Isolation and Partial Characterization of the Sialoglycoprotein Fraction of Murine Erythrocyte Ghosts

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ABSTRACT With the lithium diiodosalicylate (LIS[1]) extraction-phenol partition method, we have isolated a sialoglycoprotein fraction from DBA/2 mouse erythrocyte ghosts. We have demonstrated that the Laemmeli system for SDS PAGE can resolve this fraction into four monomers of which two (gp-2.1 and gp-3.1) appear to be authentic, whereas the other two (gp-2.2 and gp-3.2) are probably generated from gp-2.1 and gp-3.1, by limited proteolysis during the isolation procedure. All four components contain O-acetylated neuraminic acid residues, can be stained with Periodic acid–Schiff reagent (PAS) and with Coomassie Brilliant Blue (CB), and can be radioiodinated with the lactoperoxidase-glucose oxidase (LPO-GO) method. All monomers but especially gp-2.1 and gp-3.1 generate characteristic aggregates during solubilization in SDS. The aggregation is enhanced by boiling at high concentrations, and can be reversed by boiling at low concentrations. In addition, the fraction contains a diffuse component present also in ghosts which stains poorly with CB and with PAS and cannot be radioiodinated by the LPO-GO technique. SDS PAGE in the Steck and Yu gel system does not give an accurate separation of the sialoglycoprotein monomers.

In our previous work on murine erythrocyte ghosts, we detected three sialoglycoproteins by Periodic acid–Schiff reagent (PAS) staining after SDS PAGE carried out according to Steck and Yu (37), and we presented evidence for the existence and preferential localization of O-acetylated sialyl residues on two of these three sialoglycoproteins (32). When the sialoglycoproteins were subsequently analyzed on Laemmli gels (20), however, we obtained a very different pattern. The discrepancy raised the following questions: Which of the visible bands are sialoglycoprotein monomers and which are aggregates? Which of the gel systems truly separates the monomers? And, if polymerization occurs, what are the conditions that can prevent it in vitro?

Here we partially answer these questions. We eluted all resolved components from each of the two gel systems and reran each component in the same or the alternative system. Each band will be named by a prefix gp, (for glycoprotein), and a suffix: S to signify initial separation on a Steck and Yu gel; or L to signify separation on a Laemmli gel. The prefixes will always be used in the text but may be omitted from the figures.

MATERIALS AND METHODS

All reagents were analytical or reagent grade. The sources of reagents for SDS PAGE and sialic acid assays have been published (32). Lithium diiodosalicylate (LIS) was a gift from Drs. Furthmayr and Marchesi. Lactoperoxidase (LPO) and glucose oxidase (GO) (coupled to Sepharose 4B by the procedure of Cuatrecasas and Anfinsen [6, 15]) were gifts from Dr. Ann Hubbard. Trypsin (150 U/mg, treated with DPCC) and Papain (P-3125, twice recrystallized) were from Sigma Chemical Co. (St. Louis, MO); α-chymotrypsin (50 U/mg) from Worthington Biomedical Corp. (Freehold, NJ); and carrier free $K^{125}I$ from New England Nuclear (Boston, MA). Intensifying screens (Lightning Plus and Quanta II) were obtained from DuPont Instruments (Wilmington, DE), and x-ray film (X-Omat R) from Kodak (Rochester, NY).

Gel Electrophoresis

SDS PAGE was performed by solubilizing the proteins in 62.5 mM Tris-HCl, pH 6.8, 3% wt/vol SDS, 2 M urea, 2 mM EDTA, and either 20 mM dithiothreitol (DTT) or 1.5% (vol/vol) β-mercaptoethanol, heating the mixture in a boiling water bath for 5 min, and then separating its components according to Laemmli (20). Some analyses were also performed on Steck and Yu gels (37) as in Sarris and Palade (32) by solubilizing the preparations in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 3% (wt/vol) SDS, 1.5% (vol/vol) β-mercaptoethanol and 6% (vol/vol) glycerol, and heating them in a boiling water bath for 5 min before electrophoretic separation. The procedures for staining the gels with PAS and NaOH-PAS are given in Sarris and Palade (32). The composition and the buffer system of each acrylamide gel are indicated in the legend of each figure. As molecular weight markers we used: β-galactosidase from *Escherichia coli* (130,000), rabbit muscle phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), bovine chymotrypsinogen (25,700), soybean trypsin inhibitor (21,500), and human globin (16,500).

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TABLE I

Recovery of Ghost Sialyl Residues during the Isolation of the Sialoglycoprotein Fraction by LIS

		NeuNAc					
		Content					
	Dry weight	A*	B*	di‡	Recovery	Concentration§	RE ‖
	mg	*nmol*					
Ghosts	900	10,920	13,520	19	100	15	1
LIS(+) fraction	50.2	4,500	8,278	46	61	166	11
LIS(−) fraction	20.3	4,425	8,180	46	61	403	27

Sialyl residues were released by 0.025 M H_2SO_4 for 1 h at 80°C and assayed according to Warren (44) using readings at 562 and 549 nm and 2-deoxyribose and NeuNAc as standards, as previously detailed (32). All recovery figures are calculated on the basis of ghosts.

* (A and B) Sialic acid assayed before and after de-O-acetylation, respectively.

‡ *di*, de-O-acetylation index as defined in reference 32.

§ Concentration, nmoles NeuNAc/mg dry weight.

‖ *RE*, relative enrichment in sialyl residues.

Isolation of the Sialoglycoprotein Fraction

The collection and preparation of DBA/2 erythrocyte ghosts were as described in Sarris and Palade (32), except that the lysis buffer contained as protease inhibitors 0.3 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM sodium tetrathionate (STT) (21, 29, 30). The sialoglycoprotein fraction was isolated from erythrocyte (RBC) ghosts according to Marchesi and Andrews (23). The sialoglycoproteins with LIS still bound to them will be called the LIS(+) fraction, and the same preparation after removal of all detectable LIS will be designated the LIS(−) fraction.

Sialic acid content in input ghosts and derived LIS(+) and LIS(−) fractions was monitored by a modified (32) Warren assay (44). Ghosts, the LIS(+) and LIS(−) fractions from each preparation were also compared by SDS PAGE in the Steck-Yu system (37). We could not stain the sialoglycoproteins with the Coomassie Brilliant Blue (CB) procedure given in (4), but we succeeded in staining them well by fixing the gels in 25% isopropanol–10% acetic acid, removing the isopropanol with 10% acetic acid, staining overnight with 0.07% (wt/vol) CB in 10% acetic acid, and finally destaining with 10% acetic acid.[1] The sialoglycoproteins could be stained after de-*O*-acetylation (with 0.1 N NaOH–25% isopropanol) provided the alkali was removed with 25% isopropanol–10% acetic acid before applying the modified CB-staining technique. Gels were photographed and scanned as in Sarris and Palade (32).

Sialoglycoprotein Labeling with the $K^{125}I$-LPO-GO Method

The sialoglycoproteins were suspended in 100 mM sodium phosphate, pH 7.1, 10 mM glucose, and were labeled with $K^{125}I$ by the LPO-GO method, using enzymes covalently attached to Sepharose beads. After 30 min on ice, the beads were removed by centrifugation, and the supernate extensively dialysed against double distilled H_2O at 4°C to remove unreacted iodide. [^{125}I]Sialoglycoproteins thus prepared were stored in small aliquots at −20°C, and analyzed by SDS PAGE followed by CB staining and autoradiography as in Maizel (22), or with the use of intensifying screens at −70°C (39). X-ray film was developed by Kodak automatic processors in the Department of Radiology, Yale-New Haven Hospital.

Elution of [^{125}I]Sialoglycoproteins

Individual [^{125}I]sialoglycoprotein bands, located by autoradiography on wet SDS PAGE gels, were excised with razor blades and their components were eluted electrophoretically into dialysis bags (38) using the buffer of Fairbanks et al. (9) for both anodic and cathodic compartments. Recovery of sialoglycoproteins from gel slices into eluates was monitored by ^{125}I-counting in a Beckman Biogamma counter (Beckman Instruments, Inc., Palo Alto, CA) operating with 70% efficiency; it varied from 65–95% for each band in different elution experiments. The eluates were stored at −20°C. For SDS PAGE analysis, they were mixed with the appropriate solubilization buffer, heated in a boiling water bath for 5 min, and then loaded on gels.

[1] Trichloroacetic acid can substitute for acetic acid in the wash, staining and destaining steps, but it was not used because it altered significantly the dimensions of the gels.

Tryptic and Chymotryptic Digestion of [^{125}I]-Sialoglycoproteins

Individual [^{125}I]sialoglycoprotein bands, separated by SDS PAGE on 14% Laemmli gels, were located (by autoradiography) on wet gels and excised. To avoid cross-contamination between gp-2.1 and gp-2.2 and between gp-3.1 and gp-3.2, we always left behind a small strip of gel in between the bands in each pair. For the rest of the procedure we followed Alper's modification (1) of the method of Elder et al. (8). The sialoglycoproteins precipitated in the gel pieces were freed of SDS with several changes of 25% isopropanol–10% acetic acid. The acid was extracted with three washes of 25% isopropanol and the isopropanol was in turn removed with three washes of double distilled water. None of the washes eluted any radioactivity from the gel pieces. The gel fragments were then cut into smaller pieces and their digestion was carried out for 24 h at 37°C in siliconized scintillation vials with 50 μg/ml of trypsin or 40 μg/ml of chymotrypsin in 50 mM NH_4HCO_3, pH 8.0. New enzyme was added after 12 h. After 24 h, the gel pieces were removed by centrifugation and the supernatant, containing 50–90% of the input radioactivity, was recovered and lyophilized in siliconized test tubes. Incubation of gel fragments with buffer alone released only 1–2% of their radioactivity. The lyophilized material was boiled for 5 min in Laemmli solubilization buffer and re-electrophoresed on 14% Laemmli gels. Since the small peptides generated by proteolysis are soluble in the staining solutions used in Castle et al. (4), the gels were dried without fixation and the resolved peptides were localized by autoradiography at −70°C.

Papain Digestion of Eluted [^{125}I]-Sialoglycoproteins

Sialoglycoproteins separated on 14% Laemmli gels and eluted electrophoretically as described were digested with papain (45 μg/ml) for 30 min at 23°C or 37°C in the presence of SDS (5). The papain was not previously activated and there was no reducing agent present during digestion. AT the end of the incubation the samples were processed as in the section immediately above.

RESULTS

Recovery of Sialyl Residues and SDS PAGE of the Sialoglycoprotein Fraction

The sialoglycoprotein fraction isolated by LIS extraction and phenol partition (23) accounted for ~60% of the sialyl residues of the ghosts (Table I). Components carrying nonsubstituted sialyl residues were apparently lost during the extraction, since O-acetylated sialyl residues were preferentially recovered in both LIS extracts as indicated by an increase in their de-O-acetylation index. The sialic acid content of four different preparations was 191 ± 34 and 388 ± 39 nmol N-acetyl neuraminic acid (NeuNAc)/mg dry weight before and after de-O-acetylation, respectively; the de-O-acetylation index was 51 ± 5.

Sialoglycoproteins and the ghost preparations from which

they were derived were analyzed by SDS PAGE in Laemmli's system. In ghosts, PAS stained gp-3L well, but gp-1L and gp-2L poorly (Fig. 1 *A*). NaOH-PAS stained gp-1L and gp-2L more intensely, and altered the staining in the diffuse region of gp-3L by extracting its trailing end and by revealing under it several minor bands (Fig. 1 *C*). PAS stained all CB stainable components of the sialoglycoprotein fraction (Fig. 1 *B*; also Figs. 2 and 9), and NaOH-PAS greatly increased the staining of all bands, removed most of the staining of the trailing end of gp-3L, and increased the staining at its front (Fig. 1 *D*). However, in neither LIS(+) nor LIS(−) did it reveal, under the removed trail of gp-3L, the significant amounts of the components it revealed in ghosts. These components may be absent in sialoglycoprotein fractions, or present below the limit of detection of the staining procedure used. Several new bands (Fig. 1, *a*, *b*, *c*) not detectable in ghosts were present in LIS fractions; experimental results reported later on in this paper indicate that they are sialoglycoprotein aggregates.

The results presented in Fig. 1 show that the sialoglycoprotein fraction loses most of the trail of its gp-3L during gel processing for PAS-NaOH-staining, but they do not indicate what reagent or procedural step is responsible for the loss. These issues were investigated by experiments of the type presented in Fig. 2.

The trailing end of gp-3L is not destroyed by 0.1 M NaOH in solution, since de-O-acetylation of the sialoglycoproteins by 0.1 M NaOH alone, followed by SDS PAGE and PAS staining, gives a staining pattern characteristic of de-O-acetylated sialoglycoproteins without substantial removal of the trail (Fig. 2 *E*). If, however, de-O-acetylation in solution is followed by SDS PAGE and NaOH-PAS, the trail of gp-3L is practically gone (Fig. 2 *F*). Since it was not detectably removed from the gel by rinsing with 25% isopropanol (Fig. 2 *C* vs. *D*), it must be extracted from the gel by alkaline isopropanol. Some of this extraction may be due to the effect of 0.1 M NaOH on the trailing component because its loss is more pronounced when sialoglycoproteins are de-O-acetylated in solution, and then processed for SDS PAGE followed by PAS staining (Fig. 2 *C* vs. *E*).

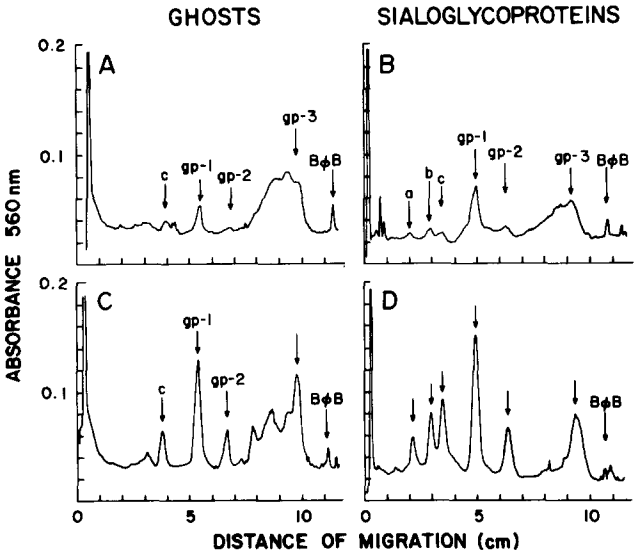

FIGURE 1 Detection of sialoglycoproteins in DBA/2 erythrocyte ghosts and derived sialoglycoprotein fraction. 320 μg of ghosts (solubilized at 3.2 mg/ml) and 100 μg of sialoglycoproteins (solubilized at 1 mg/ml) were analyzed by SDS PAGE on 10% Laemmli gels and stained with PAS (*A* and *B*), or with NaOH-PAS (*C* and *D*). *BφB*: bromphenol blue used as tracking dye.

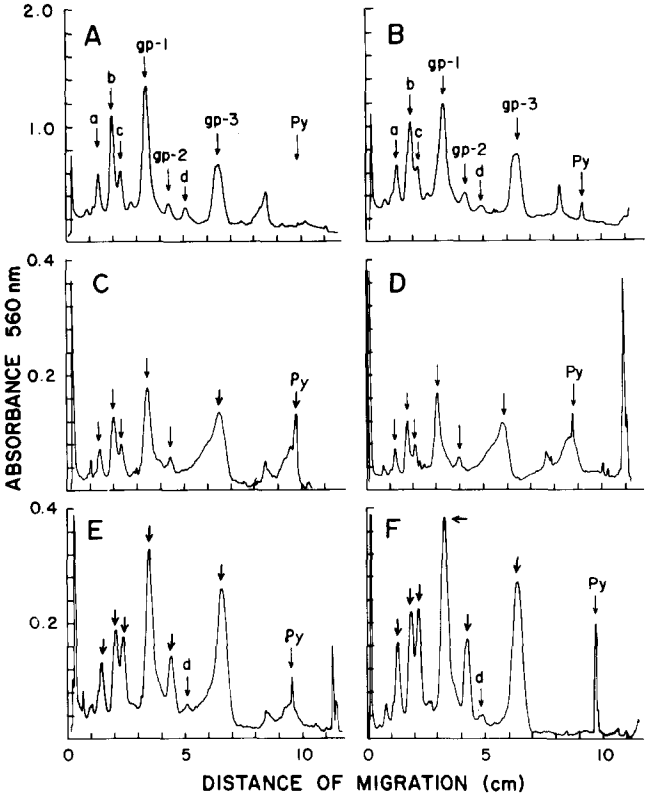

FIGURE 2 Loss of the trailing end of gp-3L in alkaline isopropanol. 150 μg of sialoglycoproteins, solubilized at 1 mg/ml by heating for 5 min in a boiling water bath, were separated by SDS PAGE on 10% Laemmli tube gels. (*A*) Stained with the modified CB procedure for sialoglycoproteins. (*B*) Fixed first in 25% isopropanol–10% acetic acid, then washed with 25% isopropanol, incubated for 45 min with 0.1 M NaOH–25% isopropanol, washed with 25% isopropanol–10% acetic acid and then stained with the CB procedure (NaOH-CB). (*C*) fixed in 25% isopropanol–10% acetic acid, then processed and stained with PAS as in (12). (*D*) fixed as in *C*, but washed after fixation with three changes of 25% isopropanol. (*E* and *F*) the sialoglycoproteins were de-O-acetylated with 0.1 M NaOH for 45 min on ice, the NaOH was neutralized with HCl, the sialoglycoproteins were solubilized at 1 mg/ml, separated by SDS PAGE and stained by PAS (*E*) or NaOH-PAS (*F*, 45 min in alkaline isopropanol). Sialoglycoprotein peaks are labeled in *A* and *B* and marked by arrows in the other panels. *Py*: pyronine (tracking dye).

The kinetics of the extraction were followed by varying the exposure to alkaline isopropranol. The trail of gp-3L was substantially lost after 15 min, in sharp contrast to *a*, *b*, *c*, gp-1L, gp-2L, and the front edge of gp-3L (Fig. 2) which showed considerable increases in staining from 0 to 45 min (Fig. 3). The leading edge of gp-3L is stained by CB, and is not extracted by alkaline isopropanol (Fig. 2 *A* and *B*); its PAS staining increases upon de-O-acetylation but the increase at the leading edge is virtually cancelled by the extraction of its trailing end, so that the overall increase of the gp-3L staining is very small (Fig. 3).

The ghosts and the cognate sialoglycoproteins were also examined in Steck and Yu gels (37), because this was the system we originally used (32). In ghosts, gp-1S and gp-2S were poorly stained by PAS (Fig. 4 *A*), but staining by NaOH-PAS was much more intense (Fig. 4 *C*). Gp-3S is the major component detectable by PAS; by NaOH-PAS it showed some-

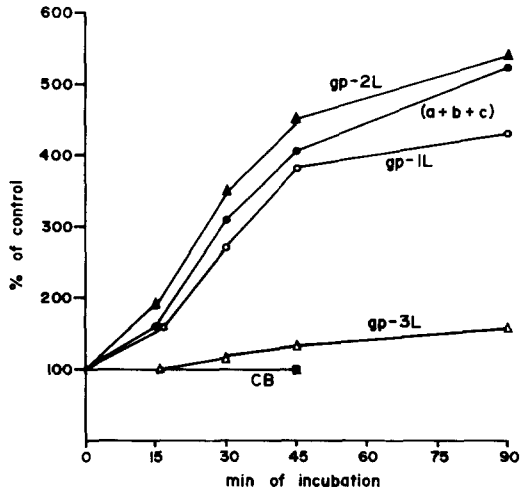

FIGURE 3 Kinetics of de-O-acetylation by alkaline isopropanol. 150 μg of sialoglycoproteins (solubilized at 1 mg/ml by heating for 5 min in a boiling water bath) were separated by SDS PAGE on 10% Laemmli tube gels. The gels were fixed and stained with PAS or with NaOH-PAS, after incubations in alkaline isopropanol for the times given on the abscissa. One gel tube was also stained with CB after 45 min in alkaline isopropanol. The areas were quantified and expressed as % of the corresponding area on a gel which had not been exposed to alkaline isopropanol. All areas are quantification of PAS staining, except for those which refer to the summated intensities of CB staining of (*a* + *b* + *c*), gp-1, gp-2, and gp-3 (they all were 100% after 45 min in alkaline isopropanol).

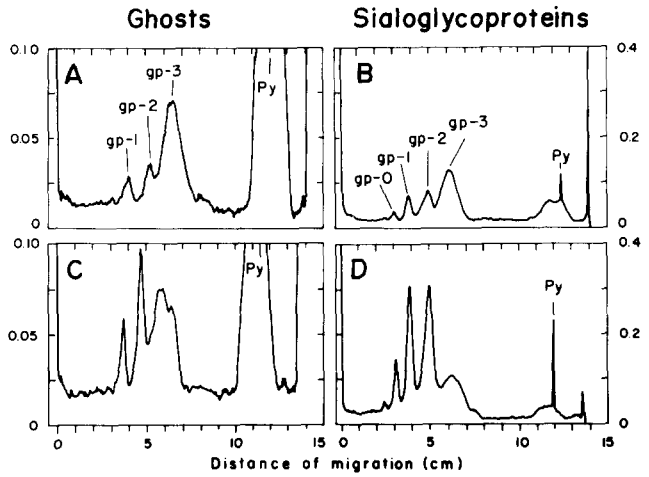

FIGURE 4 SDS PAGE of DBA/2 ghosts and of their sialoglycoprotein fraction on 5% Steck-Yu gels. Ghosts and sialoglycoproteins were solubilized at 2.0 mg/ml by heating in a boiling water bath for 5 min, and 50 μg (*A*) or 200 μg of ghosts (*C*), and 200 μg of sialoglycoproteins (*B* and *D*) were separated by SDS PAGE on 5% Steck-Yu tube gels. The gels were stained with PAS (*A* and *B*), and NaOH-PAS (*C* and *D*) after 45 min in alkaline isopropanol. Both ordinates give adsorption at 560 nm.

times small increases and occasionally a second peak appeared at its leading edge (Fig. 4*A* vs. *C*).

After SDS PAGE of the sialoglycoprotein fraction in the Steck-Yu system, PAS stained gp-1S, gp-2S, and gp-3S, as well as a new component, gp-OS (Fig. 4*B*). NaOH-PAS resulted in a large increase in the staining of gp-OS, gp-1S, and gp-2S (Fig. 4*D*), but gp-3S showed small, and variable changes (either increases or decreases). The ratios of gp-1S and gp-2S to gp-3S were much higher in the sialoglycoprotein fraction than in ghosts as seen by both PAS and NaOH-PAS.

The results of the analysis of the sialoglycoproteins in Laemmli SDS PAGE, together with the recovery of sialyl residues (Table I) suggest that an unknown number of components in the gp-3L region, carrying predominantly nonsubstituted sialyl residues, are only partially recovered in the sialoglycoprotein fraction. At least some (but not all) of these components may be represented by the trailing end of gp-31 in the Laemmli system.

Monomeric Units of the Sialoglycoprotein Fraction and Their Pattern of Aggregation

The formation of aggregates during the solubilization of human sialoglycoproteins for SDS PAGE has been documented and the parameters that influence aggregation have been partially defined; ionic strength, temperature, and protein concentration during the solubilization step are all important (10–12, 25, 34–36).

SDS PAGE demonstrated new peaks (*a*, *b*, *c*) in the sialoglycoprotein fraction which were not detectable (*a*, *b*) or not as prominent (*c*) in ghosts (Fig. 1). We suspected they were artifacts of aggregation. It was considered desirable for future work to use a gel system that presents the true picture of the sialoglycoproteins with the best possible resolution of their monomers or with minimal and well-understood artificial aggregation. Since the preparation of large amounts of murine erythrocyte ghosts for the isolation of milligram amounts of sialoglycoproteins is a tedious and expensive operation, we used a radiochemical approach to determine the best gel system and to define the monomeric units and their pattern of aggregation. We labeled the sialoglycoproteins with $K^{125}I$ with the LPO-GO methods (16), resolved individual components by SDS PAGE, eluted each band electrophoretically and analyzed the eluates by SDS PAGE in both Steck-Yu and Laemmli gels.

When the [^{125}I]sialoglycoproteins were analyzed by SDS PAGE on the Laemmli system (Fig. 5), some of the high molecular weight components (*a*, *b*, *c*) were not as prominent as in Fig. 1, but gp-1L, gp-2L, and gp-3L were well-labeled (Fig. 5, lanes *A* and *B*). Furthermore, gp-2L had two components: gp-2.1L (apparent M_r, 44,000) and gp-2.2L (apparent M_r, 43,000), barely resolved in Fig. 5, lanes *B*, *D*, and *E*, but clearly visible in Fig. 10. Gp-3L was also split into two components: gp-3.1L (apparent M_r, 27,000) and gp-3.2L (M_r, 26,000), partially resolved in Fig. 5 and well separated in Fig. 10. This splitting was not consistently demonstrable in tube gels probably because their resolving capacity was blunted by shorter migration distances, slight band curvature, and high loads of material required for PAS or CB staining. Splitting could also be demonstrated on long slab gels stained by the CB procedure for sialoglycoproteins.

We identified the monomeric units of [^{125}I]sialoglycoproteins by solubilizing the fraction under a variety of conditions and analysing the results on Laemmli gels as illustrated in Fig. 5. Solubilization at 0.5 mg/ml led to extensive aggregation at 23°C (Fig. 5, lane *A*) which was not removed by dilution to 10 μg/ml at 23°C (Fig. 5, lane *C*). Heating the sialoglycoproteins for 5 min in a boiling water bath removed only some of the bands if the concentration was 0.5 mg/ml (Fig. 5, lane *A* vs. *B*). If, however, the [^{125}I]sialoglycoproteins were diluted to 10 μg/ml and heated for 5 min in a boiling water bath, *a*, *b*, *c*, and gp-1L almost disappeared (Fig. 5, lane *D*). Since the intensities of gp-2.1L, gp-2.2L, gp-3.1, and gp-3.2L concomitantly increased, these appear to be the monomeric units which aggregate to form gp-1L, *a*, *b*, and *c* (Fig. 5). If the sialoglycoproteins

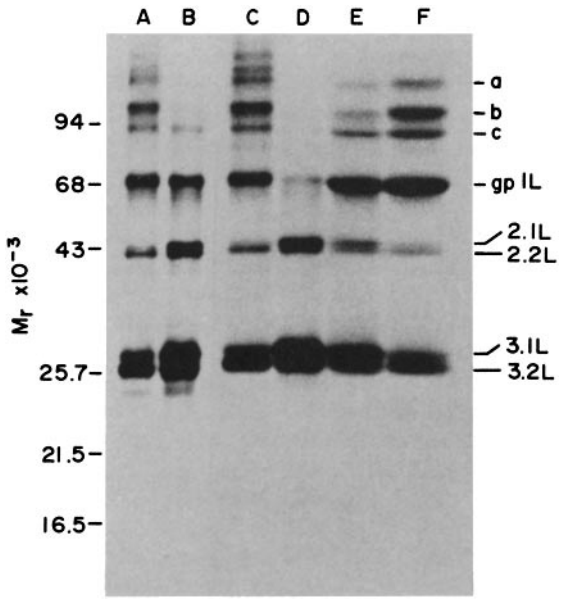

FIGURE 5 The monomeric units of the sialoglycoproteins. [^{125}I]Sialoglycoproteins were solubilized at 0.5 mg/ml (*A* and *B*), or 10 μg/ml (*C* and *D*) either at 23°C (*A* and *C*) or at 23°C followed by heating for 5 min in a boiling water bath (*B* and *D*). Samples *E* and *F* were first heated in a boiling water bath for 5 min at 10 μg/ml, then mixed with unlabeled sialoglycoproteins to a final concentration of 2.0 mg/ml and incubated at room temperature (*E*) or heated for 5 min in a boiling water bath (*F*). SDS PAGE was in a 10–15% Laemmli gradient gel.

are initially dissociated to their monomeric units by heating at low concentration, then cooled to 23°C and mixed with unlabeled sialoglycoproteins (final concentration 2.0 mg/ml), they aggregate extensively (Fig. 5, lane *E*) and heating further enhances this aggregation (Fig. 5, lane *F*). The dissociated monomers can aggregate at high concentrations at 23°C without heating, and these results are consistent with those shown in Fig. 5, lanes *A* and *B*. These findings indicate that the aggregates are formed during solubilization in the absence of heating, and that their dissociation into monomeric units requires both boiling and low protein concentrations.

We decided to investigate in further detail the differences between the patterns obtained by Steck-Yu and Laemmli systems because we wanted to establish beyond doubt the identity and interrelations of gp monomeric units and gp polymers. For this purpose, [^{125}I]sialoglycoproteins were separated by SDS PAGE in the Laemmli system, and individual bands were eluted and re-electrophoresed in the same system (Fig. 6). Under these conditions, gp-3.1L, gp-3.2L and the mixture of gp-2.1L and gp-2.2L ran true, but gp-1L dissociated in part, mainly into gp-3.1L and into some gp-3.2L. When the concentration during boiling was increased by the addition of an unlabeled sialoglycoprotein mixture, the dissociation of gp-1L was largely prevented; moreover, gp-1L aggregated to form mainly *b*, but also some *a* and *c*, and the yield of gp-3.1L was greatly reduced. Under the same conditions gp-3.1L gave rise to gp-1L and to *b*, with minor formation of *a* and *c* (Fig. 6). At high concentrations gp-3.2L aggregated somewhat (less effectively than gp 3.1) into gp-1L. When the mixture of gp-2.1L and gp-2.2L was boiled at high concentration, gp-2.1L seemed to aggregate (more than gp-2.2L) to form mostly *a* and *c*. These results confirm the earlier finding that gp-2.1L, gp-2.2L, gp-3.1L and gp-3.2L are the monomers, and they further designate gp-1L as an aggregate of gp-3.1L and of gp-3.2L. Each of the other bands (Fig. 6, *a*, *b*, *c*) contain all monomers, but each at a different ratio.

Sialoglycoproteins eluted from Laemmli gel bands were also analyzed in the Steck-Yu system (Fig. 7). Gp-3.1L and gp-3.2L run partly overlapping each other in the region of gp-3S, and gp-2.1L and gp-2.2L also overlapped each other in the region of gp-2S. Hence, this system does not resolve the components of the doublets. When gp-1L was analyzed in the same way it gave rise to gp-3S and to some gp-2S. The band eluates from Laemmli were also heated in the presence of high concentrations of unlabeled sialoglycoproteins and then analyzed by SDS PAGE in the Steck-Yu system. Gp-3.2L did not aggregate appreciably, but gp-3.1L aggregated into gp-2S and gp-1S. Gp-2.1L and gp-2.2L (eluted together) aggregated partially into gp-1S; gp-1L was resolved into gp-1S, gp-2S, and gp-3S, with the bulk of the label appearing in gp-2S. It seems, therefore, that in the Steck-Yu system, gp-1S is a mixture of gp-3.1L, gp-2.1L, or gp-2.2L (the resolution in Fig. 7 does not permit distinction between gp-2.1L and gp-2.2L). Gp-2S contains gp-2.1L, gp-2.2L, and gp-3.1L; and gp-3S consists of incompletely separated gp-3.1L and gp-3.2L. These results were confirmed by eluting the sialoglycoproteins from a Steck-Yu gel and analyzing them in a Laemmli gel after heating the samples at high or low concentrations (Fig. 8). This experiment further clarified that gp-1S contains gp-3.1L and gp-2.1L but not gp-2.2L. The experimental data so far reported suggest that all bands visible in the Steck-Yu system are heterogeneous mixtures; the suggestion was confirmed by eluting Steck-Yu gel bands and subjecting the eluates to reelectrophoresis in the

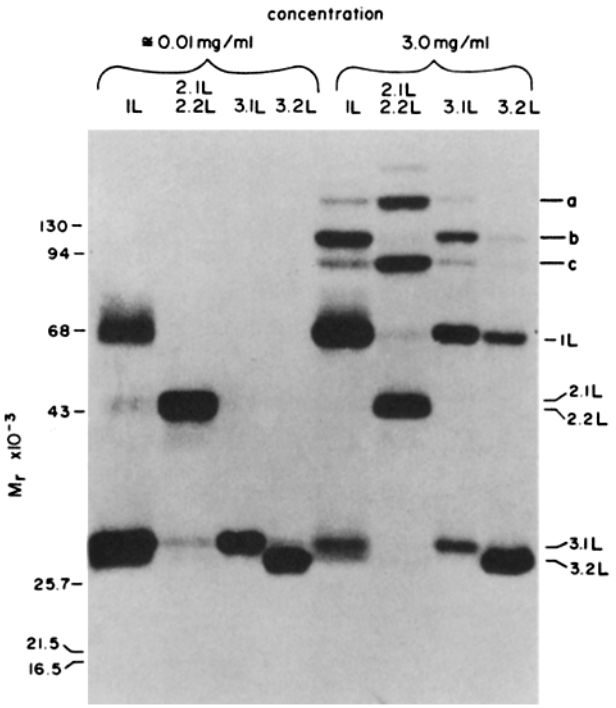

FIGURE 6 SDS PAGE of [^{125}I]sialoglycoproteins on a 10% Laemmli gel. Individual bands were eluted from a 10% Laemmli gel, and were heated for 5 min in a boiling water bath either at 10 μg/ml or at 3.0 mg/ml (by the addition of an unlabeled mixture of sialoglycoproteins) before SDS PAGE on a 10% Laemmli gel. For each band the same amount of radioactivity was loaded on the gel at low (10 μg/ml) and high (3.0 mg/ml) concentrations, but the amounts differed from one pair of bands to another.

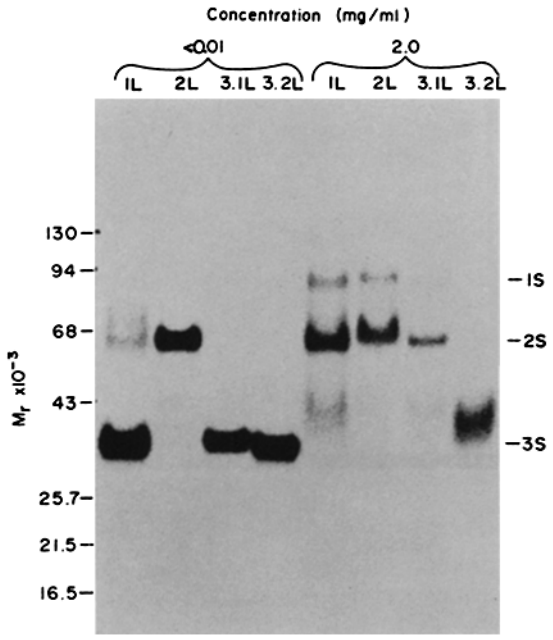

FIGURE 7 SDS PAGE of [^{125}I]sialoglycoproteins on a 5% Steck-Yu gel. The sialoglycoproteins of individual bands were eluted from a 10% Laemmli gel, and were heated for 5 min in a boiling water bath either at low concentrations (<10 μg/ml) or at 2.0 mg/ml (reached by adding an unlabeled sialoglycoprotein mixture) before SDS PAGE on a 5% Steck-Yu gel. For each band the same amount of radioactivity was loaded on the gel at low (<10 μg/ml) and at high (2.0 mg/ml) concentrations, but the amounts differed from one pair of bands to another.

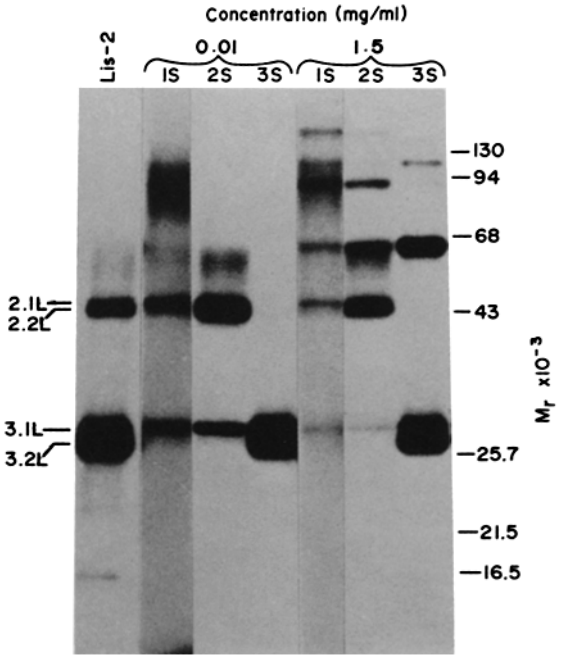

FIGURE 8 SDS PAGE of [^{125}I]sialoglycoproteins on a 10% Laemmli gel. The sialoglycoproteins of individual bands were eluted from a 5% Steck-Yu gel, and heated for 5 min in a boiling water bath at either low (10 μg/ml) or high concentration (1.5 mg/ml, obtained by the addition of an unlabeled mixture of sialoglycoproteins), before SDS PAGE on a 10% Laemmli gel. For each band the same amount of radioactivity was loaded on the gel at low and at high concentrations, but the amounts differed from one pair of bands to another. A standard [^{125}I]sialoglycoprotein fraction (LIS-2) is shown in the first (left) lane.

same system after solubilization at high as well as low concentrations (data not shown).

Relationships between Monomers: Proteolytic Degradation during the Preparation

The first three sialoglycoprotein fractions prepared by the LIS extraction-phenol partition procedure gave results identical to those in Fig. 1 when analyzed by SDS PAGE followed by PAS or NaOH-PAS staining. Moreover, SDS PAGE of [^{125}I]sialoglycoproteins from each of these preparations revealed patterns very similar to those shown in Fig. 5. However, when [^{125}I]sialoglycoproteins were dissociated to their monomeric units by heating at low concentrations before SDS PAGE, some variability in the ratios of gp-3.1L/gp-3.2L and gp-2.1L/gp-2.2L was evident among different preparations. Furthermore, a fourth sialoglycoprotein preparation (referred to as LIS 4) gave a strikingly different pattern. During its preparation the erythrocytes were lysed in the absence of STT, and phenol was removed by dialysis in the cold room (8°C), using room temperature double-distilled water. The rest of the procedure was as described in Materials and Methods. In contrast to the other preparations (represented in Fig. 9 by LIS 3) LIS 4 did not give rise to a, b, c, or gp-1L when heated at 1 mg/ml (Fig. 9). [^{125}I]Sialoglycoproteins from these two preparations were heated at 10 μg/ml (to dissociate all aggregates) and then analyzed by SDS PAGE to display all monomeric units. LIS 3 contained as usual gp-2.1L, gp-2.2L, gp-3.1L, and gp-3.2L (Fig. 10), whereas LIS 4 contained only gp-2.2L and gp-3.2L. These results raise the possibility that gp-2.2L and gp-3.2L were generated from gp-2.1L and gp-3.1L, respectively, by hydrolysis during the preparation of the last fraction.

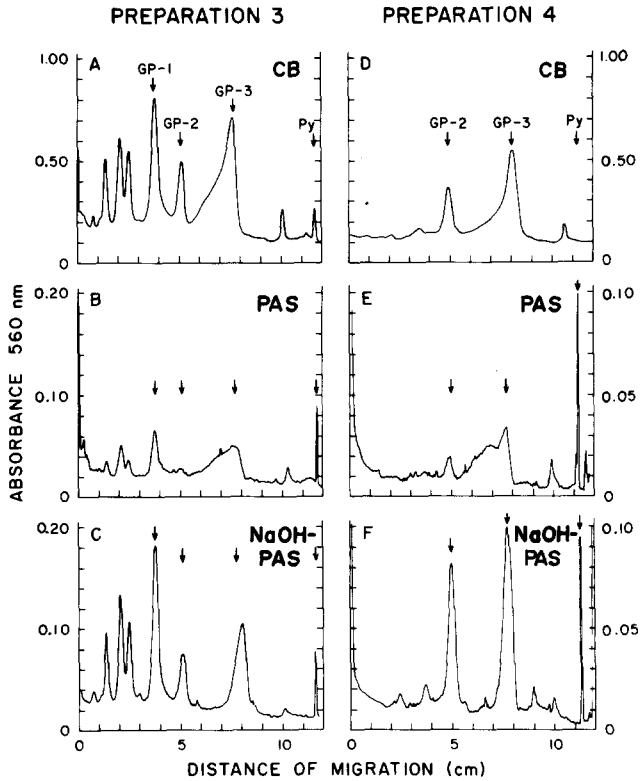

FIGURE 9 SDS PAGE of sialoglycoprotein preparations 3 and 4. Sialoglycoprotein was solubilized at 1 mg/ml by heating in a boiling water bath for 5 min, and 100-μg samples were then separated by SDS PAGE on 10% Laemmli tube gels, which were stained for protein (*A* and *D*), PAS (*B* and *E*) and NaOH-PAS (*C* and *F*).

If this were true, one would expect overlapping or even identical peptide maps for the individual bands of each doublet, and this prediction was confirmed with tryptic and chymotryptic maps of each monomer. The chymotryptic map of gp-2.1L (Fig. 11, lane *A*) was identical to the map of gp-2.2L isolated from either preparation 3 (Fig. 11, lane *B*) or preparation 4 (Fig. 11, lane *C*). Upon longer exposure, the faint peptides just below the level of 3.2 in lanes *A* and *B* (Fig. 11) became also evident, but then the larger peptides were overexposed as is the case in lane *C* (Fig. 11). Similarly, the chymotryptic peptides of gp-3.1L (Fig. 11, lane *D*) were essentially identical to the map of gp-3.2L isolated from preparation 3 (Fig. 11, lane *E*) or preparation 4 (Fig. 11, lane *F*). Again, the chymotryptic peptides of gp-1L isolated from LIS 3 (Fig. 11, lane 1L) were identical with those of gp-3.1L or gp-3.2L as expected, because gp-1L is an aggregate of gp-3.1L and gp-3.2L. These results were corraborated by tryptic peptide maps which, however, gave fewer peptides (data not shown). We have not been able to identify a missing or different peptide among these proteolytic fragments, but this result can be explained by the absence of tyrosines from the putative peptide(s).

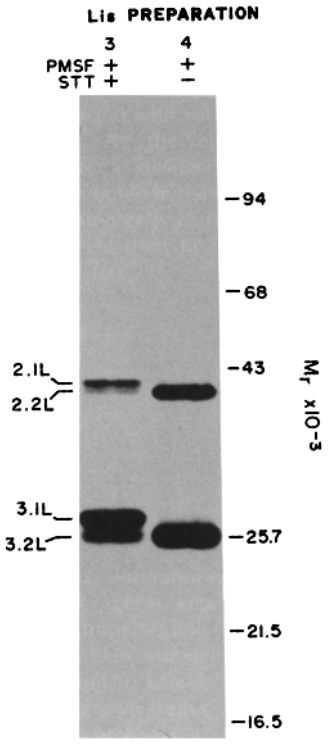

FIGURE 10 SDS-PAGE of [^{125}I]sialoglycoproteins from preparations 3 and 4. [^{125}I]Sialoglycoprotein samples from LIS preparations 3 and 4 were heated for 5 min in a boiling water bath at 10 μg/ml (to dissociate all aggregates to monomers) and were subsequently subjected to SDS PAGE on a 10–15% gradient Laemmli gel. The presence (+) or absence (−) of PMSF and of STT during the lysis step is indicated at the top of each lane.

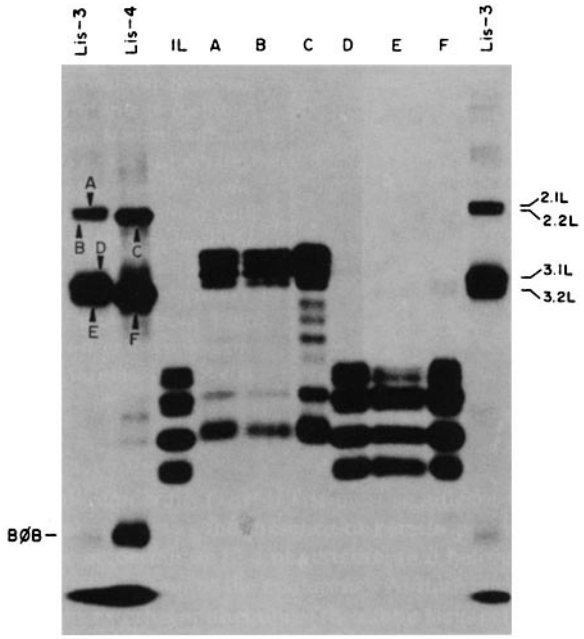

FIGURE 11 Chymotryptic peptide maps of [^{125}I]sialoglycoproteins. [^{125}I]Sialoglycoproteins from LIS preparations 3 and 4 were separated on a 14% Laemmli gel and individual bands were digested by α-chymotrypsin. The origin of each digest is as indicated in the figure (top of each lane and letters and arrowheads on the left side LIS 3 and LIS 4 lanes); except for gp-1L (1L) which was derived from LIS 3 and was solubilized at ~0.4 mg/ml. Chymotryptic digests were analyzed by SDS PAGE on a 14% Laemmli gel.

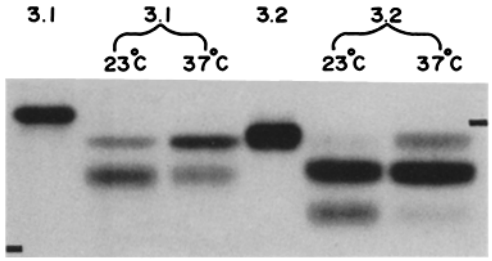

FIGURE 12 Partial papain digestion of [^{125}I]sialoglycoproteins. [^{125}I]gp-3.1 and [^{125}I]gp-3.2, eluted electrophoretically from a 14% Laemmli gel, were incubated for 30 min at 23°C or 37°C with 45 μg/ml papain. They were subsequently mixed with Laemmli solubilization buffer, heated in a boiling water bath for 5 min, and analyzed by SDS PAGE on a 14% Laemmli gel. The gels were fixed, stained and autoradiographed as given under Materials and Methods. The source of individual sialoglycoproteins is indicated at the top of the gel. Papain is unstable at 37°C; this is probably the reason for more efficient digestion at 23°C. The line on the left side of the figure marks the position of soybean trypsin inhibitor (M_r, 21,5000; the one on the right side, that of chymotrypsinogen (M_r, 25,850).

These maps suggest extensive similarity between the components of each doublet, and —together with the evidence already mentioned—are consistent with the assumption that the faster moving member of each doublet is derived from the slower one by hydrolysis, possibly by protease digestion. We were able to obtain some evidence in support of this assumption by limited papain digestion of gp-3.1L electrophoretically eluted from Laemmli gels; it generated a band comigrating with gp-3.2L and a 25,000-dalton fragment. Digestion of gp-3.2L for the same length of time left some gp-3.2 undigested, but it also generated the 25,000-dalton fragment as well as an additional 22,000-dalton fragment (Fig. 12). Aggregates can also be digested by this procedure: a gp-1L digest gave rise to gp-3.1L, gp-3.2L, and the 25,000-dalton fragment (not shown).

DISCUSSION

Extraction of murine erythrocyte ghosts by the LIS-phenol procedure results in the preferential isolation of sialoglycoproteins with O-acetylated sialyl residues. Comparative examination of the ghosts and the sialoglycoprotein fraction in the Laemmli system reveals that in the sialoglycoprotein fraction the trailing end of gp-3L is only partially recovered, and some of the minor components hidden under this trail are not detectable (Figs. 1 and 2).

SDS PAGE in the Laemmli system accurately fractionates the sialoglycoproteins into their monomeric units (gp-2.1L, gp-2.2L, gp-3.1L, and gp-3.2L). During solubilization they associate as a function of concentration to form a series of aggregates (*a*, *b*, *c*, and gp-1L). By contrast the Steck-Yu system cannot resolve gp-3.1L and gp-3.2L in the region of gp-3S; gp-2S is a complex aggregate of gp-2.1L, gp-2.2L, and gp-3.1L;

and gp-1S contains gp-2.1L, and gp-3.1L. Two of the monomers, gp-2.1L and gp-3.1L, appear to be authentic, because they can be precipitated from ghosts by a polyvalent antisialoglycoprotein serum; gp-2.2L and gp-3.2L cannot be immunoprecipitated from ghosts, probably because they are not present (A. H. Sarris and G. E. Palade, unpublished observations).

The chymotryptic peptide maps we have obtained suggest extensive similarity between gp-2.1L and gp-2.2L and between gp-3.1L and gp-3.2L. Furthermore, partial papain digestion of gp-3.1L generated gp-3.2 and a 25,700-dalton peptide, which was also generated by partial papain digestion of gp-3.2L. These results strongly suggest that gp-3.2L and gp-2.2L are generated from gp-3.1L and gp-2.1L, respectively, by proteolysis during the preparation of the sialoglycoproteins. Several neutral (3, 15, 26, 27, 41, 42) and acid proteases (14, 17, 19, 28, 31) have been detected in preparations of erythrocyte ghosts, arising probably from contaminating leukocytes (15). Some of the neutral proteases have essential sulfhydryl groups (27) and perhaps are partially inactivated by STT like other enzymes with essential sulfhydryls (21, 29, 30). However, the apparent hydrolysis of gp-2.1L and gp-3.1L could also be due to removal of oligosaccharides, phosphate, noncovalently associated phospholipid (2), or covalently attached fatty acid (33). Irrespective of its chemical nature, this modification renders the sialglycoproteins unable to aggregate during solubilization, and it deserves further investigation.

The chemical nature of the diffuse component, which is extracted from the gels by alkaline isopropanol is uncertain at present. It could be an unusual glycoprotein or a macroglycolipid similar to the one described by Dejter-Juszynski et al. (7) in chloroform-methanol extracts of human erythrocyte ghosts (12); this component carries A, B, and H determinants and is probably also present in the sialoglycoprotein fractions of human ghosts prepared by the LIS extraction-phenol partition method (40).

Our work is a novel approach to the initial characterization of the sialoglycoproteins of any erythrocyte species because the procedures used do not require large amounts of material. Furthermore, they illustrate the usefulness of peptide mapping by SDS PAGE after partial proteolysis. The results also suggest caution when the LIS extraction-phenol partition method is used for the isolation of the sialoglycoproteins of erythrocyte ghosts, because proteolysis may occur.

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