Characterization of the Oligosaccharide Units of the Bovine Erythrocyte Membrane Glycoprotein†

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ABSTRACT: The major glycoprotein of the bovine erythrocyte membrane was purified by extraction of the ghosts with lithium 3,5-diiodosalicylate followed by phenol–water extraction and acidification. The glycoprotein contains 20% protein and 80% carbohydrate by weight and gives a single band on sodium dodecyl sulfate–polyacrylamide gels with an estimated molecular weight of 230 000 daltons. The carbohydrate composition of the glycoprotein was determined to be (in residues relative to sialic acid): sialic acid, 1.0; fucose, <0.01; mannose, 0.1; galactose, 3.3; *N*-acetylgalactosamine, 0.9; and *N*-acetylglucosamine, 2.4. Pronase digestion of the isolated glycoprotein followed by Sephadex G-75 gel filtration resulted in the separation of a small pool of glycopeptides (pool III), which included all of the mannose-containing glycopeptides, from the bulk of the glycopeptide material which was in the void fractions of the column (pool I). Alkaline borohydride treatment released over 95% of the oligosaccharide units in pool I and approximately 30% of the oligosaccharide units in pool III. These oligosaccharides were isolated by gel filtration and ion-exchange chromatography. The oligosaccharides released from pool I had molecular weights of 1100–1400 daltons and contained sialic acid, galactose, and *N*-acetylglucosamine in molar ratios of 0.5–1:3:2 as well as a partial residue of *N*-acetylgalactosaminitol. The oligosaccharides released from pool III by alkali had molecular weights of 1300–1600 daltons and contained sialic acid, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylgalactosaminitol in molar ratios of 1–2:2:1:1:1. These data indicate that the majority of the oligosaccharide units of the bovine erythrocyte glycoprotein are linked O-glycosidically to the peptide backbone of the molecule.

There is now considerable evidence indicating that cell membrane glycoproteins participate in a variety of biological functions. These molecules serve as receptors for viruses, hormones, and lectins and may mediate certain types of cell–cell interactions (Hughes, 1973). Despite their prominent role in membrane-mediated phenomena, only a few membrane glycoproteins have been purified and characterized. The best studied of these is the major sialoglycoprotein of the human erythrocyte (Winzler, 1969; Segrest et al., 1973). This molecule, with a molecular weight of about 31 000, contains 60% carbohydrate in the form of multiple oligosaccharide chains located toward the amino-terminal end of the molecule (Thomas and Winzler, 1969). The structures of the two major types of oligosaccharide chains of the sialoglycoprotein have been elucidated (Kornfeld and Kornfeld, 1971; Thomas and Winzler, 1969, 1971).

In order to determine the degree to which the carbohydrate units of erythrocyte glycoproteins vary between species, we decided to isolate and characterize the bovine erythrocyte glycoprotein. The bovine erythrocyte was selected because preliminary studies revealed that the lectin binding properties of this species' cells were strikingly different from those found with human erythrocytes, indicating a major difference in their respective oligosaccharide units. In this paper we describe the isolation and composition of the major oligosaccharide units of the bovine erythrocyte glycoprotein. These oligosaccharide units are distinctly different from those found in the sialoglycoprotein of the human erythrocyte.

Experimental Procedure

Materials. Carrier-free ^{125}I was purchased from Mallinckrodt Nuclear and NaB^3H_4 from New England Nuclear Corporation. ^{14}C-Labeled *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol were prepared from their respective ^{14}C-labeled N-acetylated amino sugars, which were obtained from New England Nuclear Corporation, by treatment with 0.1 N NaOH–0.4 M $NaBH_4$ at 37 °C for 3 h. The reaction mixtures were acidified to pH 5.0 with 1 N acetic acid and dried in vacuo. The residue was dissolved in methanol, dried in vacuo to remove methyl borates, redissolved in water, and desalted over Dowex AG50X12.

Emulphogene, BC720, an alkoxypoly(ethyleneoxy)ethanol was purchased from GAF Corporation, New York, New York. 3,5-Diiodosalicyclic acid was obtained from Aldrich Chemical Company and its lithium salt purified by recrystallization from ethanol at 4 °C. DEAE-cellulose (DE-52) was purchased from Whatman Corporation.

Enzymes. Pronase was purchased from Calbiochem and Tos-PheCH_2Cl[1] trypsin from Worthington Biochemical Corporation.

Analytical Methods. Sugar compositions of erythrocyte stroma, glycoproteins, glycopeptides, and oligosaccharides were determined by gas–liquid chromatography of trimethylsilyl derivatives of the methyl glycosides released by

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[1] Abbreviations used: PAS, periodic acid Schiff's reagent; Tos-Phe CH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

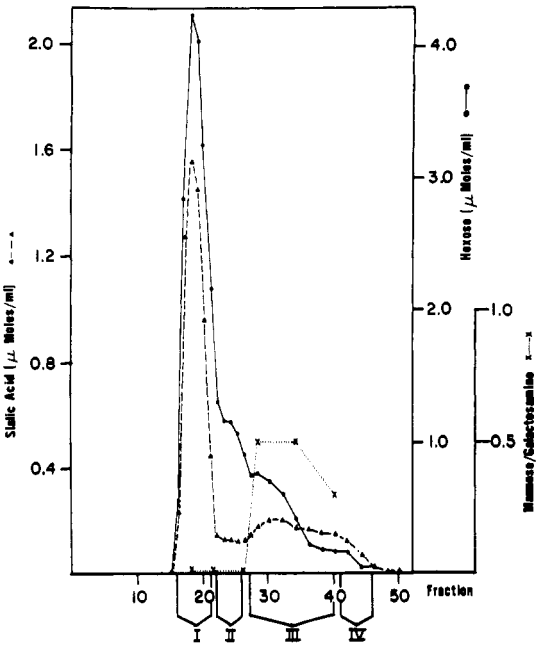

FIGURE 1: Gel filtration of a Pronase digest of bovine erythrocyte membrane glycoprotein on Sephadex G-75. The glycoprotein (465 mg) was digested with Pronase as described in Experimental Procedure, applied to a Sephadex G-75 column (2 × 75 cm), and eluted with 0.1 M pyridine acetate buffer, pH 6.4. The fractions (5 ml) were assayed for hexose and sialic acid. Selected fractions were assayed for total carbohydrate composition by gas-liquid chromatography, and the ratio of mannose to galactosamine was computed. Hexose (●—●); sialic acid (▲- - -▲); ratio mannose/galactosamine (X···X). The fractions were pooled as indicated.

methanolysis in 1.5 N methanolic HCl for 5 h at 95 °C as previously described (Baenziger, et al., 1974).

Sialic acid was determined by the method of Warren (1959), following hydrolysis in 1 N HCl for 1.5 min at 100 °C. Total hexose was assayed by the phenol-H_2SO_4 method of Hodge and Hofreiter (1962) scaled down to one-fifth volume, employing D-galactose as a standard. Amino sugars were assayed by the method of Reissig, et al. (1955). Protein was determined by the method of Lowry et al. (1951).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses. Sodium dodecyl sulfate-gel electrophoresis was performed in 5.6% gels by the method of Fairbanks et al. (1971). Protein was stained with Coomassie blue and carbohydrate with periodic acid Schiff's stain following the methods of Fairbanks et al. (1971). The gels were scanned at 555 nm in a Gilford spectrophotometric scanner at 1 cm per minute.

Lectin Binding Studies. The following lectins were purified as previously described: *Agaricus bisporus* lectin (Presant and Kornfeld, 1972), *Phaseolus vulgaris* E-PHA (Kornfeld et al., 1972), *Lens culinaris* lectin (Kornfeld et al., 1971), *Ricinus communis* agglutinin (RCA I) (Adair and Kornfeld, 1974), and wheat germ agglutinin (Marchesi, 1972). Lectins were iodinated with ^{125}I by the method of Hunter (1967) using a 10-s exposure to Chloramine-T. Specific activities ranged from 1 to 2 × 10^5 cpm per μg of protein. Lectin binding studies were performed on washed human and bovine erythrocytes as described elsewhere (Presant and Kornfeld, 1972).

Affinity Chromatography of Detergent-Solubilized Bovine Erythrocyte Glycoproteins. An affinity adsorbent was prepared by conjugating RCA I to Sepharose 2B using a modification of the method of Cuatrecasas (1970) as previously described (Adair and Kornfeld, 1974). The RCA I-Sepharose contained 10 mg of lectin per milliliter of Sepharose. Bovine erythrocyte membranes (25 ml at 10 mg/ml of protein) were stirred into 175 ml of 0.5% Emulphogene-5 mM phosphate, pH 8.0 at 4 °C. The solution was swirled occasionally for 30 min, followed by centrifugation at 12 000*g* for 1 h at 4 °C. The supernatant fluid, containing 82% of the membrane sialic acid and 80% of the RCA I binding sites, was passed through the lectin affinity column (1.9 × 13 cm) which had been equilibrated with 0.5% Emulphogene-5 mM phosphate, pH 8.0. The column was washed with 1 M NaCl-0.5% Emulphogene-5 mM PO_4, pH 8.0, to remove nonspecifically adsorbed material before elution with 0.1 M lactose-0.5% Emulphogene-5 mM PO_4, pH 8.0. The eluate was extensively dialyzed against buffer without lactose to remove the haptene sugar. The lactose eluted and the nonadsorbed glycoprotein were each assayed for haptene inhibitory activity against RCA I as previously described (Adair and Kornfeld, 1974).

Preparation of Bovine Erythrocyte Membrane Glycoprotein Oligosaccharides. Step 1: Isolation of the Glycoprotein. Fresh pooled blood, anticoagulated with heparin, was obtained from Black Angus and Hereford calves at a local slaughter house. The erythrocytes were washed three times with 3 volumes of 0.9% NaCl to remove the plasma and buffy coat. Erythrocyte stroma were prepared by hemolyzing washed cells with 20 volumes of 10 mM Tris-0.1 mM EDTA, pH 7.4. The ghosts were sedimented at 14 500*g* for 30 min at 4 °C and washed three times in the hemolyzing buffer. The erythrocyte glycoprotein was then extracted from the ghosts with recrystallized 0.25 M lithium 3,5-diiodosalicylate and further purified by partition in phenol-H_2O and acidification as described by Marchesi and Andrews (1971). The final step of phospho-cellulose chromatography was omitted because in preliminary experiments it produced no purification above that obtained by acidification to pH 3.5 with citric acid. Following acidification, a fine precipitate formed which was removed by centrifugation, at 45 000*g* for 45 min. The supernatant fluid containing the glycoprotein was then extensively dialyzed against 1 mM Tris buffer, pH 7.0, and stored at −20 °C until used. The yield of glycoprotein was 70% as calculated from the recovery of sialic acid.

Step 2: Pronase Digestion of the Glycoprotein. The glycoprotein (465 mg) was incubated with 3% (w/w) Pronase in 0.05 M Tris buffer, pH 8.2, containing 2 mM $CaCl_2$ in a final volume of 4.5 ml. The incubation was carried out for 48 h at 37 °C under a toluene atmosphere. An additional 2% Pronase was added after 17 and 39 h and the pH of the reaction was adjusted back to 8.2 with NaOH at these times. At 48 h the reaction mixture was centrifuged to remove a fine precipitate which was devoid of hexose and the supernatant was applied to a Sephadex G-25 column (2 × 85 cm). The column was eluted with H_2O, and those fractions containing hexose, as detected by phenol-H_2SO_4 method, were combined and subjected to repeat Pronase digestion for an additional 30 h. The product of this second digestion was applied to a Sephadex G-75 column (2 × 75 cm) and eluted with 0.1 M pyridine acetate buffer, pH 6.4. The fractions (5 ml) were assayed for hexose and sialic acid, as shown in Figure 1. Aliquots of selected fractions from 18 to 40 were assayed for total sugar content by gas-liquid chromatography and the ratio of mannose to *N*-acetylgalactosamine was plotted (Figure 1). There were at least two populations of glycopeptides in the digest since the

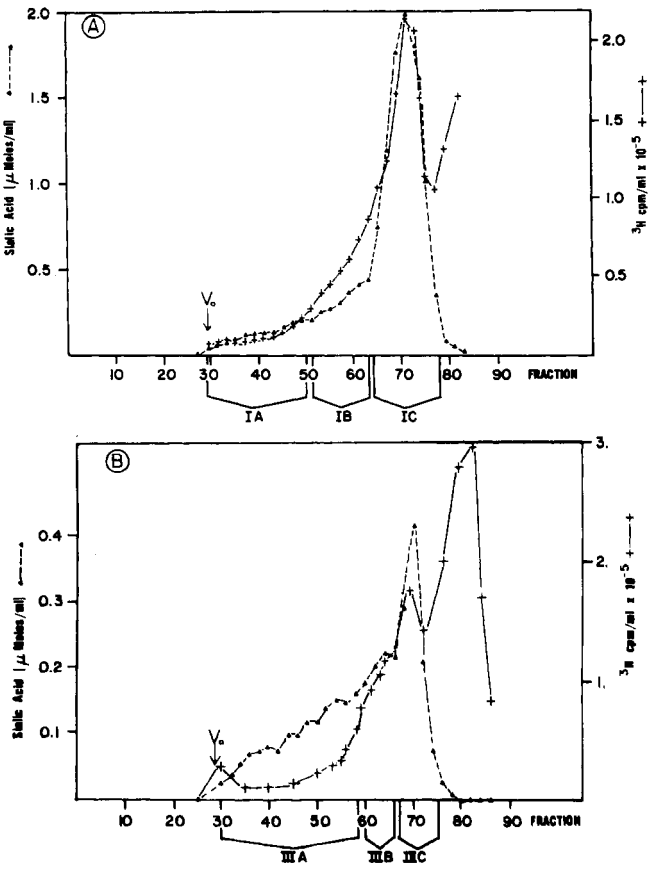

FIGURE 2: Sephadex G-50 fractionation of the products of alkaline-NaB^3H_4-treated glycopeptides. The glycopeptides of pool I (Figure 2A) and pool III (Figure 2B), derived from Sephadex G-75 fractionation of the Pronase digested erythrocyte membrane glycoprotein (Figure 1), were subjected to alkaline NaB^3H_4 treatment as described under Experimental Procedure. The reaction products were passed over a Sephadex G-50 column (1.5 × 53 cm) which was eluted with water. The fractions (1 ml) were assayed for sialic acid and radioactivity content. Fractions were pooled as indicated. Sialic acid (▲- - -▲); ^3H (+—+).

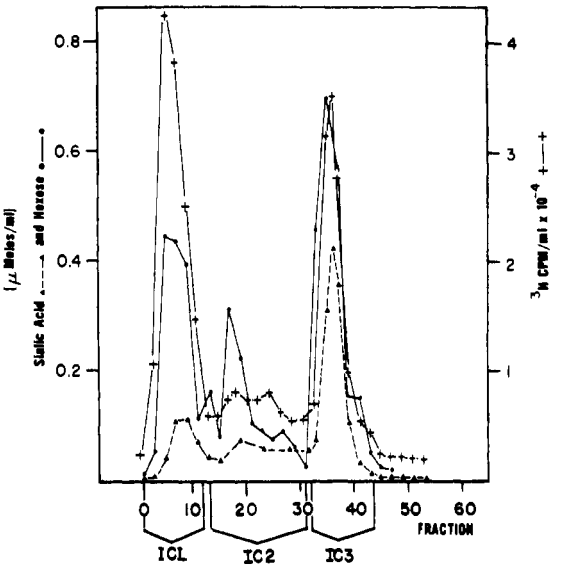

FIGURE 3: DEAE-cellulose chromatography of the oligosaccharides in Sephadex G-50 pool IC (Figure 2A). The pooled fractions were loaded onto a DEAE-cellulose column (0.9 × 10 cm) equilibrated with 4 mM phosphate buffer, pH 6.8. After washing the column with 36 ml of the starting buffer, the column was eluted with a linear gradient (50 ml each chamber) of 4–100 mM phosphate buffer, pH 6.8. Aliquots of the 2-ml fractions were assayed for hexose (●—●), sialic acid (▲- - -▲), and counted for incorporated ^3H (+—+). The fractions were pooled as indicated.

pools designated I and II contained sialic acid, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine, while pools III and IV contained these sugars as well as mannose. The fractions were pooled as indicated in Figure 1 and lyophilized.

Step 3: Alkaline Borohydride Treatment. The pooled, lyophilized fractions I and III were dissolved in 2 ml of 0.1 N NaOH–0.4 M NaB^3H_4 and incubated in the dark at room temperature under a N_2 atmosphere. To determine when the reaction had gone to completion, aliquots were removed at time 0 and at 24-h intervals thereafter, acidified with glacial acetic acid, and dried under vacuum. Methanol was added and methyl borates were removed by evaporation under vacuum. This was repeated and the residue was counted in 10 ml of Bray's solution in a Packard Model 2000 liquid scintillation counter. When the uptake of ^3H became constant after 120 h, the samples were brought to pH 5 to destroy the excess NaB^3H_4 and treated as above.

Step 4: Gel Filtration and Ion-Exchange Chromatography. The alkaline-treated samples were applied to a Sephadex G-50 column (1.5 × 53 cm) and eluted with water. The fractions were assayed for sialic acid and ^3H content. Over 95% of the sialic acid present in pool I was now greatly retarded on the column (Figure 2A). This material (pool IC of Figure 2A) was rerun on the Sephadex G-50 column, and fractions were assayed for sialic acid and ^3H as before. Aliquots of selected fractions of the sialic acid-containing oligosaccharide peak were assayed for total carbohydrate composition by gas–liquid chromatography. While the ratio of galactose to *N*-acetylgalactosamine was constant throughout the peak, heterogeneity for sialic acid was detected. For this reason pool IC was further fractionated on DEAE-cellulose as shown in Figure 3. Two distinct species were isolated and termed IC1 and IC3. IC1 and IC3 were individually re-fractionated on DEAE-cellulose, pooled, and stored at −20 °C.

After alkaline treatment, the mannose-rich pool III material was passed over the Sephadex G-50 column as shown in Figure 2B. The bulk of sialic acid containing material was also highly retarded. Aliquots of selected fractions were assayed for total carbohydrate composition by gas–liquid chromatography and based on these results (Table V), the fractions were pooled as noted in Figure 2B. The most highly retarded fractions which contained *N*-acetylgalactosaminitol (pool IIIC) were refractionated over the Sephadex G-50 column.

Susceptibility of the Purified Bovine Erythrocyte Membrane Glycoprotein to Degradation by Trypsin and Pronase. Purified glycoprotein (4 mg) was incubated with Tos-PheCH_2Cl-trypsin (100 units) in 2 ml of 0.1 M phosphate buffer, pH 7.0, in a toluene atmosphere at 37 °C for 48 h. Glycoprotein (4 mg) was treated with 5% Pronase (w/w) in 2 ml of 50 mM Tris–2mM $CaCl_2$, pH 8.2, at 37 °C, in a toluene atmosphere for 48 h. The pH was adjusted to 8.2 and an additional 5% (w/w) Pronase added at 24 h. Reactions were terminated by boiling and the reaction mixtures were concentrated threefold. Three gels, each containing 50 μl of one of the digests or native glycoprotein plus protein molecular weight markers (30 μg/marker), were electrophoresed simultaneously. The gels were stained with periodic acid Schiff's reagent and Coomassie blue.

Table I: Lectin Binding Sites on Bovine and Human Erythrocytes.[a]

Lectin	Bovine Erythrocytes[f]	Human Erythrocytes[f]
P. vulgaris E-PHA	0.04	0.52[b]
L. culinaris PHA	0.02	0.55[c]
RCA I	0.85	1.2[d]
Wheat germ agglutinin	3.3	8.2[d]
A. bisporus PHA	4.6	6.8[e]

[a] Erythrocytes were incubated with ^{125}I-labeled lectins, and the number of binding sites per cell was calculated as previously described (Presant and Kornfeld, 1972). Each value represents the average of at least two determinations. [b] (Kornfeld and Kornfeld, 1971). [c] (Kornfeld et al., 1971). [d] (Adair and Kornfeld, 1974). [e] (Presant and Kornfeld, 1972). [f] Sites/cell × 10^6.

Results

Lectin Binding to Bovine Erythrocytes. Our initial interest in the bovine erythrocyte glycoprotein arose because the binding of ^{125}I-labeled lectins to bovine erythrocytes was strikingly different from that found with human erythrocytes, as shown in Table I. Bovine erythrocytes bind less than 10% the number of *P. vulgaris* E-PHA and *L. culinaris* PHA molecules as do human erythrocytes. Taking into consideration the 30% smaller volume of bovine erythrocytes, the number of *A. bisporus* PHA and RCA I binding sites are approximately the same per unit area of cell surface for the two cell types, while wheat germ agglutinin binding sites are decreased by 50% on bovine erythrocytes. The decreased number of binding sites for the various lectins were reflected by a decreased ability of the solubilized bovine erythrocyte glycoprotein to inhibit hemagglutination by these lectins (data not shown).

Glycoprotein and Carbohydrate Composition of Bovine Erythrocyte Ghosts. Our initial experiments were performed to provide information about the total carbohydrate content of bovine erythrocyte ghosts and the number of glycoprotein molecules they contain. As shown in Figure 4c, when bovine erythrocyte ghosts were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate, a number of major protein components were detected. This protein pattern is remarkably similar to that obtained with human erythrocyte ghosts (Figure 4a). Staining the gels with periodic acid Schiff reagent to detect glycoproteins (Figure 4d), revealed that bovine ghosts had only one major glycoprotein which barely entered a 5.6% polyacrylamide gel. This pattern is quite different from that observed with human erythrocyte ghosts where three periodic acid Schiff position bands were seen (Figure 4b). Since the periodic acid Schiff's reagent may not react with all membrane glycoproteins, additional polyacrylamide gels of whole bovine erythrocyte ghosts were sliced into 1-cm sections and subjected to acid hydrolysis followed by direct amino sugar analysis, as previously described (Adair and Kornfeld, 1974). All of the amino-sugar-containing glycoprotein was detected in the first 1-cm gel slice suggesting that, in the case of bovine erythrocyte ghosts, the PAS stain accurately reflected the location of glycoprotein in the polyacrylamide gels.

To determine whether the single glycoprotein band seen on sodium dodecyl sulfate–polyacrylamide gels did indeed represent a single homogeneous glycoprotein, the technique of affinity chromatography on RCA I-Sepharose was employed. The membrane glycoproteins of human erythrocytes can be separated into two fractions on RCA I-Sepharose: the major sialoglycoprotein which does not bind and the RCA I receptor glycoproteins which adsorb and can be eluted by the haptene sugar lactose (Adair and Kornfeld, 1974). When bovine erythrocyte ghosts were solubilized with the nonionic detergent Emulphogene and passed over an RCA I-Sepharose affinity column, as described in Experimental Procedure, 13% of the sialic acid and hexose containing material adsorbed to the column and was quantitatively eluted with lactose (Table II). The nonadsorbed and the lactose eluted glycoprotein had identical carbohydrate compositions and served equally well as haptene inhibitors of the *R. communis* lectin (Table II). Although the affinity column was overloaded, the presence of a significant amount of a second glycoprotein would have altered the composition and/or the specific inhibitory activity seen in the two fractions. These results provide further evidence for the presence of only a single glycoprotein species in the bovine erythrocyte membrane.

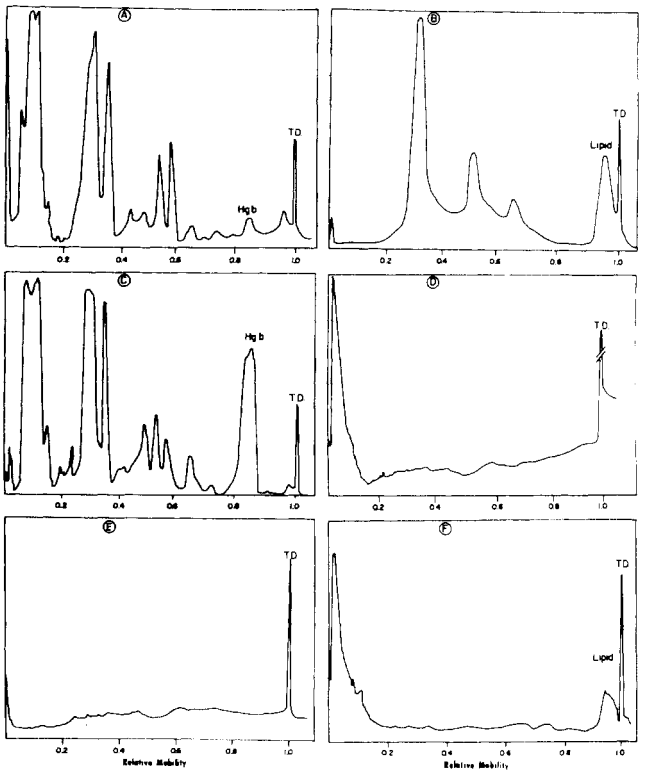

FIGURE 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described in Experimental Procedure. Washed erythrocyte ghosts (70–200 μg of protein) or isolated membrane glycoproteins (40 μg) were electrophoresed in 5.6% gels and were stained for protein with Coomassie blue and for carbohydrate with periodic acid Schiff's reagent (PAS) as described by Fairbanks et al. (1971). The gels were scanned on a Gilford spectrophotometer at 555 nm. Human erythrocyte ghosts stained with (A) Coomassie blue and (B) PAS. Bovine erythrocyte ghosts stained with (C) Coomassie blue and (D) PAS. Isolated bovine erythrocyte membrane glycoprotein stained with (E) Coomassie blue and (F) PAS.

The carbohydrate composition of bovine erythrocyte ghosts is shown in Table III along with the composition of human erythrocyte ghosts for comparison. The membranes of both cell types contain similar amounts of sialic acid, galactose, glucose, and *N*-acetylgalactosamine while bovine membranes contain less mannose and fucose and more *N*-acetylglucosamine.

Characterization of the Purified Bovine Erythrocyte Glycoprotein. The bovine erythrocyte glycoprotein, isolated

Table II: Fractionation of Bovine Erythrocyte Glycoprotein on RCA I-Sepharose.[a]

Fraction	Sialic Acid (μmol)	Hexose (μmol)	Lectin Inhibitory Activity TOTAL IU[b]	IU/μmol of sialic acid
Emulphogene extract	18.5	47		
Nonadsorbed material	15.8	37.4	44 240	2.8×10^3
Lactose eluate	2.4	5.8	6 240	2.6×10^3
% in eluate	13.2%	13.4%	12.4%	

[a] Twenty-five milliliters of erythrocyte ghosts were solubilized with 0.5% Emulphogene in 5 mM phosphate buffer, pH 8.0, and passed through the RCA I-Sepharose column as described in Experimental Procedure. [b] One inhibitory unit (IU) is defined as that amount of material which inhibits the binding of [^{125}I]lectin by 50% in a standard assay system.

Table III: Carbohydrate Composition of Bovine and Human Erythrocyte Ghosts and Their Sialoglycoproteins.

Sugar Residues[a]	Bovine Erythrocyte Ghosts	Bovine Erythrocyte Isolated Sialoglycoprotein	Human Erythrocyte Ghosts	Human Erythrocyte Isolated Sialoglycoprotein
Sialic acid	1.0	1.0	1.0	1.0
Fucose	<0.1	<0.01	0.46	0.15
Mannose	0.24	0.10	0.61	0.19
Galactose	2.70	3.30	2.8	0.72
Glucose	0.38	<0.01	0.39	<0.002
N-Acetylglucosamine	2.28	2.40	1.1	0.30
N-Acetylgalactosamine	1.36	0.88	1.4	0.57

[a] Sialic acid arbitrarily set equal to 1.0. The concentration of sialic acid in bovine erythrocyte ghosts is 0.17 μm/mg of protein vs. 0.1 μm/mg of protein in human erythrocyte ghosts.

Table IV: Composition of DEAE-Cellulose Pools IC1 and IC3.

Sugar	Pool IC1 μm/ml	Pool IC1 Residues/molecule	Pool IC3 μm/ml	Pool IC3 Residues/molecule
Sialic acid	0.28	0.5	0.31	1.1
Galactose	1.90	3.1	0.84	2.9
N-Acetylglucosamine	1.22	2.0	0.56	2.0
N-Acetylgalactosaminitol	+[a]		+	

[a] *N*-Acetylgalactosaminitol detected, but not quantitated by gas–liquid chromatography (see Results).

by the lithium diiodosalicylate solubilization procedure described in Experimental Procedure, behaved as a homogeneous high molecular weight molecule when subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 4f). The purified glycoprotein migrated in the same position as the PAS positive band in the whole membranes and failed to stain with Coomassie blue (Figure 4e), a finding that is typical of glycoproteins containing high percentages of sugar (Fairbanks et al., 1971). No other proteins were detected in the preparation.

The amino acid composition of the purified bovine erythrocyte glycoprotein is similar to that of the human erythrocyte sialoglycoprotein (unpublished observation), while the carbohydrate compositions are quite different (Table III). Using these data it was calculated that the bovine glycoprotein contains 80% carbohydrate and 20% protein by weight. The data in Table III also show that the carbohydrate composition of the bovine glycoprotein is similar to that of whole ghosts, indicating that the glycoprotein can account for most of the carbohydrate of the bovine erythrocyte ghosts. The glycolipids of the membrane presumably contain the rest of the sugar, including the glucose residues.

The bovine erythrocyte glycoprotein was relatively resistant to digestion with proteolytic enzymes. Following treatment with trypsin, the purified glycoprotein was altered very little as shown by its mobility on polyacrylamide gels in sodium dodecyl sulfate and by its appearance in the void volume of a Sephadex G-150 column. However, after digestion with Pronase there was a 45% reduction in the size of the glycoprotein as revealed by its migration on polyacrylamide gels, and 44% of the sialic acid and 34% of the hexose containing material were retarded on a Sephadex G-75 column (Figure 1).

The fractions from the Sephadex G-75 column were combined as pools I, II, and III as indicated in Figure 1 and subjected to further fractionation procedures so that purified oligosaccharides could be obtained. Virtually all of the mannose-containing oligosaccharide material appeared in the most retarded fractions of the Sephadex G-75 column, labeled pool III.

Purification of the Oligosaccharide Units in the Sephadex G-75, Pool-I Material. The high molecular weight glycopeptide material in pool I was next subjected to alkaline treatment in the presence of tritiated borohydride. When this material was passed over a Sephadex G-50 column (Figure 2A), greater than 95% of the sialic acid containing oligosaccharides were now highly retarded, indicating that these oligosaccharides had been linked to the peptide backbone by alkali-labile *O*-glycosidic linkages. The retarded material (pool IC) was rerun on Sephadex G-50 and then subjected to ion-exchange chromatography on DEAE-cellulose. As shown in Figure 3, this procedure yielded two major peaks of carbohydrate material, termed IC-1 and IC-3. Both peaks were labeled with tritium, indicating that the oligosaccharide chains contained sugar alcohols at their reducing ends.

The carbohydrate compositions of pools IC-1 and IC-3 are shown in Table IV. Both oligosaccharide fractions contain 3 residues of galactose, 2 residues of *N*-acetylglucosamine, and a partial residue of *N*-acetylgalactosaminitol. Pool IC-3 has, in addition, 1 full residue of sialic acid which accounts for its binding to DEAE-cellulose. No amino acids were detected in either fraction on amino acid analysis. Based on these compositions, the calculated molecular weights of IC-1 and IC-3 are 1110 and 1401, respectively, which agree well with the molecular weights of 1100 and 1250 which were estimated by gel filtration on a calibrated Sephadex G-50 column (Bhatti and Clamp, 1968).

Table V: Carbohydrate Composition of Selected Sephadex G-50 Fractions of Alkaline-Borohydride-Treated, Pool-III Material.[a]

Sugar	Fraction Number (μmol/ml)				
	35	54	64	70	82
Sialic acid	0.5	1.5	2.1	4.2	0
Mannose	0.4	1.1	3.0	0	0
Galactose	2.5	4.3	6.9	4.0	0
N-Acetyl-glucos-amine	1.9	7.6	4.2	1.2	0
N-Acetyl-galactos-amine	0.4	4.4	1.8	2.1	0
N-Acetyl-galactos-aminitol	0	0	0.8	1.7	0
N-Acetyl-glucosam-initol	0	0	0	0	0

[a] Total carbohydrate was determined by gas-liquid chromatography. The fractions analyzed were from the Sephadex G-50 column shown in Figure 2B.

Table VI: Carbohydrate Composition of Refractionated Pool III-C Material.[a]

Sugar	Pool III C-1		Pool III C-2	
	μmol[b]	Residues[c]	μmol[b]	Residues[c]
Mannose	<0.1	(0)	0	(0)
Galactose	1.09	(2)	2.06	(2)
N-Acetyl-galactos-aminitol	0.45	(1)	0.99	(1)
N-Acetyl-galactos-amine	0.48	(1)	0.99	(1)
N-Acetyl-glucos-amine	0.32	(1)	0.73	(1)
Sialic acid	1.0	(2)	1.0	(1)

[a] Fractions 67-75 of the Sephadex G-50 column (pool III-C of Figure 2B) were pooled, concentrated, and rerun on the Sephadex G-50 column. The material separated into two sialic acid containing peaks which were pooled and analyzed for total carbohydrate composition by gas-liquid chromatography. [b] Micromoles of sugar per micromole of sialic acid. [c] Residue number to the nearest integer.

To confirm that the ^3H was actually incorporated into *N*-acetylgalactosaminitol, the oligosaccharides were hydrolyzed in 4 N HCl for 4 h at 100 °C, lyophilized, resuspended in 0.3 N HCl, and passed over a Dowex 50 column as described by Gardell (Gardell, 1953). Under these conditions, a single tritiated hydrolysis product was obtained which eluted from the column coincident with authentic galactosaminitol. In a separate experiment the oligosaccharides were hydrolyzed in acid and passed over a Dowex 50 column, and the peak fractions of tritium-labeled hydrolysis product were recovered and identified by gas-liquid chromatography as galactosaminitol. The lack of a full residue of *N*-acetylgalactosaminitol was most likely due to sugar destruction during the alkaline treatment step (Lloyd and Kabat, 1969; Ballou, 1954).

Nature of the Oligosaccharide Units in the Sephadex G-75, Mannose-Rich Pool-III Material. The mannose-rich, Sephadex G-75, Pool-III glycopeptides (Figure 1) were subjected to alkaline treatment in the presence of tritiated borohydride. When these glycopeptides were then filtered over Sephadex G-50, a complex elution profile resulted, with over half of the sialic acid containing material being retarded greater than 2 void volumes (Figure 2B). Selected column fractions were assayed for total carbohydrate composition by gas-liquid chromatography and the results are shown in Table V. The mannose-containing oligosaccharides were found between fractions 35 and 64, with the higher molecular weight molecules being devoid of either *N*-acetylgalactosaminitol or *N*-acetylglucosaminitol, suggesting that they were N-glycosidically linked to the peptide backbone. Unfortunately the small amounts of these larger glycopeptides precluded further purification procedures.

The highly retarded oligosaccharide material which peaked in fraction 70 was found to contain sialic acid, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylgalactosaminitol but no mannose. This material (approximately 30% of the total sialic acid) was pooled as noted in Figure 2B (fraction IIIC) and re-run on Sephadex G-50 where it was resolved into two peaks, termed IIIC1 and IIIC2. The material in each peak was analyzed by gas-liquid chromatography, and their respective carbohydrate compositions are shown in Table VI. Both oligosaccharides appear to have been originally O-glycosidically linked to the peptide backbone, accounting for the residue of *N*-acetylgalactosaminitol following alkaline-borohydride treatment. The two oligosaccharides probably differ only by a sialic acid residue and resemble the pool IC oligosaccharides except for the presence of 1 residue each of *N*-acetylglucosamine and *N*-acetylgalactosamine rather than 2 residues of *N*-acetylglucosamine and none of *N*-acetylgalactosamine.

Discussion

Previous investigators have compared human and bovine erythrocyte membrane proteins and have noted similarities between the major classes of proteins separable on sodium dodecyl sulfate-polyacrylamide gels (Capaldi, 1972; Kobylka et al., 1972). In contrast to these similarities, there are several major differences between the glycoproteins of the two species of erythrocytes. Firstly, bovine erythrocytes appear to have only one major glycoprotein which contains virtually all of the carbohydrate of the membrane, excluding the glycolipids. This supposition is supported by the PAS staining pattern and the direct amino sugar analysis of polyacrylamide gels of whole bovine erythrocyte ghosts and by the fact that different species of glycoproteins could not be separated by affinity chromatography on an RCA I-Sepharose column. Furthermore the purified glycoprotein which migrates as a single band on sodium dodecyl sulfate-polyacrylamide gels can account for almost all of the membrane sialic acid, galactose, *N*-acetylglucosamine and most of the mannose and *N*-acetylgalactosamine. In contrast, human erythrocyte membranes contain at least two major classes of glycoproteins which differ significantly in carbohydrate composition and are separable on a RCA I-Sepharose affinity column (Adair and Kornfeld, 1974).

The bovine erythrocyte membrane glycoprotein has previously been isolated by the chloroform-methanol-water extraction technique and found to be much larger than the major human erythrocyte sialoglycoprotein (Capaldi, 1973). Molecular weight estimates of its size vary from 180 000 to 285 000 daltons compared with about 31 000

daltons for the human sialoglycoprotein (Capaldi, 1973). To date it has not been possible to dissociate the bovine erythrocyte membrane glycoprotein into subunits. Capaldi has published an amino acid analysis of the bovine glycoprotein which varies from the present data chiefly in its lower content of threonine and arginine (Capaldi, 1973). This discrepancy may be explained by a higher content of contaminating protein in that preparation since the material contained 62% carbohydrate by weight whereas in the present report the glycoprotein contains 80% carbohydrate by weight. The amino acid content of the bovine glycoprotein bears a strong similarity to its human counterpart with both glycoproteins having a high content of serine, threonine, and glutamic acid (Capaldi, 1973 and unpublished observations).

The major objectives of this study were to analyze the carbohydrate units of the bovine erythrocyte glycoprotein and to compare them with the oligosaccharide units present on the human erythrocyte glycoproteins. The bovine glycoprotein resembles the human erythrocyte sialoglycoprotein in that it is very rich in carbohydrate (80% by weight vs. 60% by weight in the case of the human sialoglycoprotein) and it contains predominantly oligosaccharide units which are linked O-glycosidically to the peptide backbone. If one assumes that most of the *N*-acetylgalactosamine residues in the bovine glycoprotein are involved in O-glycosidic linkages to serine and/or threonine residues of the peptide backbone and that all of the mannose residues of the molecule are located in oligosaccharide units that are linked N-glycosidically to the peptide backbone (and that each of these oligosaccharide units contains an average of two mannose residues), then one can calculate that there are approximately 17 O-glycosidically linked oligosaccharides per N-glycosidically linked oligosaccharide. This compares with 6 O-glycosidically linked chains per N-glycosidically linked chain on the human erythrocyte sialoglycoprotein (Presant and Kornfeld, 1972). In both glycoproteins *N*-acetylgalactosamine residues are involved in the O-glycosidic linkages. In spite of these similarities in the overall makeup of the two species molecules, the composition of the O-glycosidically linked oligosaccharide units is very different. Thus the majority of these molecules present on the bovine glycoprotein contain sialic acid, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine in a molar ratio of 0.5–1:3:2:1 while the O-glycosidically linked molecules of the human erythrocyte sialoglycoprotein contain sialic acid, galactose, and *N*-acetylgalactosamine in a molar ratio of 1–2:1:1 (Thomas and Winzler, 1969). Even the minor species of the bovine glycoprotein O-glycosidically linked oligosaccharides have a composition quite distinct from that of the human sialoglycoprotein counterpart (pool III C material, Table VI). We conclude that the O-glycosidically linked oligosaccharide units of the major glycoproteins of bovine and human erythrocytes must have strikingly different structures.

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