglutinating activity.

A crude myxovirus (B-Lee) solution was a gift from the late Professor A. Gottschalk and was centrifuged and filtered before use.

All other general reagents were obtained from Merck Ltd. (Darmstadt).

Preparation of glycoprotein fractions

Milk fat globule membranes, isolated as previously described [2], were washed three times with distilled water and once with physiological saline and then extracted with 90 % phenol as described for erythrocytes by Uhlenbruck et al. [6].

Chemical treatments

Desialylation of glycoproteins and oligosaccharides. Desialylated glycoproteins and oligosaccharides were prepared by mild acid hydrolysis (0.05 M H_2SO_4, 80 °C for 1 h) followed by neutralisation with Dowex 1 (OH^- form) resin. The resin was removed by centrifugation (2500×*g*, 5 min) and washed three times with distilled water, following which the combined supernatant and washings was lyophilized. Incubation with neuraminidase was found to be equally effective as the acid hydrolysis procedure in all cases tested.

Alkaline borohydride treatment. Alkaline borohydride treatment of glycoprotein samples was performed by dissolving glycoprotein (1–2 mg) in 0.05 M NaOH containing 1.0 M $NaBH_4$ (2 ml) and incubating for 24 h in the dark at 50 °C. Tre-

halose (30 μg) was added as an internal standard and excess borohydride destroyed by adjusting to pH 6.0 with Dowex 50 (H^+ form) resin until no more gas was evolved. After centrifugation (2500 $\times g$, 5 min) the supernatant was removed and the resin washed three times with distilled water. The combined supernatant and washings was lyophilized and boric acid was removed by repeated evaporation with methanol.

Alkaline treatment. Glycoprotein (10 mg) in 0.02 M $Ba(OH)_2$ solution was heated at 80 °C for 15 min, cooled and neutralised with 0.05 M H_2SO_4. The precipitate of $BaSO_4$ was removed by centrifugation (4000 $\times g$, 15 min) and the supernatant concentrated (1 ml) and examined by paper chromatography [7, 8]. Human erythrocyte membrane glycoprotein and *N*-acetyl-D-galactosamine were separately treated with alkali under the above conditions and the products used as chromatographic standards.

Periodate oxidation. Periodate oxidation of native or desialylated glycoprotein was performed by dissolving the sample (4–5 mg) in 0.02 M sodium acetate buffer (2 ml, pH 4.5) containing 10 μmol $NaIO_4$. The reaction mixture was cooled in ice and left in the dark at 0 °C for 20 h, after which excess sodium borohydride was added to reduce any remaining periodate. The excess borohydride was then destroyed by adjusting to pH 6.0 with 2.0 M acetic acid, followed by dialysis overnight at 4 °C and lyophilization.

Sephadex chromatography of alkaline borohydride-treated glycoprotein

Glycoprotein (10 mg) that had been treated with alkaline borohydride and freed of boric acid was dissolved in water (2 ml) and applied to a column of Sephadex G-25 (1 $\times$ 100 cm) previously equilibrated with 0.1 M acetic acid. The column was eluted with 0.1 M acetic acid at 4 °C and fractions were collected and assayed for sialic acid and hexose.

Lyophilized fractions eluted from the Sephadex G-25 column were dissolved in water (2 ml) and applied to a column of Sephadex G-15 (1 $\times$ 100 cm) that had also been previously equilibrated with 0.1 M acetic acid. The column was eluted with 0.1 M acetic acid and fractions collected which were assayed for sialic acid and hexose.

Analytical procedures

Sialic acid. Total sialic acid was released by hydrolysis in 0.05 M H_2SO_4 for 1 h at 80 °C and assayed by the method of Aminoff [9] using *N*-acetyl-neuraminic acid as a standard. Free sialic acid was assayed directly without prior hydrolysis.

Hexoses and hexosamines. Total hexose was determined colorimetrically by the cysteine-sulphuric acid method [10] using D-galactose as a standard.

Individual hexoses and hexosamines in carbohydrate-containing fractions were determined by hydrolysis followed by gas-liquid chromatography. Glycoprotein samples (0.5–1.0 mg) were hydrolysed in 3 M HCl for 4 h at 100 °C, after which erythritol (30 μg) was added as an internal standard. The solution was neutralised by the addition of Ag_2CO_3 until gas ceased to be evolved and the suspension removed by centrifugation (2500 $\times g$, 10 min), washed with distilled water and recentrifuged. The washing and centrifugation was twice repeated and the combined supernatant and washings were lyophilized. The lyophilizate was reacetylated by incubation with acetic anhydride for 16 h at room temperature [11], relyophilization and overnight storage in vacuo over $CaCl_2$.

The dried free monosaccharides were trimethylsilylated for 30 min under conditions described by Sweeley et al. [12], except that hexamethyldisilazane and trimethylchlorosilane were used in the reaction mixture.

Aliquots of the trimethylsilylated material (1–2 μl) were chromatographed on a Hewlett-Packard 7620A Research Chromatograph on columns of coiled glass (6 foot × 0.25 inch) packed either with 3.1 % silicone gum rubber SE-30 on Gas Chrom Q (80–100 mesh) or with 3 % OV-17 on Gas Chrom Q (Serva Ltd.) using a temperature programme from 125 to 230 °C (2 °C per min) with a nitrogen flow rate of 45 ml/min.

Sugar amounts were calculated automatically by a Hewlett-Packard HP 3380A integrator, pre-programmed with appropriate correction factors or alternatively by measuring peak heights.

Disaccharides. Free disaccharides were assayed by gas chromatography as described above for free monosaccharides except that they were trimethylsilylated for 2 h and chromatographed isothermally at 250 °C with trehalose as an internal standard.

Immunological assays

Haemagglutination inhibition tests were performed using a Cooke serial dilution microtitre system (Cooke Instruments, Zollikon, Switzerland). Native and desialylated glycoprotein solutions, at a concentration of 5 mg/ml, were used for inhibition studies.

Desialylated erythrocytes were prepared from outdated human blood (Blood bank, Medical University of Cologne) by incubation of a 2 % suspension in physiological saline (20 ml) with 200 μl of neuraminidase solution for 1 h at 37 °C, following which the cells were washed three times with physiological saline. 2 % suspensions were used for immunological assays.

EXPERIMENTAL AND RESULTS

Preparation of glycoprotein fractions

Samples of the phenol-extracted glycoprotein were run on sodium dodecyl sulphate polyacrylamide gels using the discontinuous system of Laemmli [13] (Fig. 1). Three major bands and traces of two minor bands were detected, all of which stained for both carbohydrate and protein. In addition a diffuse fast-running band stained for carbohydrate only (Fig. 1, gel B). This component was not removed by extraction with chloroform/methanol or for a second time with phenol, indicating that it is tightly bound to glycoprotein.

The total carbohydrate composition of the glycoprotein fraction is shown in Table I.

Alkali-labile oligosaccharides from the glycoprotein fraction

Identification of disaccharide. The lyophilized glycoprotein fraction was treated with alkaline borohydride, desialylated and assayed gas chromatographically for reduced disaccharide. A single peak (retention times 1.66 (SE-30) and 1.87 (OV-17) relative to trehalose) was detected, which co-chromatographed with a standard of β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosamine that had been subjected to the above

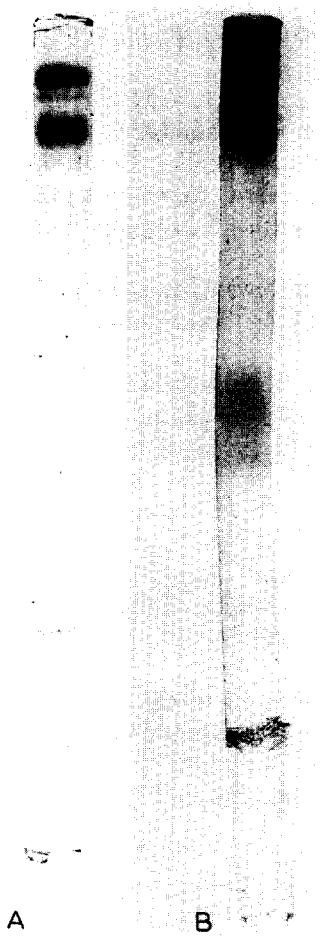

Fig. 1. Sodium dodecyl sulphate gel electrophoresis patterns of the glycoprotein fraction using 10 % acrylamide containing gels. Samples (75 μg) of lyophilized glycoprotein were prepared as described by Dahr et al. [14] for erythrocyte proteins. Gel A was stained for protein with Coomassie brilliant blue and gel B for carbohydrate with periodate-Schiff reagent [14, 15].

TABLE I

CARBOHYDRATE COMPOSITION OF GLYCOPROTEIN FRACTION

Values are averages of 6 batches. Variation was less than 10 % of each value.

Monosaccharide	mg/100 mg glycoprotein	μmol/100 mg glycoprotein
Fucose	0.55	3.35
Mannose	3.74	20.8
Galactose	8.64	48.0
N-Acetylgalactosamine	4.51	20.4
N-Acetylglucosamine	3.12	14.1
Glucose	4.68	26.0
Sialic acid	9.40	30.3

alkaline borohydride procedure. The reduced disaccharide comprised 4.6 % by weight of the total glycoprotein fraction and accounted for approximately one half of the total *N*-acetylgalactosamine.

Alkaline borohydride treatment of the glycoprotein fraction followed by gas chromatography without prior desialylation gave no peak corresponding to the reduced disaccharide, which is presumably all substituted by sialic acid in the native glycoprotein. When the above alkaline borohydride-treated glycoprotein fraction was analysed directly for free monosaccharides by gas chromatography, small amounts of *N*-acetylgalactosaminitol (comprising approx. 1 % by weight of the reduced disaccharide) were detected, suggesting that some unsubstituted *N*-acetyl galactosamine is linked directly to the protein. The peak was not increased when a desialylation step was introduced.

Sephadex chromatography of alkali-labile oligosaccharides. Passage of the alkaline borohydride treated glycoprotein fraction through a column of Sephadex G-25 allowed the eluate to be pooled into three fractions (Fig. 2), which were then lyophilized. After desialylation, only fraction B was found to contain the reduced disaccharide. This fraction was lyophilized and passed through a column of Sephadex G-15, the eluate from which was pooled into two fractions (Fig. 3). Samples from fraction B1 (Fig. 3) were assayed for total sialic acid and, after desialylation, for reduced disaccharide. The reduced disaccharide β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosaminitol was found to be present to the extent of 1 mol per 2.18 mol of sialic acid. The reduced disaccharide could not be detected unless the sample wa first desialylated and no free sialic acid was present.

Periodate oxidation of glycoprotein fraction

Native glycoprotein was oxidised with periodate, treated with alkaline borohydride, desialylated and assayed for reduced disaccharide. β-D-Galactosyl-(1 → 3)-*N*-acetyl-D-galactosaminitol was detected to the extent of 60 % of that of a control from which the periodate step was omitted. When the glycoprotein was desialylated prior

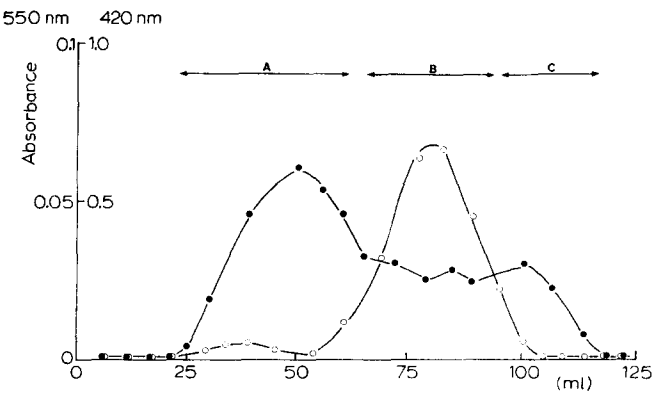

Fig. 2. Fractionation on Sephadex G-25 of the products from alkaline borohydride treatment of the glycoprotein fraction. The boric acid-free product in water (2 ml) was applied to a column (1 × 100 cm) of Sephadex G-25, equilibrated at 4 °C with 0.1 M acetic acid. The column was eluted at 4 °C with 0.1 M acetic acid with a flow rate of 10 ml/h. Samples of fractions (1 ml) were analysed for hexose ($A_{420\,nm}$, ●) and sialic acid ($A_{550\,nm}$, ○). Fractions were pooled as indicated and lyophilized.

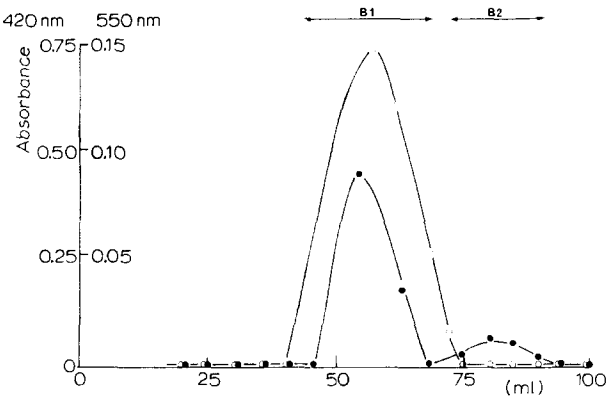

Fig. 3. Fractionation on Sephadex G-15 of fraction B (Fig. 2) from Sephadex G-25. Lyophilized Fraction B in water (2 ml) was applied to a column (1 × 100 cm) of Sephadex G-15 equilibrated at 4 °C with 0.1 M acetic acid. The column was eluted at 4 °C with 0.1 M acetic acid with a flow rate of 10 ml/h. Samples of fractions (1 ml) were analysed for hexose ($A_{420\,nm}$, ●) and sialic acid ($A_{550\,nm}$, ○). Fractions were pooled as indicated and lyophilized.

to periodate oxidation, no disaccharide was found, indicating that in the native glycoprotein at least 60 % of the alkali-labile disaccharide is protected from periodate oxidation by sialic acid.

Alkaline treatment of glycoprotein fraction

Alkaline treatment of *N*-acetylgalactosamine produced zones corresponding

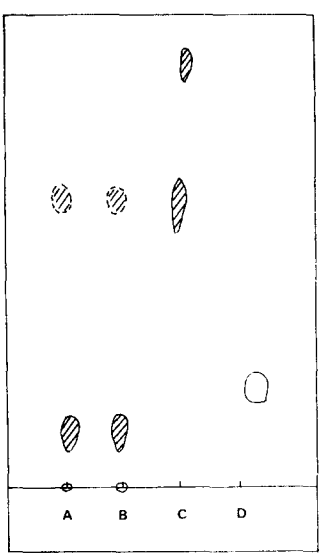

Fig. 4. Tracing of chromatogram of products of alkaline hydrolysis of (A) human erythrocyte membrane glycoproteins, (B) milk fat globule membrane glycoprotein and (C) *N*-acetylgalactosamine. (D) Untreated standard of sialic acid. Spots shown: ● gives a reddish-purple colour with Ehrlich spray [7] in the cold, ○ gives a violet colour with orcinol trichloroacetic acid spray [8]. Chromatograms were run on Whatman No. 1 paper in *n*-butanol/pyridine/water (6:4:3, v/v/v) [7].

TABLE II

INHIBITION OF AGGLUTININS BY MILK FAT GLOBULE MEMBRANE GLYCOPROTEIN

Values are expressed as their reciprocals. Values represent the minimum amount of inhibitor required to bring about inhibition of four agglutination doses of agglutinin. Glycoprotein samples at a concentration of 5 mg/ml were used against (1) desialylated human erythrocytes of group O; (2) desialylated human erythrocytes of group NN or MN; (3) human erythrocytes of group NN or MN; (4) human erythrocytes of group A; (5) human erythrocytes of group O. n.t., not tested.

Glycoprotein	Source of Agglutinin						
	A. hypogoea (1)	Human anti-T (1)	*V. graminea* (2)	Rabbit anti-N (3)	*H. pomatia* (4)	*C. hortensis* (4)	Myxovirus (5)
Native glycoprotein	2^5	2^0	ϕ	ϕ	2^7	2^9	2^6
Desialylated glycoprotein	2^9	2^8	2^4	n.t.	2^7	2^9	ϕ
Alkaline borohydride-treated glycoprotein	n.t.	ϕ	ϕ	n.t.	2^2	2^5	n.t.
β-D-Galactosyl(1 → 3)-*N*-acetyl-D-galactosamine	2^5	2^3	ϕ	n.t.	ϕ	ϕ	n.t.

to Chromogens I and III [16], which were visualised on paper chromatograms using the direct Ehrlich spray [7]. Alkaline treated fractions from milk fat globule membrane and erythrocyte membrane both showed traces of Chromogen I together with a major direct-Ehrlich-positive spot with R_F 0.1 (Fig. 4). Material migrating with the same R_F has been obtained from alkali-treated bovine salivary mucoprotein and identified as sialyl-chromogen I derived from a 6-α-*O*-sialyl-*N*-acetylgalactosamine residue in the mucoprotein [7]. The zone has also been previously identified in alkali-treated erythrocyte membrane glycoprotein [8] and its production from milk fat globule membrane glycoprotein indicates the presence in this glycoprotein also of a 6-*O*-sialyl-*N*-acetyl-D-galactosamine residue.

When the alkali-treated membrane glycoprotein fraction was subjected to mild acid hydrolysis prior to chromatography, the spot with R_F 0.1 disappeared and was replaced by zones corresponding to Chromogen I and sialic acid, thus confirming the identity of the R_F 0.1 spot as sialyl-Chromogen I.

Immunological assays

Desialylated glycoprotein fraction inhibited haemagglutination of desialylated erythrocytes both by human serum anti-T antibody and by the lectin from *A. hypogoea*, both of which are reported [5, 17] to be specific for the disaccharide β-D-galactosyl (1 → 3)-*N*-acetyl-D-galactosamine (T-antigen). Desialylated glycoprotein also inhibited haemagglutination of desialylated group NN or MN erythrocytes, by the extract from *V. graminea*, known to be specific for blood group N [18] and to require the presence of the disaccharide moiety β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosamine for activity.

Native glycoprotein did not inhibit the haemagglutination activity of the *V. graminea* extract and only weakly that of anti-T antibody but did moderately inhibit the activity of the lectin from *A. hypogoea*. Alkaline borohydride treatment of desialylated glycoprotein abolished the inhibitory activity against anti-T antibody and against *V. graminea* extract (Table II).

Agglutinins from *H. pomatia* and *C. hortensis*, specific for terminal *N*-acetylgalactosamine and terminal α-linked *N*-acetylgalactosamine respectively [4], were both strongly inhibited by native glycoprotein. The inhibition was not increased following desialylation of the glycoprotein and was considerably reduced followed alkaline borohydride treatment (Table II).

DISCUSSION

Phenol extraction of milk fat globule membrane gave a fraction which showed three major bands on sodium dodecyl sulphate gel electrophoresis together with two minor bands. As all bands stained for both protein and carbohydrate, it is apparent that the phenol extraction procedure is relatively specific for glycoprotein in this system. The glycoprotein fraction contains the same six monosaccharide residues that were detected in glycopeptides cleaved from the milk fat globules by pronase [2] and in addition contains glucose.

Alkaline borohydride treatment of the glycoprotein fraction released a carbohydrate fraction which, following removal of sialic acid, was found to contain the reduced disaccharide β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosaminitol. The re-

duced disaccharide was identified by gas chromatographic comparison with a standard, with which it co-chromatographed on two different columns. The presence of β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosamine in the glycoprotein fraction is supported by haemagglutination inhibition studies in which desialylated glycoprotein was shown to inhibit the activity of anti-T antibody and the lectins from *A. hypogoea* and *V. graminea*, all of which are specific for structures containing the disaccharide. The loss of inhibitory activity against anti-T antibody and *V. graminea* following alkaline borohydride treatment of the glycoprotein emphasises the alkaline lability of the disaccharide-protein linkage.

β-D-Galactosyl(1 → 3)-*N*-acetyl-D-galactosamine is known as the Thomsen-Friedenreich antigen (T-antigen) [20] and has been found attached to protein chains by alkali-labile linkages in glycoproteins from a variety of sources including submaxillary gland [21], brain [22], Antarctic fish [23] and human erythrocyte membrane [24]. The T-antigen has also been demonstrated in non-human erythrocyte membrane glycoproteins with only trace amounts in bovine erythrocytes (manuscript in preparation). The disaccharide is usually substituted by sialic acid or fucose, although unsubstituted T-antigen has been demonstrated in tumour cells [25].

β-D-Galactosyl(1 → 3)-*N*-acetyl-D-galactosaminitol was only detected following desialylation of glycoprotein components from milk fat globule membrane, indicating that in this membrane the T-antigen is always substituted by sialic acid. The isolation, following alkaline borohydride treatment of the glycoprotein fraction, of a tetrasaccharide containing β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosaminitol substituted by two molecules of sialic acid, is in accordance with the evidence of a similar tetrasaccharide in pronase-cleaved glycopeptides [2] and suggests that such a tetrasaccharide may be the major source of the alkali-labile disaccharide in this glycoprotein. The results of periodate oxidation of native and chemically treated glycoprotein show that at least 60 % of the β-D-galactosyl(1 → 3)-*N*-acetyl-D-galactosamine residues are protected by sialic acid from destruction by periodate. The true figure is likely to be considerably higher than 60 % as the periodate oxidation procedure involves a dialysis step which is not included in the control experiment and which can lead to loss of material. Protection against periodate, of the galactosyl residue of the disaccharide, implies that this moiety is either substituted at C3 or disubstituted. Both substitution patterns are clearly possible in a tetrasaccharide containing the disaccharide plus two molecules of sialic acid and the results of alkaline treatment of the glycoprotein fraction are relevant in this context.

Paper chromatography following treatment of the glycoprotein fraction with $Ba(OH)_2$ demonstrated the presence of sialyl-Chromogen I as the major component, with only traces of Chromogen I itself. Alkaline treatment of *N*-acetyl-D-galactosamine is known to produce Chromogens I and III (Fig. 5, R=H) which can be visualised on paper chromatograms using the direct Ehrlich spray [7]. Sialyl-Chromogen I must have arisen from 6-*O*-sialyl-*N*-acetyl-D-galactosamine residues as this chromogen cannot be produced from a 4-substituted galactosamine and C3 may be either substituted or free as this substituent is eliminated during the reaction [7]. Gas chromatography of alkaline-treated glycoprotein provided evidence for the presence in the native glycoprotein of single *N*-acetylgalactosamine units linked directly to protein, although in very low amounts, but not for sialyl-*N*-acetylgalactosamine. It appears, therefore, that the 6-*O*-sialyl-*N*-acetyl-D-galactosamine must have arisen

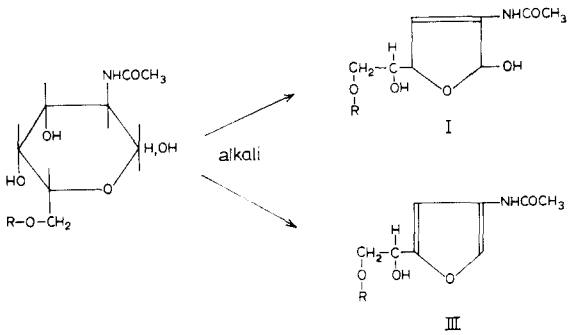

Fig. 5. Formation of chromogens after alkaline treatment of *N*-acetylgalactosamine derivatives after Gottschalk and Graham [7].

from a C3 substituted derivative and in view of the proven existence of β-D-galactosyl-(1 → 3)-*N*-acetyl-D-galactosamine in the glycoprotein, the obvious choice for the precursor of sialyl-Chromogen is the 6-*O*-sialyl derivative of the latter disaccharide. If this trisaccharide is indeed present in the isolated tetrasaccharide, then, in order to satisfy the periodate oxidation evidence, the second sialic acid residue must be attached to C3 of the galactosyl moiety. The tetrasaccharide structure consistent with all these data is shown in Fig. 6, in which the sialyl linkages are shown as α in view of the known specificity of neuraminidase [26], which was as effective as acid hydrolysis in cleaving the sialic acid residues in all cases tested.

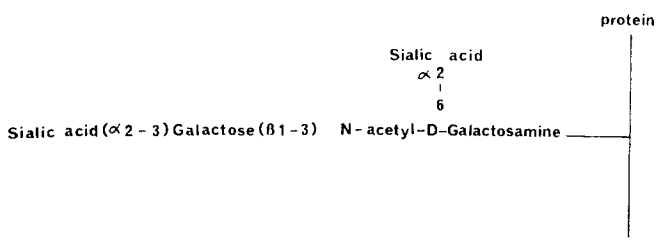

Fig. 6. Proposed structure of alkali-labile tetrasaccharide from milk fat globule membrane glycoprotein.

Traces of Chromogen I itself were detected following alkaline treatment of the glycoprotein, which could have arisen from protein-linked unsubstituted *N*-acetyl-D-galactosamine residues, the presence of which was demonstrated by gas chromatography and confirmed by haemagglutination inhibition studies using agglutinins from *H. pomatia* and *C. hortensis*. Alternatively, degradation of the sialyl-Chromogen could have occurred to a minor extent in the presence of alkali giving rise to trace amounts of Chromogen.

The structure isolated from milk fat globule membrane (Fig. 6) is presumably also on bovine mammary cell plasma membrane [27, 28] and shows for the first time that the structure is not specific to the membrane of the erythrocyte [24].

The inhibition of myxoviral haemagglutination by native glycoprotein but not by desialylated glycoprotein confirms evidence from other sources [19] that sialic acid is an essential component of the myxoviral binding site.

It is of interest to compare the antigenicity of bovine milk fat globule membrane with that of bovine erythrocytes, as some significant differences are apparent. The bovine erythrocyte, which has been desialylated by neuraminidase, is agglutinated neither by anti-T serum nor the agglutinin from *H. pomatia* [20], suggesting the absence, or presence in small amounts, of galactosamine-containing alkali-labile oligosaccharide chains. Glycoproteins from the milk fat globule membrane, however, strongly inhibit *H. pomatia* agglutinin and, after desialylation, also strongly inhibit anti-T serum. It is of interesting immunological significance that an animal can express human blood group A-like activity on a cell such as the mammary secretory cell (milk fat globule membrane) and lack such a specificity on its erythrocytes.

Milk fat globule membrane glycoproteins also show certain similarities to another primate blood group substance, blood group N substance. While *V. graminea* lectin, which is considered to be blood group N specific, is inhibited by milk fat globule membrane glycoproteins, rabbit anti-N antibodies were not inhibited, suggesting that, although a structure is present on the glycoprotein displaying certain similarities to blood group N substance (as detected by *V. graminea*), the protein core is not sufficiently identical enough to allow the expression of "true" blood group N activity.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB/68/IV). Thanks are expressed to Dr. Wolfgang Dahr for gifts of human erythrocyte membrane glycoprotein and anti-N serum. Drs. Wolfgang Dahr and Wolfgang Glöckner are thanked for helpful discussion and Frau Dorit Karduck for technical assistance.

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