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Biochemical and Morphological Properties of Bovine Erythrocyte Membrane Glycoproteins

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The major and minor sialoglycoproteins of the bovine erythrocyte have been solubilized and extensively purified. A comparison of composition revealed that the major glycoprotein had 77% carbohydrate and 23% peptide, and the minor one had 27% carbohydrate and 73% peptide. Molar ratios of sugars were related, however, the major glycoprotein had twice as much galactose and sialic acid as did the minor glycoprotein. Molecular weights, estimated from retardation coefficients of mobility in sodium dodecyl sulfate gel electrophoresis, were 55,000 for the major glycoprotein and 34,000 for the minor glycoprotein. The glycoproteins were studied by electron microscopy before and after delipidation and after ultracentrifugation. The major glycoprotein, prior to delipidation, formed large micelles. After delipidation, the major glycoprotein could not be visualized suggesting that it did not form aggregates in aqueous solution. The minor glycoprotein was visualized as rather uniform spherical aggregates (62 Å average diameter) which tended to form short chains and small clumps. These characteristic aggregates were seen both before and after delipidation. After ultracentrifugation, fixation and sectioning both glycoproteins appeared to have formed microcrystalline arrays with average periodicity of 49 Å.

Key words: erythrocyte, membranes, glycoproteins, electronmicroscope, gel electrophoresis, bovine

In earlier reports from our laboratory, we described the isolation of infectious mononucleosis heterophile antigen from bovine erythrocytes [1,2]. This antigen was extracted from the membranes with hot 75% ethanol. The partially purified antigen was a glycoprotein of apparent molecular weight (MW_{app}) 25,000 containing 20% carbohydrate. Other laboratories have described isolation of glycoproteins from the bovine erythrocyte. Capaldi [3] reported extraction of bovine erythrocyte membranes with chloroform:methanol:water (6:3:1) and purification from the aqueous phase of a 180,000 MW_{app} glycoprotein which contained 38% protein and 62% carbohydrate. More recently Emerson and Kornfeld [4] using lithium 3,5-diiodosalicylate (LIS) ex-

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traction followed by phenol-water extraction and Hamazaki et al [5] using hot aqueous phenol extraction described isolation of a larger (>200,000 MW_{app}) glycoprotein which was 80% carbohydrate. According to Emerson and Kornfeld this large-molecular-weight glycoprotein accounted for almost all of the bovine membrane sialic acid, galactose and N-acetylglucosamine. In contrast we had observed that nearly 30% of membrane sialic acid was associated with the smaller heterophile antigen active glycoprotein [6]. Merrick et al reported isolation of both the heterophile antigen type of small MW_{app} (26,000) glycoprotein from bovine membranes and also the large-molecular-weight type glycoprotein [7]. However, they reported only 9.3% carbohydrate for the minor glycoprotein and the molar ratios of sugars which they found for the major glycoprotein were very different from those reported by Hamazaki et al [5] or by Emerson and Kornfeld [4]. In the present report we have prepared both the minor heterophile antigen active glycoprotein and the major "high-molecular-weight" from the erythrocyte membranes of a single bovine, purified these glycoproteins, and characterized them as to composition and morphology.

MATERIALS AND METHODS

Analytical Procedures

Polyacrylamide gel electrophoresis (PAGE) studies were done in 0.1% sodium dodecyl sulfate (SDS) in 0.01 M phosphate buffer, pH 7.0, as previously described [8]. Sialic acids were determined by the thiobarbituric acid method as described by Aminoff [9] after hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 hr. N-Glycolylneuraminic acid (Sigma) served as a standard. Glycoprotein was treated with neuraminidase (Calbiochem) at 37°C for 24hr at an enzyme to substrate ratio of 200 units/mg glycoprotein then heated at 100°C for 3 min. The free sialic acid was separated from the residual glycoprotein on Sephadex G-25. Neutral sugars and amino sugars were determined by gas–liquid chromotography of the corresponding alditol acetates following hydrolysis in sealed, evacuated tubes in 2–6 N HCl for 2–6 hr at 100°C, reduction and acetylation as previously described [10]. Amino acids were determined after hydrolysis in 6 N HCl 110°C in sealed, evacuated tubes for 24 hr with a JEOL 5AH amino acid analyzer. Thin-layer chromatography (TLC) was performed on silica gel (Uniplates: Analtech). The following solvent systems were used (all ratios given by volume): (A) chloroform:methanol:water (65:25:4) and (B) chloroform:methanol:ammonium hydroxide (65:25:4). Spots were stained with iodine vapor, as a general lipid stain and by a resorcinol stain for gangliosides or sialic acid derivatives or by a molybdate–mercury stain for phospholipids. These were done as described by Kates [11]. Agar gel diffusion studies were carried out as previously described [1]. Sera were obtained from patients with heterophile antibody positive, clinically confirmed infectious mononucleosis.

Preparation of Membranes

Bovine blood was collected at the time of slaughter into anticoagulant citrate solution and transported to the laboratory on ice. The red blood cells were separated from plasma and "buffy coat" by repetitive (four times) centrifugation and washing with 0.15 M NaCl in 0.05 M phosphate buffer, pH 7.4. The cells were lysed and washed with cold 0.005 M sodium phosphate, pH 8.0. The membranes were pelleted at 13,000g for 60 min at 4°C. The supernatant was aspirated and the loosely packed membrane

layer was decanted from the red pellet. This procedure was repeated until the membranes were creamy white and the material freeze-dried.

Preparation of 75% Ethanol Extract

A portion of the membrane prepared from a single bovine was freeze-dried and then extracted (as shown in Fig. 1) sequentially, under reflux with acetone, 100% ethanol, and finally with 75% ethanol. The material extracted with 75% ethanol was concentrated to small volume by rotary evaporation and freeze-dried. The yield was 1–2% of the dry weight of the membranes.

Preparation of LIS–Phenol Extract

A portion of the membrane prepared as above was added to 0.3 M LIS in 0.05 M Tris–HCl, pH 7.5 (25 mg membrane protein/ml), and stirred for 15 min at room temperature and then at 4°C for an additional 15 min (Fig. 2). The mixture was centrifuged at 45,000g for 75 min. The supernatant was decanted and stirred with an equal volume of 50% phenol for 15 min at 4°C. The phenol suspension was centrifuged in 250-ml glass bottles in a swinging-bucket rotor (IEC) at 4°C for 4 hr at 1,300g and left to stand overnight, before removal of the aqueous phase which was dialyzed against running tap water for 3 days, against two changes of deionized water overnight and freeze-dried. The yield was 4–5% of the dry weight of the membranes.

Purification of the Crude Extracts

Both extracts were carried through several purification steps (as shown in Fig. 3). Phosphocellulose chromatography was carried out to remove contaminating substances less acidic than the glycoproteins. Delipidation was done by sequential ether:ethanol (4:1) and chloroform:methanol (2:1) extraction. Thin-layer chromatog-

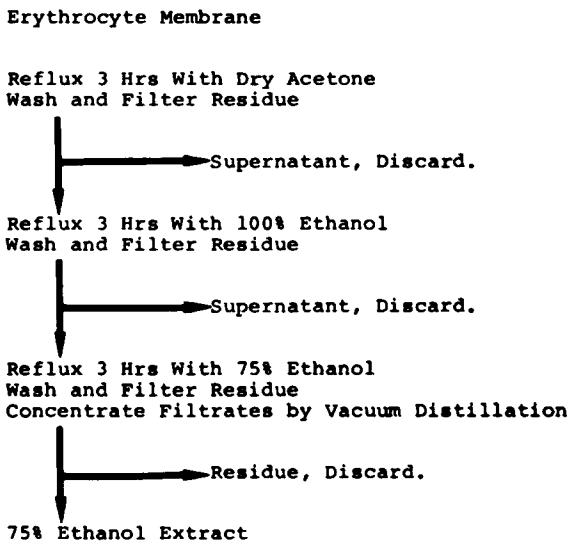

Fig. 1. Preparation of GPI from hemoglobin stroma by extraction with 75% ethanol.

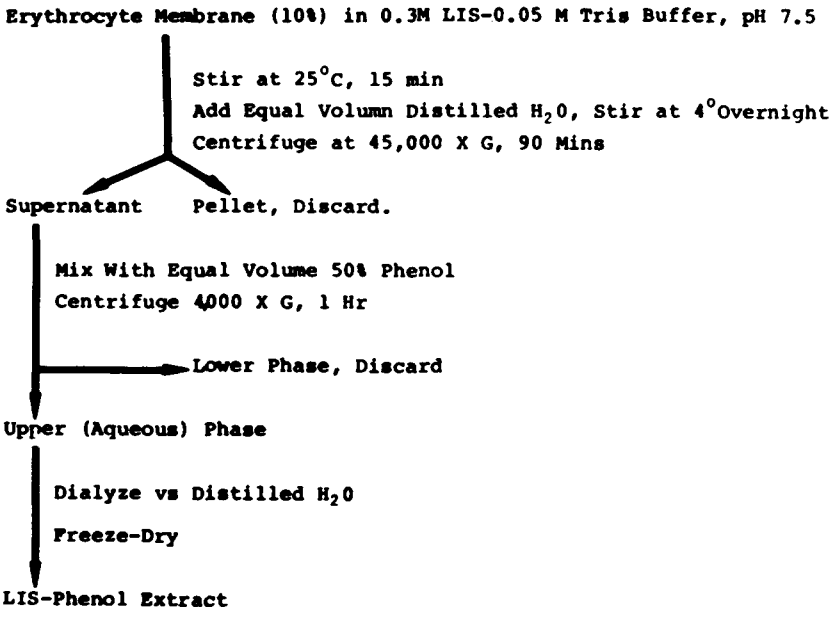

Fig. 2. Preparation of GPII from hemoglobin free stroma by extraction with LIS-phenol.

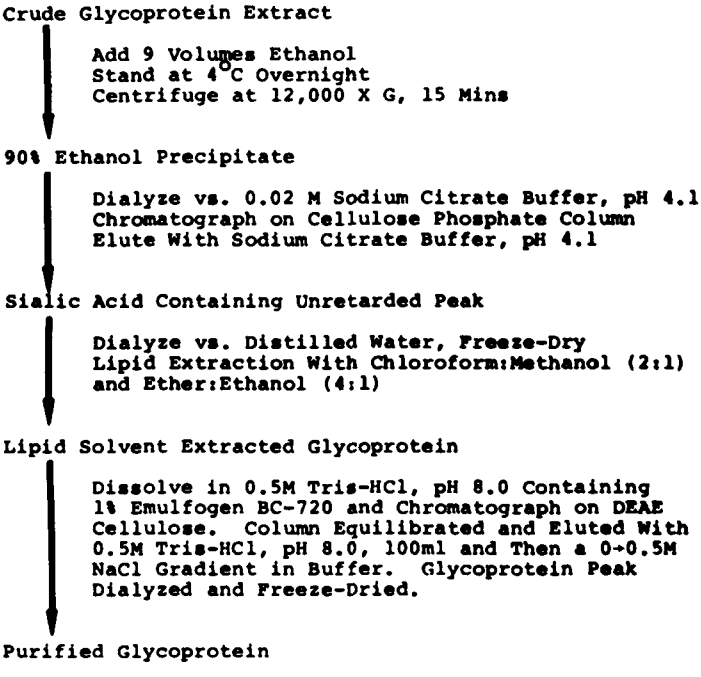

Fig. 3. Purification of GPI and GPII.

raphy of these extracts from the 75% ethanol soluble material in solvent A showed by I_2 visualization a streak of material from the origin to R_f = 0.55 as well as a discrete spot at R_f 0.59. The material between R_f 0.03–0.14 stained with the molybdate reagent. In solvent B these extracts by I_2 visualization streaked from the origin thru R_f 0.25 and stained intensely with the molybdate reagent at R_f s 0.21 and 0.25. Additionally there was an intense I_2 staining spot at R_f = 0.3 which also stained with molybdate and a faint I_2 spot at R_f 0.58 which did not stain for the phospholipid. Results of thin-layer chromatography of the lipid solvent extracts of the LIS–phenol prepared crude glycoprotein in solvent A showed, by I_2 visualization, a streak of material from the origin thru R_f 0.07 which stained with molybdate reagent and a spot with R_f 0.6 which did not stain with molybdate. In solvent B there was again, by I_2 staining, a streak through R_f 0.08 only the tip of which stained with the molybdate reagent and a spot at R_f 0.41, which stained with both I_2 and molybdate reagent. Both glycoproteins were dissolved in 1% Emulphogene and chromatographed on DEAE-cellulose as suggested by Dejter-Juszynski et al for the removal of complex glycolipids [12]. In both preparations, the glycoprotein was recovered from the column in a single peak upon application of a salt gradient. Also in both a small amount of material was unretarded by the DEAE column and emerged with the detergent. These materials did not absorb at 280 mm and did contain some sialic acid (2.7% of the original sialic acid of the 75% ethanol-extracted material and 0.7% of total sialic acid the LIS–phenol preparation). Both were examined by thin-layer chromatography. In solvents A and B the material removed from the 75% ethanol-soluble glycoprotein did not migrate appreciably above the origin. It stained with I_2 and resorcinol but not for phospholipid. The sample isolated from the LIS–phenol prepared glycoprotein showed essentially the same migration and staining patterns. However, the intensity of the staining was less than the material removed from the 75% ethanol-solubilized glycoprotein, particularly with the resorcinol reagent. Dejter-Juszynski et al report that the macroglycolipid which they isolated from human erythrocyte glycoprotein by this type of chromatography did not migrate from the origin [12]. We also examined by TLC both of the delipidated glycoprotein in solvents A and B. We were unable to detect anything by I_2 or phosphate staining in either purified glycoprotein preparation.

Electron Microscopic Studies of the Bovine Erythrocyte Glycoprotein

For negative staining, glycoproteins were prepared fresh with sterile distilled water at 1 mg/ml. Droplets were placed on a 300-mesh grid coated with 0.75% collodion film. Both uranyl acetate and phosphotungstic acid (PTA) were used, but it was observed that 1% PTA, pH 6.7, gave best contrast for negative staining. For thin sectioning, glycoproteins were centrifuged at 40,000 rpm (72 hr) in a Beckman Spinco ultracentrifuge in a SW50L rotor. The top 90% of the supernatant fluid was carefully removed without mixing the bottom layer of the centrifuge tube and 2.5% glutaraldyde (in 0.05 M sodium cacodylate buffer, pH 7.4) was layered into the tube. The tubes were incubated at 4°C overnight for fixation, postfixed with 1% OsO_4 (in above buffer), dehydrated in an ethanol series, and embedded in Araldite. Sections were cut with glass knives on a Sorvall MT2-B ultramicrotome, collected on uncoated or collodion coated grids, and examined in a Philips EM 300 after "staining" in saturated 50% ethanolic uranyl acetate and lead citrate. The electron microscope was calibrated with a Fullam's carbon grating replica prior to taking the micrographs.

RESULTS

Comparison of Compositions

Analytical results are shown in Table I. The major bovine erythrocyte glycoprotein (GPII) which was isolated by LIS-phenol extraction had almost exactly three times as much total carbohydrate content as did the minor erythrocyte glycoprotein (GPI) solubilized by hot 75% aqueous ethanol. GPII had twice as much mannose and sialic acid as did GPI and four times as much galactose and glucosamine. Amino acid compositions were also distinct. For example, GPI had twice as much methionine and more glycine and proline than did GPII. The major glycoprotein contained, however, five times as much tyrosine as did GPI and twice as much threonine and serine.

Agar Gel Immunodiffusion

When compared in agar gel diffusion tests (Fig. 4) a major difference in receptor property was noted. The GPI formed a precipitation line with antibody in the serum of a patient with infectious mononucleosis (IM). After treatment of GPI with neuraminidase a less copious precipitation formed which was spurred over by the line given by the native GPI. In contrast, GPII failed to form a detectable line of precipitation with the antibody in IM serum either before or after neuraminidase treatment.

TABLE I. Comparative Compositions for Bovine Erythrocyte Glycoproteins

Constituent	Glycoprotein I	Glycoprotein II
	gm/100 gm Glycoprotein	
Carbohydrate	26.7	76.7
Peptide	73.3	23.3
	Molar ratios	
N-Glycolylneuraminic acid	1.0	1.0
Galactose	1.5	3.2
Mannose	0.1	0.1
N-Acetylglucosamine	1.1	2.4
N-Acetylgalactosamine	0.5	0.7
	mole/100 mole amino acid	
Aspartic acid	6.2	5.9
Threonine	8.8	15.3
Serine	7.5	14.4
Glutamic acid	16.4	13.6
Proline	13.9	9.4
Glycine	9.9	5.9
Alanine	5.0	5.7
Valine	5.0	6.0
Methione	1.8	0.9
Isoleucine	5.3	3.1
Leucine	7.7	5.2
Tyrosine	0.5	2.5
Phenylalanine	3.0	1.9
Histidine	1.1	1.3
Lysine	2.6	2.4
Arginine	4.8	6.4

Polyacrylamide Gel Electrophoresis

The results of electrophoresis of GPII and GPI in SDS PAGE are shown in Figure 5. Both glycoproteins stained with periodic acid–Shiff reagent (shown). GPI also stained well with Coomassie blue, but GPII did not. The relative mobility of the two glycoproteins were determined at four acrylamide concentrations. When the results were plotted in a Ferguson plot [13] and compared to a set of standard peptides, it was clear that the two bovine erythrocyte glycoproteins behaved in opposite and anomalous ways from each other and the marker proteins (Fig. 6). Extrapolation of the lines to zero gel concentration yielded a free mobility value for GPII which was one-half that of the standard molecular weight proteins. The free mobility of GPI was higher than that of the standards. Clearly, estimation of molecular weights from RM at a single gel concentration would lead to a gross overestimation of the subunit

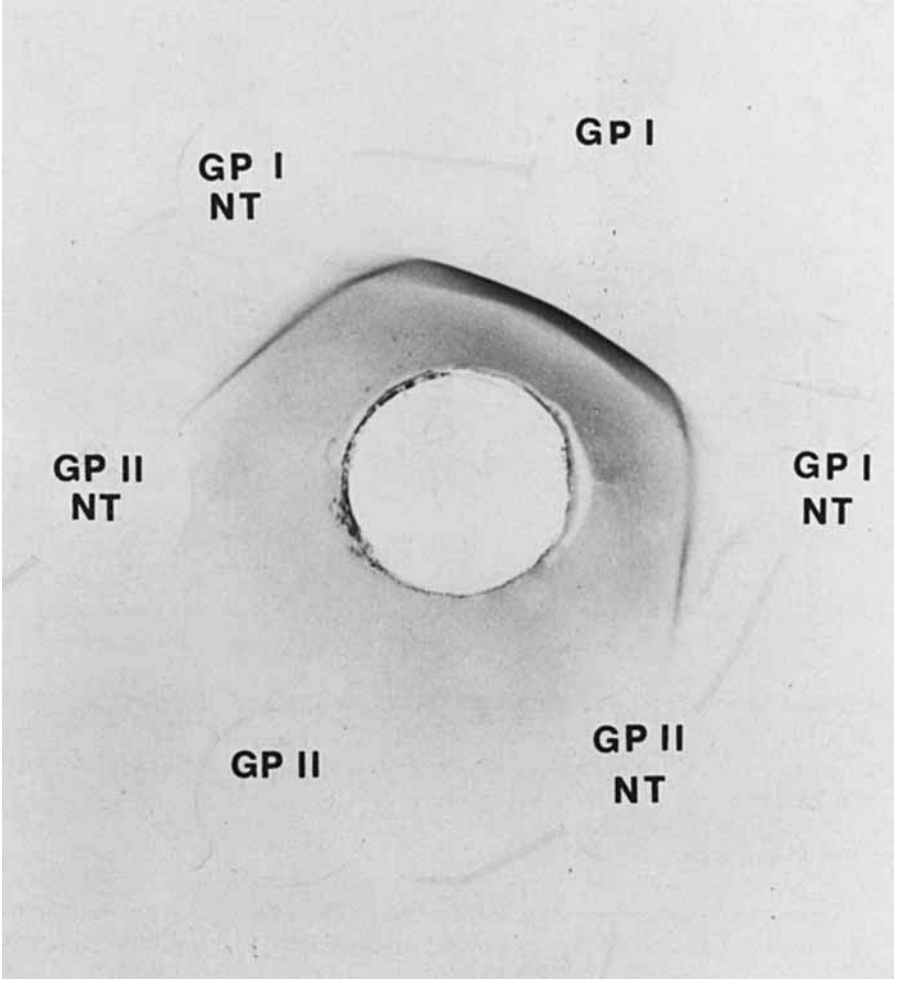

Fig. 4. Agar gel immunodiffusion: Center well IM serum. GP NT is neuraminidase-treated material.

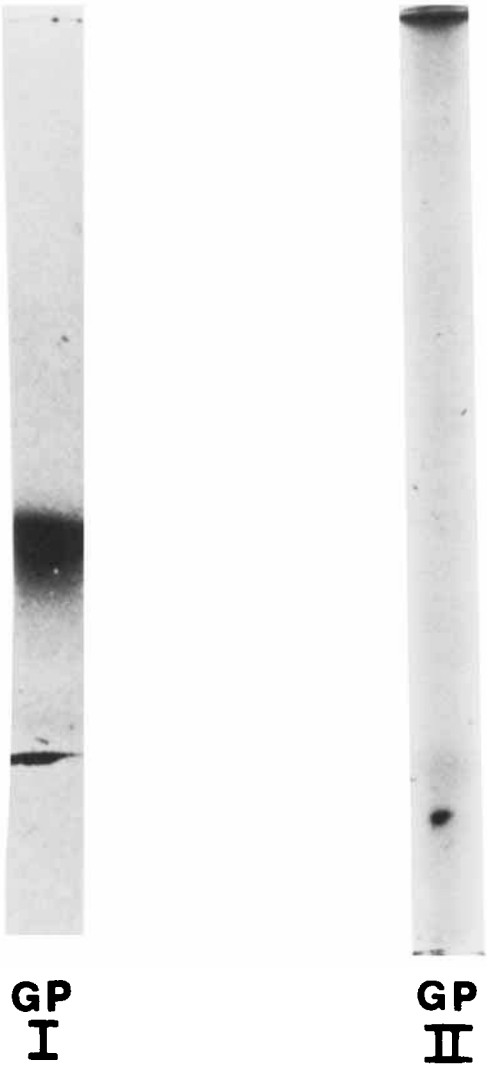

Fig. 5. Electrophorograms of GPI and GPII on SDS-PAGE and stained with PAS.

molecular weight of GPII and a lesser but perhaps significant underestimation of the size of GPI. Therefore, the molecular weights were estimated from the retardation coefficients ($-K_R$), calculated from the slopes of the lines in the Ferguson plots and given in Table II.

Electron Microscopy

The morphology of the two glycoproteins in freshly prepared aqueous solutions was examined by electron microscopy of negatively stained preparations from droplets placed on grids. In samples of GPII studied prior to the extraction with lipid solvents (Fig. 3), large amorphous globules were seen (Fig. 7A). After extraction of the glycoprotein with chloroform:methanol and ether:ethanol and DEAE-chromatography in

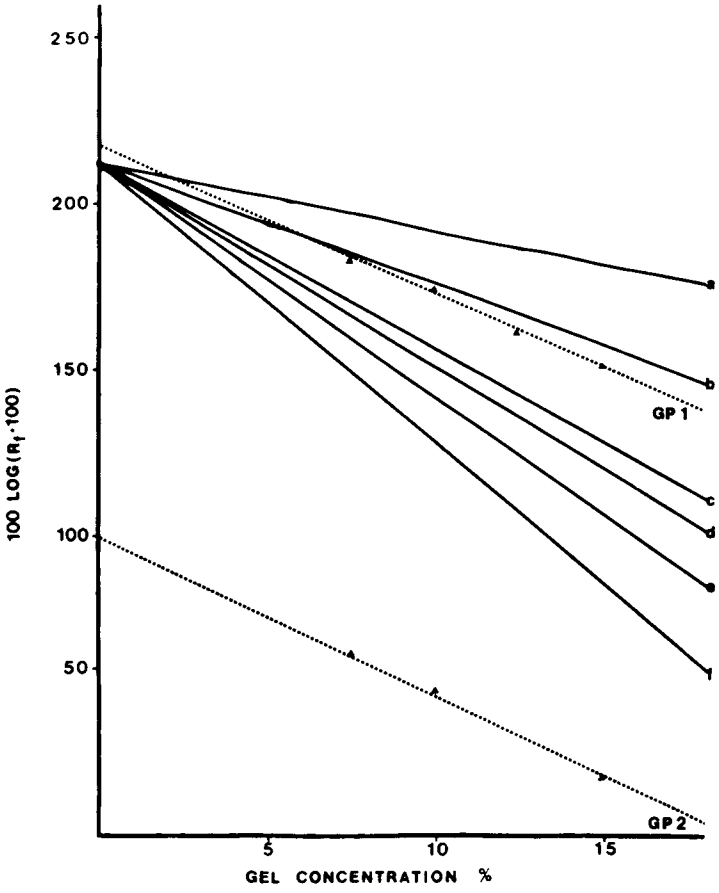

Fig. 6. Ferguson plot. A function of log relative mobility as plotted for gel concentration. The standard proteins are lysozyme (a); Bence Jones protein (b); ovalbumin (c); and fibrinogen γ, β, α chains (d, e, f).

TABLE II. Apparent Molecular Weight of Subunit and Aggregates of Bovine Erythrocyte Membrane Glycoproteins

Glycoprotein	Subunit molecular weight			Aggregate molecular weight		
	$-K_R$[a]	Y[b] intercept	MW_{app}[c]	diameter[d]	$\bar{\nu}$[e]	MW_{app}[f]
GPI	4.4	217.0	33,800	(62 ± 8)	0.70 cm³/gm	107,000
GPII	6.4	100.2	54,800	–	–	–

[a] $-K_R$ (a retardation coefficient).
[b] y-Intercept (which is a measure of elecrophoretic mobility at 0 gel concentration).
[c] Molecular weight estimation from $-K_R$ determined as previously described from SDS-PAGE analysis [8].
[d] This is the average ± 1 standard deviation of 100 diameters measured on photomicrographs.
[e] Partial specific volume ($\bar{\nu}$) calculated from composition of GPI using the values of Cohn and Edsall [14] for amino acids and of Gibbons [15] for sugars.
[f] The MW_{app} was calculated from the diameter and $\bar{\nu}$ using: $MW_{app} = \frac{(6 \times 10^{23})(4/3\pi r^3)}{\bar{\nu}}$.

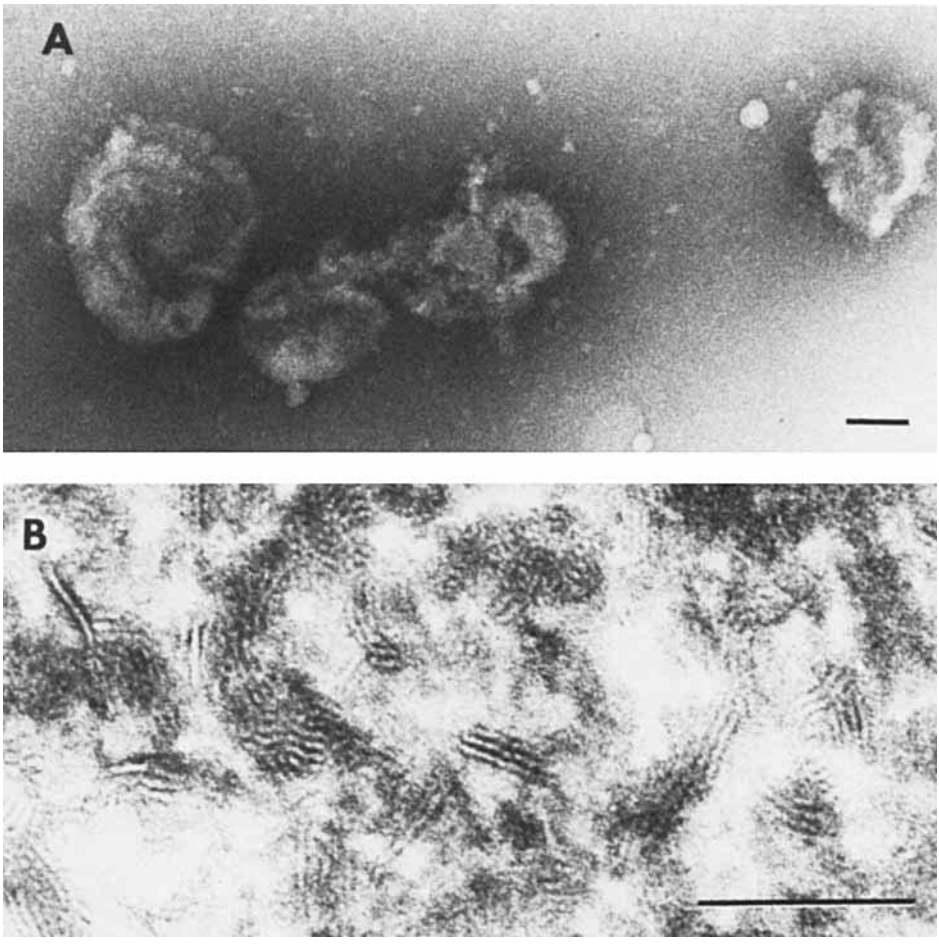

Fig. 7. Electronmicrograph of GPII. Bar = 1000 Å. Panel A shows negative staining GPII prior to lipid extraction. Panel B shows GPII, thin-sectioned after centrifugation.

Emulphogene, we were unable to visualize this glycoprotein suggesting that it does not aggregate after delipidation. In contrast, the appearance of GPI in this type of negatively stained preparation is shown in Figure 8A. Small rather uniform, compact globular aggregates were seen which tended to form short chains and clumps. From the average diameter (62 ± 8 Å) and the partial specific volume (0.69) the molecular weight of the aggregate was estimated to be 107,000. The aggregates of GPI were not different before or after delipidation. Another type of experiment was performed on both glycoproteins. Aqueous solutions of the delipidated glycoprotein were subjected to high-speed centrifugation in order to effect a high concentration of the glycoprotein in the bottom of the tube. The concentrated glycoprotein was then fixed and then sectioned with the results seen in Figures 7B and 8B. The ordered "microcrystalline" arrays had similar periodicities in both GPI (49 ± 3.3 Å) and GPII (50 ± 4.6 Å).

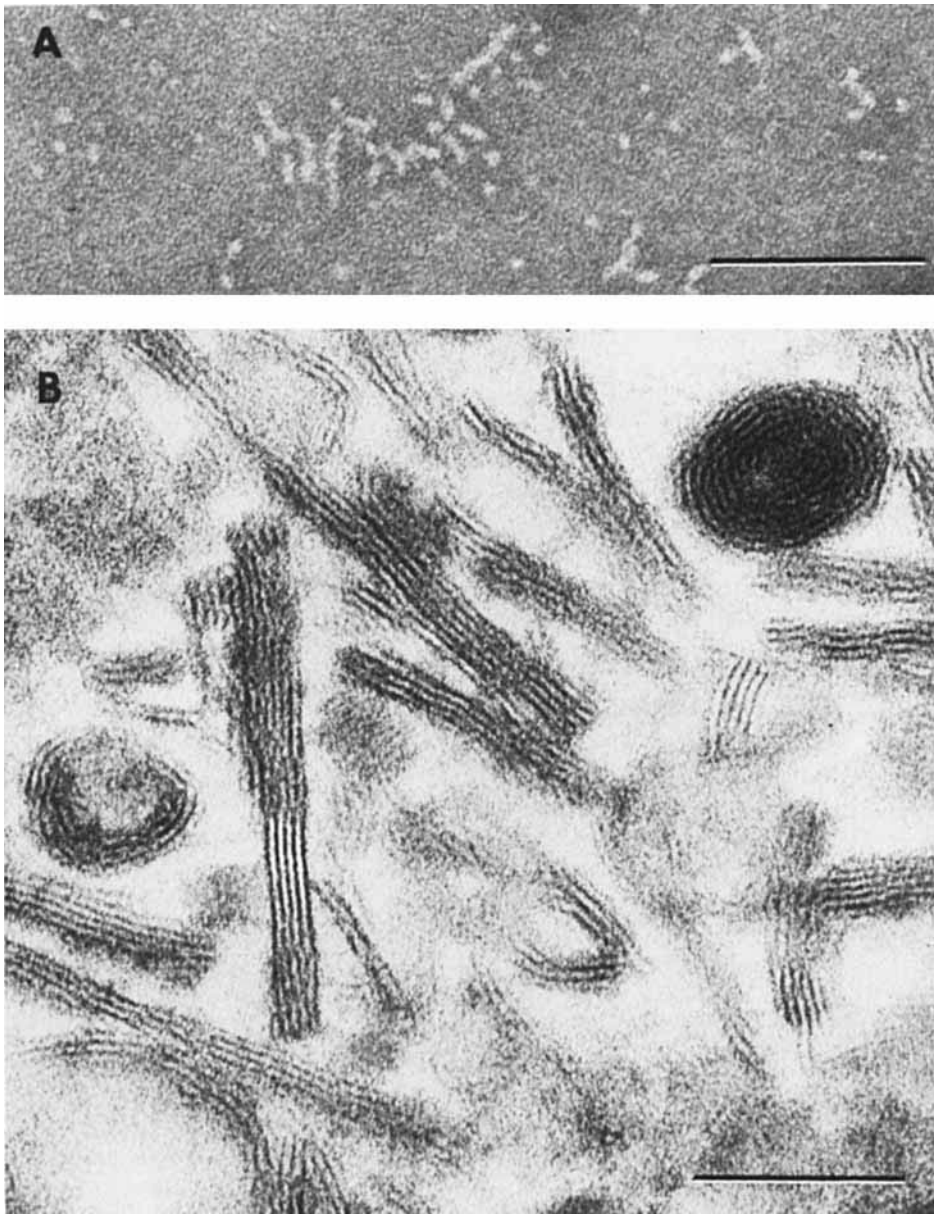

Fig. 8. Electronmicrograph of GPI. Bar = 1000 Å. Panel A shows negative staining of GPI. Panel B shows GPI, thin-sectioned after centrifugation.

DISCUSSION

In contrast to the human, horse, sheep, and goat erythrocyte membranes, the bovine glycoprotein does not have a "glycophorin"-type sialoglycoprotein; ie, a molecule containing 50–60% carbohydrate and a subunit molecular weight of 25,000–30,000 [8,10,11,16]. The major membrane glycoprotein in this type of red cell is a heavily glycosylated (80%) glycoprotein which was described by Capaldi in 1973 [3], and further characterized by Emerson and Kornfeld as containing "almost all of the membrane sialic acid, galactose, N-acetylglucosamine and most of the mannose and N-acetylgalactosamine" [4]. In 1971 we reported the isolation of bovine erythrocyte glycoprotein which was less glycosylated than glycoproteins extracted from sheep or horse red cell membrane. This glycoprotein had receptor activity for the heterophile antibody of infectious mononucleosis. We had reported that this glycoprotein had a subunit MW_{app} of ~25,000 [1,8].

Emerson and Kornfeld reported a MW_{app} of 230,000 from SDS-PAGE, for the glycoprotein they studied, and Capaldi reported a value of 150,000 from gel filtration in SDS, 180,000 from sedimentation equilibrium studies in 6 M guanidinium HCl and 285,000 from SDS-PAGE. Clearly at least two different types of glycoproteins are present in the membrane of the bovine erythrocytes. In an earlier report we described isolation by aqueous ethanol extraction and by LIS-phenol extraction and the partial purification of these glycoproteins from the bovine red cells [17]. In the present study more extensive purification and characterization were carried out.

Beginning with LIS-phenol extraction we isolated and purified a glycoprotein (GPII) with 80% carbohydrate. The hot organic extraction method of Fletcher and Woolfolk [1] was the initial solubilization step of bovine red cell membranes which yield a highly purified glycoprotein (GPI), with receptor properties for the heterophile antibody of infectious mononucleosis. It was also possible to isolate this glycoprotein by 75% ethanol extraction of the membrane residue left after LIS-phenol extraction and to solubilize GPII from membranes previously extracted sequentially by hot organic solvents. Comparison of the properties of GPI and GPII revealed major differences. On SDS-PAGE GPII, as had previously been reported, behaved as a very-large-molecular-weight molecule, and GPI as a much smaller molecule. When the RM of both glycoproteins were determined at several gel concentrations, it became apparent that the behaviors of GPI and GPII deviated from those of standard peptides and from each other. We have made previous notes of the difficulty associated with using SDS-PAGE data to estimate molecular weight, or even to enumerate membrane glycoproteins [8,10]. However, careful studies, using multiple buffer systems, SDS concentrations and acrylamide concentrations, can make this method at least as reliable as other empirical methods [18]. For example, a recent study by Leach et al [19] recommended gel chromatography in 6 M guanidinium hydrochloride as the most reliable empirical method for many glycoproteins. They note, however, that glycopolypeptides rich in sialic acid may exhibit deviant behaviors or resist denaturation in guanidinium hydrochloride—as is the case with human glycophorin [20] or horse erythrocyte glycoprotein [21]. Therefore, we estimated the apparent subunit size of GPI and GPII by measuring mobility in SDS-PAGE at graduated gel concentrations. Ferguson plots [13] showed that GPII had much lower (one-half) net surface charge density (the mobility, or y intercept, determined by extrapolation of the curve to zero gel concentration) than the standard peptides used as molecular weight markers. GPII, in contrast had Y_0 higher than that of the molecular weight markers. The slopes of the curves

($-K_R$) are proportional to the effective radius [22] and molecular weight [23]. MW_{app} of GPII estimated in this way was 55,000 daltons, three times smaller estimates than from gel chromatography data and five times smaller than SDS-PAGE estimations from RM. The molecular weight of GPI was estimated to be 34,000, a value 25% higher than that previously estimated.

The two glycoproteins had distinctive amino acid compositions. The peptide molecular weight of GPI was almost two times (25,000) higher than that of GPII (13,000). In contrast GPII had three times more sugar than GPI; for example, GPII had ~21 residues of sialic acid per mole and 66 mole of galactose as compared to 10 and 15 for GPI. Both molecules had detectable mannose—1mol/mol for GPI and 2 mol/mol for GPII—suggesting that at least one asparagine-linked chain occurs per molecule. Probably the great majority of oligosaccharide chains in both molecules are of the alkaline-labile (O-linked) type. The carbohydrate composition we found for GPII was closely similar to that described by Emerson and Kornfeld [4] and different from that reported by Capaldi [3], by Merrick et al [7], and by Hamazaki et al [5]. The amino acid composition for GPII we found was similar (except for serine and glutamic acid) to that reported by Hamazaki et al and rather unlike that described by Capaldi. Our preparation of GPI appears to be related to a bovine erythrocyte glycoprotein studied by Merrick et al except that it has nearly twice as much carbohydrate as the material analyzed by that group [7].

The morphology of these two glycoproteins were also studied by electron microscopy. Electron micrographs of GPI showed small spherical aggregates of 62 Å diameter which tended to form short chains. Electron micrographs of crude preparations of GPII showed large globules which show projections around their peripheries—suggesting that GPII is orientated in the vesicles with the carbohydrate extending out. In contrast GPII after removal of contaminating lipid, did not appear to form aggregates in dilute solution since even a dimer would have been within our range of visualization. The most accepted model for erythrocyte membrane glycoproteins is that of an asymmetric, amphipilic molecule (having both hydrophobic and hydrophilic portions) [24]. When amphiphilic molecules are dissolved in water they can achieve separation of their hydrophobic portions from water by self-aggregation (micelle formation) [4]. It would appear that GPI easily forms this type of micelle—and that this self-association results from the inherent properties of the molecule itself—and not contaminating lipids. The forms of micelles assumed by amphiphilic molecules can be small spheres, but also disks, long cylinders, or bilayers [25]. Reiss-Husson and Luzzati [26,27] have used X-ray diffraction to study ionic micelles in the absence of added salt and observed a transition from small globular micelles to rodlike micelles as the concentration of amphiphilic molecules increased [27]. When the concentration is sufficiently high, liquid crystalline phases are formed with an ordering of the rodlike micelles into parallel structures [25,27]. Further increase in concentration and decrease in water content leads to a lamellar phase consisting of extended bilayers. In an effort to create a very concentrated solution we subjected both glycoproteins to prolonged ultracentrifugation and fixed only the material at the bottom of the centrifuge tubes which was sectioned and "stained." Both glycoproteins, but more extensively GPI, formed laminar sheets which were both extended and parallel bilayers and stacks arranged into cylinders and multiwalled vesicles. This indicates that GPII, a very extensively glycosylated glycoprotein, can behave as an amphiphilic molecule under the proper conditions. This also may be the first demonstration of liquid crystal formation by membrane glycoproteins.

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