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Identification of Differences between the Surface Proteins and Glycoproteins of Normal Mouse (Balb/c) and Human Erythrocytes

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Summary. The topography of the external surface of the Balb/c mouse erythrocyte has been investigated and compared to the human erythrocyte by using a series of protein radiolabeling probes. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the pattern of Coomassie Blue stained proteins was very similar for mouse and human erythrocyte ghosts, as was the distribution of radioactivity in protein bands after lactoperoxidase catalyzed radioiodination. The mouse erythrocyte glycoproteins identified by periodic-acid-Schiff and 'Stains-All' reagents, sialic acid analysis of gel slices, binding of ^{125}I-wheat germ agglutinin and ^{125}I-concanavalin A to the gels, and glycoprotein radiolabeling techniques, differed markedly from the sets of proteins labeled by radioiodination, and also differed from the human erythrocyte glycoproteins. Instead of the PAS I to PAS IV series of sialoglycoproteins characteristic of human erythrocytes, the mouse erythrocyte possesses a broad band of sialoglycoproteins with several peaks ranging in mol wt from 65,000 to 32,000. The same group of sialoglycoproteins were labeled by the periodate/$B^3H_4^-$ technique specific for terminal sialic acid, and the galactose oxidase/$B^3H_4^-$ method (plus neuraminidase) specific for galactosyl/N-acetylgalactosaminyl residues penultimate to sialic acid. These results emphasize the necessity to employ a variety of protein radiolabeling probes based on different labeling specificities, to study the membrane topography of cells which are poorly understood compared to the human erythrocyte membrane.

A variety of techniques have been used to specifically label and identify the surface proteins and glycoproteins of the human erythrocyte [reviewed in 11, 43, 82, 89]. Some of these techniques have been extended to comparative studies of the surface proteins of bovine and equine erythrocytes [13], and erythrocytes of rabbit [22] and rat [23, 57]. The proteins and glycoproteins of the purified erythrocyte membranes from various species have also been compared [18, 33, 36, 46, 47, 49, 52, 91]. However, the proteins and glycoproteins of the mouse erythrocyte membrane, and their topographical distribution in the membrane have only been described briefly [14, 16, 32].

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The mouse is a particularly convenient animal for laboratory studies on the biochemistry and immunology of intraerythrocytic infections involving various species of *Plasmodium* and *Babesia*. Knowledge of the surface molecules on the *normal* mouse erythrocyte is important in these studies for three reasons: (i) the invasive form of these parasites, the merozoite, attaches to the erythrocyte surface in a process which involves specific recognition, prior to entry and subsequent growth and multiplication within the host cell [55]. The surface proteins and glycoproteins on the mouse erythrocyte must be identified as part of our goal to identify and isolate the receptor molecules on the host cell and merozoite surface, (ii) there is evidence that infection of red blood cells with *Plasmodium* or *Babesia* species results in parasite-dependent changes in surface proteins and antigens of infected cells [12, 17, 86, 90]. Isolation of such antigens, if they can be shown to be specific for the surface of the infected cell, may lead to a vaccine which would enable the host's immune system to recognize and effect removal of infected cells. In order to identify parasite-dependent protein antigens on the surface of infected cells, knowledge of the normal mouse erythrocyte surface molecules is essential, and (iii) uninfected red blood cells from infected blood are also reported to express altered surface antigens [75]. Identification of changes on the surface of these cells, by comparison with the erythrocytes from normal animals, may contribute to our understanding of the excessive anemia which develops during these infections, and which may involve loss and destruction of uninfected as well as infected red blood cells [75].

In this study we compare the proteins of human and mouse erythrocytes labeled by lactoperoxidase-catalyzed radioiodination together with the surface glycoproteins labeled by the following two techniques: (i) periodate oxidation followed by tritiated borohydride reduction of terminal sialic acid residues on surface sialoglycoproteins [4, 50], and (ii) oxidation of terminal (or penultimate) galactose/N-acetylgalactosamine residues on surface glycoproteins by galactose oxidase (+neuraminidase), followed by tritiated borohydride reduction [27]. These glycoprotein labeling techniques have been developed and optimized in model studies using the human erythrocyte [25, 26, 29, 61, 83], and we compared the results for the human cell with those for the mouse erythrocyte. The glycoprotein and protein composition of mouse and human erythrocyte membranes have also been compared by gel electrophoresis followed by protein/glycoprotein staining or binding of radioiodinated lectins.

Materials and Methods

Chemicals and Materials

All human erythrocytes were obtained from an adult donor (blood group O). Mouse erythrocytes were obtained from 6–8 week old inbred Balb/c mice derived from a specific pathogen-free facility but maintained conventionally [56]. Human or mouse blood was obtained immediately before experiments by bleeding into Alsever's solution. Erythrocytes were washed at least three times at 4° in human or mouse tonicity phosphate buffered saline (respectively: 20 mM sodium phosphate, 0.120 M NaCl, pH 7.3, designated NaCl/PO_4:A[1]; 20 mM sodium phosphate, 0.149 M NaCl, pH 7.3, designated NaCl/PO_4:B; as appropriate, and depleted of leucocytes by passage through a sulphoethyl cellulose column as previously described [39]. Leucocyte contamination was reduced to $<0.01\%$ of the total cells and platelets were absent.

Lactoperoxidase was supplied by Calbiochem, San Diego, Calif. (B grade, 20.3 I.U./mg at 30°, 5 mg/ml) and glucose oxidase (Type V from *Aspergillus niger*, 200 U/mg, 6.35 mg/ml) was from Sigma Chemical Co., St. Louis, Mo. Both of these enzymes were assayed as described previously [40]. Galactose oxidase (416 U/mg protein) was obtained from Kabi, Stockholm, Sweden, and *Vibrio cholerae* neuraminidase (500 BW units/ml) was from Behringwerke, Marburg-Lahn, Germany. The proteolytic activity in these enzymes was assayed using azoalbumin (Sigma Chemical Co.) as substrate[2] at enzyme concentrations tenfold higher than used for cell labeling reactions. After 20 min at 25° no proteolytic activity was detected in any of these enzymes (minimum activities of α-chymotrypsin and trypsin detectable were 1 mU and 0.57 U, respectively). Protease (*Streptomyces griseus* Protease, Type VI) was obtained from Sigma Chemical Co.

Carrier free ^{125}I as $Na^{125}I$ (100 mCi/ml) and tritiated sodium borohydride (NaB^3H_4) (5.0 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, and handled as described elsewhere [28]. Greater than 90% of the tritium was released from the NaB^3H_4 by 10 min treatment with 0.01 N H_2SO_4. ^{125}I was counted in a Packard 5375 Auto-Gamma scintillation spectrometer. ^3H radioactivity was solubilized with Soluene (Packard Instrument Co., Ill.) prior to addition of scintillation cocktail, then counted in a Packard Tri-Carb scintillation spectrometer.

Sodium dodecyl sulfate (SDS), Special Purity grade, was from British Drug Houses, Australia Acrylamide, N,N′-methylenebisacrylamide, Coomassie Brilliant Blue R-250, thiobarbituric acid and "Stains All" (SA:1-ethyl-2-[3-(ethylnaphtho [1,2d] thiazolin-2-ylidene)-2-methyl propenyl]-naphtho [1,2d]-thiazolium bromide), were obtained from Eastman Kodak Co., Rochester, N.Y. 2,5-Diphenyloxazole (PPO) and *p*-bis [2-(5-phenyloxazolyl)] benzene (POPOP) were obtained from New England Nuclear, Boston, Mass. Concanavalin A (Con A), wheat germ agglutinin (WGA) and soybean agglutinin (SBA) were from Pharmacia, Uppsala, Sweden. Crystalline N-acetylneuraminic acid, methyl-α-D-mannopyranoside, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were supplied by Sigma Chemical Co. All reagents, inorganic chemicals, and solvents were analytical reagent grade. Glass-distilled water was used for all aqueous solutions.

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[1] *Abbreviations used:* SDS, dodecyl sulfate sodium salt; NaCl/PO_4:A, human tonicity phosphate buffered saline; NaCl/PO_4:B, mouse tonicity phosphate buffered saline; SA, "Stains All", 1-ethyl-2-[3-(1-ethylnaphtho [1, 2d] thiazolin-2-ylidene)-2-methylpropenyl]-naphtho [1, 2d]-thiazolium bromide; PAS I, PAS II, PAS III and PAS IV, the periodic acid – Schiff stained glycoproteins of human red cell membranes; Band 3, Band 3 of the human red cell membrane proteins [45]; Con A, concanavalin A; WGA, wheat germ agglutinin; SBA, soybean agglutinin.

[2] R.J. Howard, P.M. Smith, and G.F. Mitchell (*Aust. J. Exp. Biol. Med. Sci., in press*).

Surface Labeling of Erythrocyte Protein and Glycoproteins

Radioiodination

This was performed using the optimal conditions for specific labeling of mouse erythrocyte surface proteins [3]. Erythrocytes (10^9 cells) were incubated (10 min at room temperature) in 1 ml of $NaCl/PO_4$:A or B plus 500 μCi $Na^{125}I$, 20 mM glucose, 5.9 mU glucose oxidase, and 3.2 mU lactoperoxidase. Radioiodination was terminated by addition of 10 vol cold buffer, and the cells washed three times at 4° before membrane preparation or solubilization of intact cells in electrophoresis sample buffer. The amount of radioactivity incorporated into hemoglobin during this labeling reaction was measured by using 14% acrylamide gels to separate hemoglobin from other proteins and counting gel slices containing hemoglobin [4].

Periodate/NaB^3H_4 labeling

Washed erythrocytes (10^9 cells) were pelleted and incubated in 90 μl $NaCl/PO_4$:A or B plus 10 μl of 20 mM KIO_4 which had been dissolved immediately prior to use in the same buffer. After incubation for 20 min at room temperature in darkness, the samples were washed twice by centrifugation at 4° with 10 ml of buffer and reduced with 50 μCi of NaB^5H_4 for 30 min at room temperature in darkness, in a total volume of 100 μl of buffer. These reaction conditions are very similar to those used previously to label the human erythrocyte [26, 29, 50, 61]. Cells were washed 3 times as described above and a subsample solubilized directly in SDS sample buffer for electrophoresis. Membranes were prepared from the remaining cells by hypotonic lysis, washed, and then solubilized in the same way for electrophoresis.

Galactose oxidase/NaB^3H_4 labeling

The conditions used for this labeling method were very similar to those employed previously [25, 27, 83]. Washed erythrocytes (10^9 cells) were incubated in 50 μl Dulbecco's $NaCl/PO_4$ adjusted to human or mouse tonicity (respectively: 0.120 M NaCl, 3 mM KCl, 8 mM sodium phosphate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3, designated Dulbecco's $NaCl/PO_4$:A; 0.151 M NaCl, 3 mM KCl, 8 mM sodium phosphate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3, designated Dulbecco's $NaCl/PO_4$:B), with 0.6 U of galactose oxidase for 30 min at room temperature in darkness. When neuraminidase treatment was used, 1.9 U neuraminidase were added with the galactose oxidase. The samples were washed and reduced with NaB^3H_4, then washed and solubilized as described above for the periodate labeling method.

Enzyme pre- or post-treatment

Washed radiolabeled cells were incubated for 30 min at 37° in Dulbecco's $NaCl/PO_4$:A or B in a total volume of 1 ml per 10^9 cells with 0.25 mg *Streptomyces griseus* protease. The cells were washed twice at 4° in $NaCl/PO_4$:A or B plus 0.5% wt/vol bovine albumin and 5 mM EDTA, then twice in $NaCl/PO_4$:A or B and solubilized in electrophoresis sample buffer.

Washed erythrocytes (10^9 cells) were incubated in 200 μl Dulbecco's $NaCl/PO_4$:A or B with 2.6 U or neuraminidase for 30 min at 37°. After two washes in $NaCl/PO_4$:A or B at 4°, the cells were pelleted for labeling by the periodate/NaB^3H_4 method.

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[3] *See* footnote 2, p. 173.

[4] *See* footnote 2, p. 173.

SDS-Polyacrylamide Gel Electrophoresis and Analysis of Gels

Erythrocytes or erythrocyte membranes were pelleted, and SDS sample buffer [48] which includes 3% wt/vol SDS and 5% vol/vol 2-mercaptoethanol, added in a ratio of 100 μl per 10^8 cells. Samples were immediately vortexed and heated for 5 min at 100°, then stored at −20° until electrophoresis. Prior to electrophoresis, aliquots were removed and an equal volume of fresh SDS sample buffer added and the sample reheated 5 min at 100°. Cylindrical gels were loaded with up to 5×10^7 solubilized cells and slab gels with up to 2×10^7 solubilized cells. Polyacrylamide gel electrophoresis in the presence of SDS and estimation of protein molecular weights was performed according to Laemmli and Favre [48] using cylindrical gels (11 cm × 4 mm ID), or slab gels in an apparatus from Savant Instruments, Inc. Slab gels were 1.5 mm thick and 135 mm wide, with a 25 mm deep stacking gel and 110 mm deep separation gel. The ratio of acrylamide to N,N′-methylenebisacrylamide was 20/1 unless indicated otherwise. Stacking gels were 3% acrylamide and the separation gels were 8%, 10%, or 14% acrylamide.

Gels to be stained for glycoproteins or protein, or to be analyzed for the distribution of ^{125}I-radioactivity, were fixed in 7% acetic acid. Gels which were examined for the distribution of ^{3}H-radioactivity were placed directly in dimethyl sulfoxide and dehydrated with a change of dimethyl sulfoxide prior to radioactivity analysis. Preliminary experiments established that fixation of tritiated proteins in gels with aqueous methanol or glutaraldehyde, prior to dehydration in dimethyl sulfoxide, had no effect on the results.

Protein staining with Coomassie Brilliant Blue R-250 and destaining was performed as described by Fairbanks, Steck and Wallach [21]. Glycoproteins were stained by the periodic-acid-Schiff (PAS) procedure of Glossman and Neville [33] or with "Stains All" (SA) reagent by a rapid method of King and Morrison [45] employing 0.01% SA in 2-propanol at 37°. The SA method stains sialoglycoproteins blue, with nonglycosylated proteins poorly stained (red) on a red background, and allows gel scanning at 560 nm to detect sialoglycoproteins alone. Coomassie Blue and PAS stained gels were scanned at the same wavelength using a Canalco Model J Microdensitometer. Cylindrical gels were analyzed for the distribution of ^{125}I of ^{3}H radioactivity by slicing frozen gels into 1 or 2-mm segments and counting or, alternatively, by longitudinal gel slicing and autoradiography of the middle 1-mm thick slice. Slab gels were analyzed by autoradiography [84] or fluorography [5]. Analysis of ^{125}I radioactivity in gel slices was performed by direct counting in a γ counter. ^{3}H radioactivity in gel slices was counted in a liquid scintillation counter after digestion of slices in 200 μl Soluene (Packard) (24 hr × 22°) and addition of 3 ml scintillation cocktail.

Lectins were iodinated with ^{125}I by the method of Hunter and Greenwood [41]. Specific activities ranged from 4×10^5 to 6×10^5 cpm per μg protein.

The binding of lectins to separated proteins was determined by binding ^{125}I-lectins to membrane proteins after electrophoresis on 8% acrylamide gels, exactly as described by Gurd and Evans [35].

Other Methods

Erythrocyte membranes were prepared by hypotonic lysis in 20 mosM sodium phosphate pH 7.4, and washed 3 to 5 times until free of hemoglobin [19]. Acid-insoluble material in cells or membranes was precipitated and washed with 7% wt/vol trichloroacetic acid. Protein was measured according to Hartree [38] with bovine serum albumin as standard. Sialic acid was quantitated by a fluorimetric method [37] using crystalline N-acetylneuraminic acid as standard. When gel slices were assayed for sialic acid, a gel slice (about 0.1 ml in volume) was soaked in 1 ml 0.1 M H_2SO_4 for 2 hr, heated for 1 hr at 80° and then shaken overnight at 22°. Portions of the supernatant were assayed for sialic acid as described above.

Results

Mouse erythrocyte membrane proteins identified as surface proteins have been denoted *a* to *j* in order of increasing apparent molecular weight (M_r) on SDS-gel electrophoresis. (The properties of these proteins and their molecular weights are summarized in Table 5.)

Comparison of Proteins and Glycoproteins of Human and Mouse Erythrocyte Membranes

Mouse and human erythrocyte membranes exhibited similar complexity in patterns of Coomassie Blue stained proteins separated by gel electrophoresis, with approximately 30 bands visible (Fig. 1). Several bands from the mouse erythrocyte membrane were tentatively identified (as shown in Fig. 1) on the basis of extensive homology in these patterns. Figure 2 shows the glycoproteins of these membranes stained with the PAS and SA reagents. The mouse membrane lacked the intensely stained PAS I band characteristic of the human membrane, and contained up to four poorly stained bands; four visible after PAS staining and two visible after SA staining. The M_r of these mouse membrane glycoproteins (*b, c, d* and *f*) were 92,000, 89,000, 65,000 and 43,000, respectively. There were also carbohydrate staining bands ahead of the dye front which did not stain with Coomassie Blue. When gels containing electrophoresed erythrocyte membrane proteins were sliced and assayed for sialic acid, reproducible patterns for the human and mouse cell membranes were obtained (Fig. 3). Comparison of Figs. 2 and 3 shows that the sialic acid-containing bands for the human membrane, except for PAS III and a 20,000-dalton band, were also PAS stained. Only one

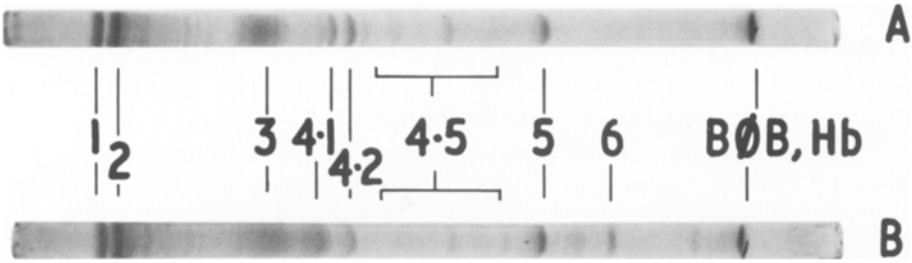

Fig. 1. SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins stained for protein with Coomassie Blue. (*A*): Mouse erythrocyte ghosts. (*B*): Human erythrocyte ghosts. Bands numbered according to the nomenclature of Fairbanks, Steck and Wallach [45] (50 μg membrane protein was electrophoresed)

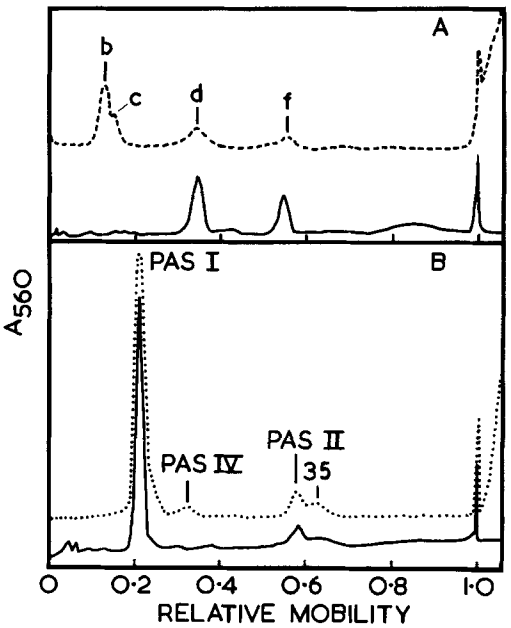

Fig. 2. Densitometric tracing of SDS-polyacrylamide gel electrophoretic patterns for mouse (*A*) and human (*B*) erythrocyte ghosts stained with periodic acid-Schiff (PAS: ---) and "Stains All" (SA:——) stains for carbohydrate. (100 μg membrane protein was electrophoresed)

band (Band *f*) among the mouse membrane proteins which contained sialic acid was also stained by the PAS technique. Bands *e*, *g*, *h*, *i* and *j* contained sialic acid but were PAS-negative. Sialic acid analysis of washed erythrocyte membranes indicated a significantly lower content in the mouse compared to the human membranes (27 μg N-acetylneuraminic acid/mg protein and 37 μg N-acetylneuraminic acid/mg protein, respectively). The distribution of total sialic acid between proteins separated by electrophoresis and the non-Coomassie Blue staining material at and just beyond the dye marker position (Fig. 3) was also different for mouse and human erythrocyte membranes. Approximately 25 to 30% of the total sialic acid of the human erythrocyte membrane was present in material with mobility >1.0, whereas the corresponding value for the mouse erythrocyte was 60 to 65%. The nature of this material is in doubt, however, as other sugars, tracking dye, and polyunsaturated fatty acids which migrate in this region interfere with sialic acid assays [63, 71].

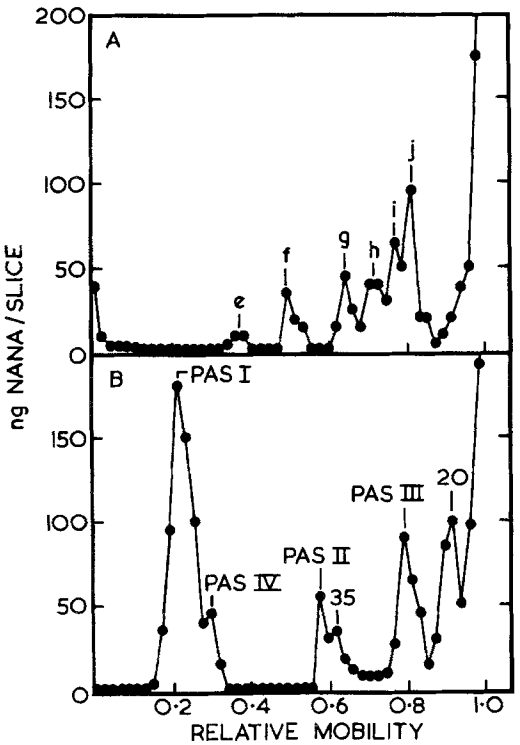

Fig. 3. Sialic acid determination in gel slices after separation of mouse (*A*) and human (*B*) erythrocyte membrane proteins by electrophoresis on cylindrical 8% acrylamide gels in the presence of SDS. The frozen gels were sliced into approximately 2-mm thick slices and the total sialic acid was determined in each slice after acid hydrolysis. (50 μg membrane protein was electrophoresed)

Lactoperoxidase Catalyzed Radioiodination of Surface Proteins

The distribution of radioactivity in proteins of mouse and human erythrocytes labeled by the lactoperoxidase method under optimal conditions for mouse erythrocytes[5] is shown in Fig. 4. On gels with a 40:1 ratio of acrylamide to bis-acrylamide, three bands were labeled on the human erythrocyte; however, gels with a 20:1 ratio resolved the major band into two labeled species identified as Band 3 and PAS I. In contrast, the major band labeled on mouse erythrocytes, Band *b* with M_r of 92,000, was not split on gels with a 20:1 ratio of acrylamide to bis-acrylamide. The minor labeled bands on the mouse erythrocyte, *a*, *d*, *e* and *g*, with M_r of 108,000, 65,000, 57,000 and 36,000, were also different from

[5] *See* footnote 2, p. 173.

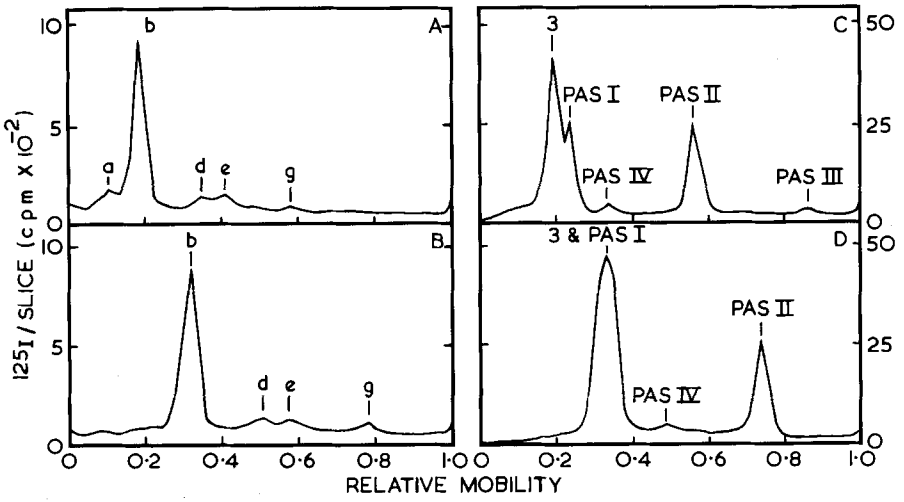

Fig. 4. SDS-polyacrylamide gel electrophoresis of ^{125}I-labeled cells. (*A–B*): Mouse erythrocytes; (*C–D*): human erythrocytes. Gels were 8% acrylamide with a ratio of acrylamide to bis-acrylamide of 20:1 in *A* and *C* and 40:1 in *B* and *D*. 1-mm thick slices of gel were analyzed for radioactivity

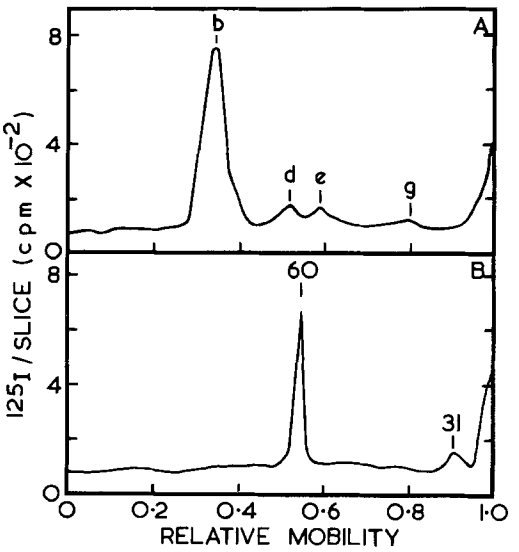

Fig. 5. Treatment of ^{125}I-labeled mouse erythrocytes with protease: SDS-polyacrylamide gel electrophoresis. (*A*): Untreated cells. (*B*): Cells treated with *S. griseus* protease. 1-mm thick slices of gel were analyzed for radioactivity

the minor labeled bands, PAS IV, PAS II and PAS III, on the human erythrocyte. Under the conditions of lactoperoxidase catalyzed radioiodination used, hemoglobin constituted less than 5% of the total iodinated protein indicating specific labeling of surface proteins. This was

confirmed by examination of the susceptibility of the iodinated proteins on intact cells to digestion by *S. griseus* protease. Less than 0.1% of the cells lyzed during this treatment. The results in Fig. 5, show that all of the radioactivity in peaks *b*, *e* and *g*, was removed, with two new bands (with M_r of 60,000 and 31,000) appearing after protease treatment. The amount of radioactivity in the 60,000 mol wt band indicates it was derived from Band *b*. Due to the similar relative mobilities of the 60,000 mol wt band, and Band *d* on untreated cells, it is not possible to comment on the susceptibility of band *d* to the protease.

Erythrocyte Glycoproteins Labeled by Periodate-NaB^3H_4

The incorporation of tritium into mouse and human erythrocyte membranes after periodate and NaB^3H_4 treatment, which labels sialic acid, is compared in Table 1. In the absence of periodate treatment tritium incorporation was decreased by approximately 83% and 95% for the mouse and human erythrocytes, respectively. Prereduction with $NaBH_4$ had no significant effect on tritium uptake or the profile of labeled proteins on gel electrophoresis (results not shown). Neuraminidase pretreatment of mouse erythrocytes reduced the periodate-dependent uptake of tritium by 83%, indicating at least this extent of specific labeling of sialic acid in the periodate-dependent radiolabeling. Figure 6 shows the distribution of tritium in labeled proteins separated gel electrophoresis. The sialoglycoproteins of the human erythrocyte, PAS I, PAS

Table 1. Incorporation of tritium from NaB^3H_4 into erythrocyte membranes after periodate treatment[a]

Pretreatment	Treatment	Membranes	
		Mouse	Human
		(cpm/10^9 cells)	
None	None/NaB^3H_4	43,800	120,000
None	Periodate/NaB^3H_4	260,000	2,300,000
$NaBH_4$ 10 min	Periodate/NaB^3H_4	250,000	2,300,000
$NaBH_4$ 10 min	Periodate/NaB^3H_4	200,000	2,500,000
Neuraminidase	Periodate/NaB^3H_4	80,000	ND

[a] Erythrocytes were pretreated with $NaBH_4$ (4 mg/ml) in NaCl/PO_4: A or B, as appropriate, at a cell density of 2×10^9/ml, in darkness at 22°, and then washed twice before periodate treatment. Neuraminidase pretreatment and other methods are described in *Materials and Methods*.

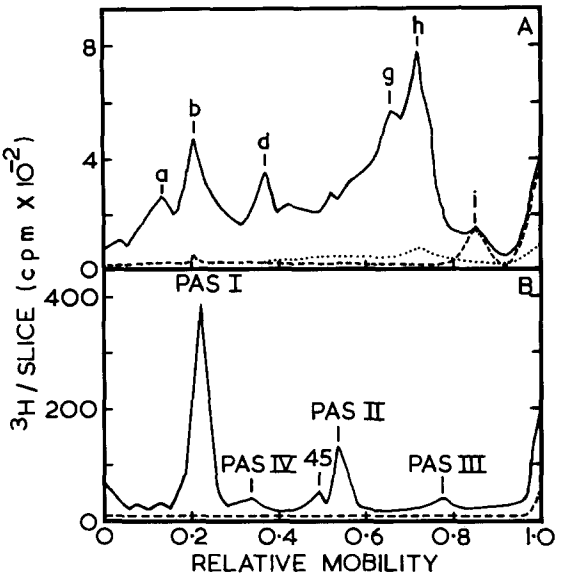

Fig. 6. SDS-polyacrylamide gels of periodate/NaB^3H_4-labeled erythrocytes. (*A*): Mouse erythrocytes; (*B*): human erythrocytes. The distribution of radioactivity in 1-mm thick gel slices is shown for cells treated with periodate (——) or incubated in the absence of periodate (– – –) (2×10^7 solubilized cells loaded per gel). The effect of treatment of labeled mouse erythrocytes with *S. griseus* protease is also shown (...) in *A*. The distribution of radioactivity after treatment of mouse erythrocytes with neuraminidase followed by periodate/NaB^3H_4 was very similar to the minus periodate control

II, PAS III, and PAS IV, were labeled by periodate and NaB^3H_4 treatment, together with a band of material which migrated beyond the dye marker. In contrast, the pattern of radioactivity of mouse erythrocyte sialoglycoproteins consisted of several poorly labeled broad overlapping bands (*see also* Fig. 8). Distinct peaks of radioactivity were observed for the mouse erythrocyte corresponding to Bands *a, b, d, g, h* and *i* in Fig. 6 (with M_r of 108,000, 92,000, 65,000, 36,000, 32,000 and 28,000). The most heavily labeled bands of the mouse cell membrane migrated beyond the dye marker, with approximately 5 times as much radioactivity as Band *h*, the most heavily radiolabeled protein. It must be emphasized that parallel gels of the periodate/NaB^3H_4 labeled mouse membrane which were stained for protein with Coomassie Blue exhibited excellent resolution of stained proteins similar to Fig. 1. Treatment of periodate/NaB^3H_4 labeled mouse erythrocytes with protease, under conditions which did not cause cell lysis, released 67% of the membrane-associated radioactivity, corresponding to release of 79% of the label incorporated in periodate-dependent reactions (Table 2). Protease treatment also

Table 2. Effect of protease on tritium incorporated into mouse erythrocyte membranes labeled by Periodate/NaB^3H_4

Treatment	Post treatment	Membranes (cpm/10^9 cells)
Periodate/NaB^3H_4	None	275,000
Periodate/NaB^3H_4	Protease	92,300

Table 3. Incorporation of tritium from NaB^3H_4 into erythrocyte membranes after galactose oxidase treatment

Treatment	Membranes	
	Mouse	Human
	(cpm/10^9 cells)	
None/NaB^3H_4	47,800	90,300
Galactose oxidase + NaB^3H_4 [a]	46,100	102,000
Galactose oxidase + neuraminidase + NaB^3H_4 [b]	45,000	87,800
Galactose oxidase/NaB^3H_4	88,400	563,000
Galactose oxidase + neuraminidase/NaB^3H_4	169,000	899,000
Neuraminidase/NaB^3H_4	49,000	98,700

[a] Washed erythrocytes (10^9 cells) were incubated in 100 µl Dulbecco's NaCl/PO_4:A or B with 0.6 U galactose oxidase and 50 µCi NaB^3H_4 for 30 min in darkness at 22°.

[b] 1.9 U neuraminidase added.

reduced the radioactivity in membrane proteins separated by acrylamide gel electrophoresis to background levels (Fig. 6). The proteins labeled during periodate-dependent uptake of tritium are therefore surface membrane sialoglycoproteins.

Erythrocyte Glycoproteins Labeled by Galactose Oxidase-NaB^3H_4

Treatment of human or mouse erythrocytes simultaneously with NaB^3H_4 and galactose oxidase plus neuraminidase, for 30 min at 22°, resulted in background levels of tritium incorporation equal to the minus-enzyme control (Table 3). However, when the enzymic oxidation step and two rapid washes at 4 °C preceded NaB^3H_4 reduction, tritium label was taken up in excess of background levels. Galactose oxidase treatment alone resulted in approximately twice as much label incorporated into mouse erythrocytes as the minus-enzyme control. Addition of neuraminidase enhanced tritium uptake an additional twofold. Neuraminidase treatment alone had no effect on tritium incorporation.

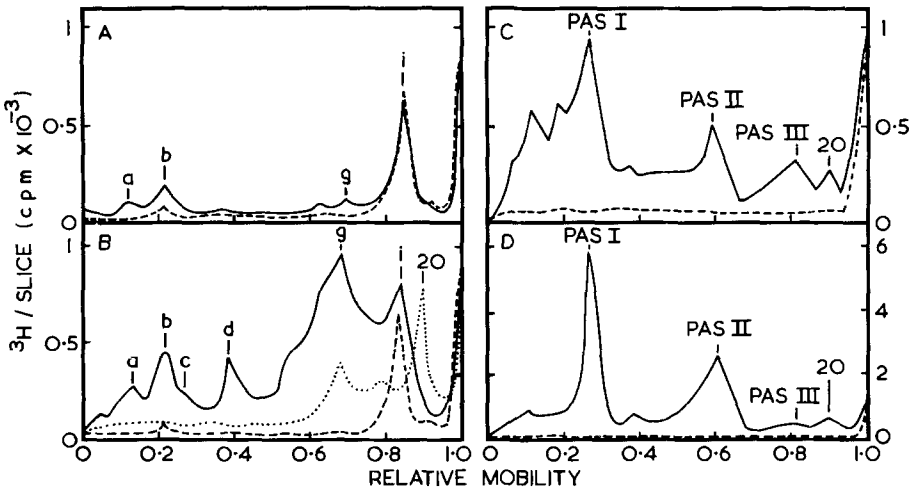

Fig. 7. SDS-polyacrylamide gels of galactose oxidase/NaB^3H_4 treated erythrocytes. (*A*): Mouse erythrocytes labeled with galactose oxidase treatment. (*B*): Mouse erythrocytes labeled after galactose oxidase and neuraminidase treatment. (*C*): Human erythrocytes labeled after galactose oxidase treatment. (*D*): Human erythrocytes labeled after galactose oxidase and neuraminidase treatment. In each case the distribution of radioactivity is shown for treated cells (——) and cells labeled after incubation in the absence of the enzymes (– – –) (5×10^7 solubilized cells loaded per gel). The effect of incubating mouse erythrocytes labeled by galactose oxidase and neuraminidase treatment with *S. griseus* protease is shown in *B* (...). Incubation of mouse or human erythrocytes with neuraminidase alone, followed by NaB^3H_4, gave distributions of radioactivity identical to the minus-enzyme control treatments

The human erythrocyte membrane took up significantly more label than the mouse erythrocyte membrane after labeling of terminal or penultimate galactose oxidase sensitive residues (Table 3). The distribution of radioactivity in mouse and human membrane proteins labeled by the galactose oxidase method is shown in Fig. 7. Galactose oxidase/NaB^3H_4 treatment labeled Bands *a, b* and *g* of the mouse erythrocyte poorly. Although Band *i* incorporated radioactivity, it was labeled in an enzyme-independent reaction (Fig. 7*A*). Addition of neuraminidase enhanced the labeling of Bands *a, b* and *g*, and also resulted in labeling of Band *d* and perhaps band *c* as a shoulder on Band *b* (Fig. 7*B*). The labeling of human erythrocyte glycoproteins by these two methods is shown for comparison in Fig. 7*C* and *D*. Protease treatment of intact mouse erythrocytes labeled by the galactose oxidase plus neuraminidase/NaB^3H_4 reaction resulted in removal of 42% of the acid-insoluble membrane radioactivity (Table 4). The gel electrophoresis profile of radiolabeled proteins after protease treatment indicated complete removal

Table 4. Effect of protease on tritium incorporated into mouse erythrocyte membranes labeled by galactose oxidase plus neuraminidase/NaB^3H_4

Treatment	Post treatment	Membranes (cpm/10^9 cells)
Galactose oxidase + neuraminidase/NaB^3H_4	None	173,000
Galactose oxidase + neuraminidase/NaB^3H_4	Protease	100,000

of Bands *a, b, c* and *d*, from the cell, and removal of approximately 60% of the label in Band *g* (Fig. 7*B*). A labeled band with M_r of 20,000 produced from protease treatment probably represents a partial degradation product of higher molecular weight labeled species. A large proportion of the radioactivity incorporated into mouse erythrocyte membranes after galactose oxidase treatment, with or without neuraminidase, was in non-Coomassie Blue staining material at or beyond the dye marker. The label in this region was not removed by protease treatment of the cells. The distributions of radioactivity in mouse erythrocyte proteins labeled by their sialic acid residues (periodate/NaB^3H_4) or galactosyl/N-acetylgalactosaminyl residues penultimate to sialic acid (galactose oxidase and neuraminidase/NaB^3H_4) were compared by slab gel electrophoresis and fluorography (Fig. 8). Neuraminidase treatment of glycoproteins labeled by the latter technique resulted in a small decrease in relative mobility of Bands *a, b* and *d* which was difficult to detect by transverse slicing of cylindrical gels. The mobility of Band *i*, which was labeled by NaB^3H_4 treatment alone, was not affected by neuraminidase. Although separate bands with the mobilities of *g* and *h* were labeled by lactoperoxidase catalyzed radioiodination, it appears that the glycoprotein bands migrating at these positions are interconvertible as a result of neuraminidase treatment. The apparent molecular weights of the labeled glycoprotein bands differed on polyacrylamide gels of different acrylamide concentrations and different ratios of acrylamide to bis-acrylamide. Their molecular weights are therefore provisional.

Binding of Iodinated Lectins to Erythrocyte Glycoproteins

^{125}I-WGA bound to at least five components of the mouse erythrocyte membrane, identified by their apparent molecular weights as *a, b, d, g* and *h* (Fig. 9). This figure also shows that ^{125}I-Con A bound to bands

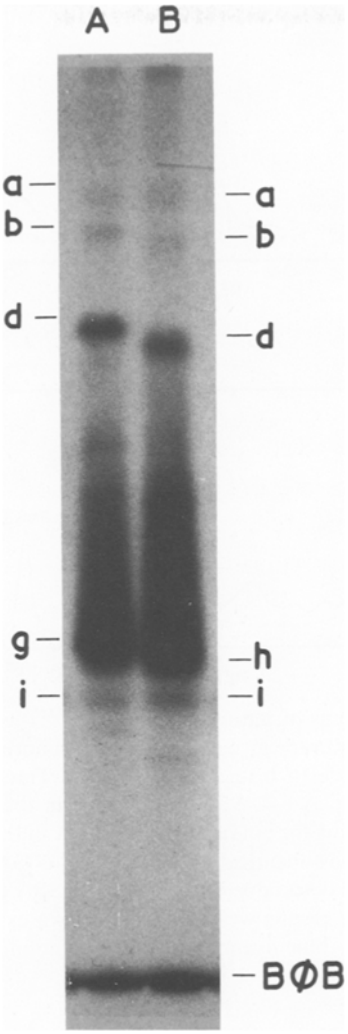

Fig. 8. Fluorography patterns of mouse erythrocyte glycoproteins labeled by the galactose oxidase plus neuraminidase/NaB^3H_4 and periodate/NaB^3H_4 methods, and separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide salb gels (40:1 acrylamide to bis-acrylamide). 1×10^7 solubilized cells were loaded in each gel slot. (*A*): Galactose oxidase plus neuraminidase/NaB^3H_4 labeling. (*B*): Periodate/NaB^3H_4 labeling. BΦB: Bromophenol blue dye marker

a, *b* and *h*, but not to other regions of the gel. When incubations of ^{125}I-lectins were conducted in the presence of 2.5% wt/vol of the appropriate hapten, only background levels of bound lectin were observed. The results of parallel binding studies with the human erythrocyte glycoproteins are shown in Fig. 9*B* for comparison. In this case, ^{125}I-WGA

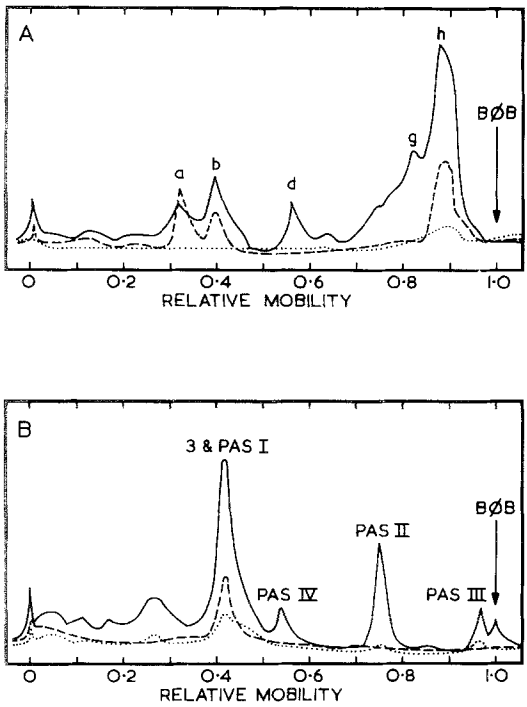

Fig. 9. Densitometric tracings of autoradiographs of ^{125}I-lectin binding to mouse (*A*) and human (*B*) erythrocyte membrane glycoproteins electrophoresed on 8% acrylamide cylindrical gels (ratio of acrylamide to bis-acrylamide 40:1). Tracings are shown for binding of ^{125}I-WGA (——), ^{125}-I-Con A (– – –) and ^{125}I-WGA in the presence of 2.5% N-acetyl-D-glucosamine (. . .). The tracing for binding of ^{125}I-Con A in the presence of 2.5% methyl-α-D-mannopyranoside was almost identical to that for ^{125}I-WGA in the presence of N-acetyl-D-glucosamine. There was no detectable binding of ^{125} I-SBA to mouse or human erythrocyte glycoproteins. Very similar results were obtained in two experiments. *BΦB*: bromophenol blue dye marker, relative mobility 1.0

bound to the major glycoprotein bands of the human erythrocyte membrane, PAS I to IV, with some binding also to unidentified high molecular weight species with relative mobilities <0.3. A low level of ^{125}I-Con A binding to the PAS I plus Band 3 peak was also observed.

Discussion

In this paper we have analyzed the proteins and glycoproteins of the mouse erythrocyte membrane, and have identified which of these species are external, using protein and carbohydrate radiolabeling probes in combination with proteolytic treatment of intact labeled cells. Parallel

Table 5. Relative abundances of proteins and glycoproteins of human and mouse erythrocytes detected by cell surface labeling and protein staining techniques[a]

Erythrocytes	Band designation[b]	Mol wt ($\times 10^{-3}$)[c]	External proteins identified by radiolabeling				Membrane proteins		
			Tyrosine[d] labeling	Gal/GalNAc labeling		Sialic acid labeling	PAS stain	"Stais All" stain	Sialic acid determination
				Terminal	Penultimate				
Human	3	89	*S*						
	PAS I	83	*W*	*W*	*S*	*S*	*S*	*S*	*S*
	PAS IV	68	*W*			*W*	*W*		*W*
		45				*W*			
	PAS II	42	*S*	*W*	*S*	*S*	*W*	*W*	*W*
		35					*W*	*W*	*W*
	PAS III	26	*W*	*W*	*W*	*W*			*S*
		20		*W*	*W*				*S*
Mouse	*a*	108	*W*	*W*	*W*	*W*			
	b	92	*S*	*W*	*W*	*W*	*W*		
	c	84			*W*		*W*		
	d	65	*W*		*W*	*W*	*W*	*W*	
	e	57	*W*						*W*
	f	48	*W*				*W*	*W*	*W*
	g	36	*W*	*W*	*S*	*W*			*W*
	h	32	*W*			*W*			*W*
	i	28			*W*	*W*			*W*
	j	23							*S*

[a] The relative levels of radioactivity, protein stain or sialic acid in different bands for the human erythrocyte were denoted by *S* (strong) and *W* (weak) and the proteins of the mouse erythrocyte compared with these levels

[b] Designation for the human erythrocyte according to Fairbanks *et al.* [45]. Mouse erythrocyte proteins (excluding those stained by Coomassie Blue alone) were denoted *a–j* in order of increasing mol wt

[c] Incomplete resolution of erythrocyte membrane proteins by the acrylamide gel system used precludes the association of each of the protein species identified in this table with a single protein of each mol wt class. Molecular weights were determined on 8% acrylamide gels with a 20:1 acrylamide to bis-acrylamide ratio.

[d] Some of these results are described elsewhere (Footnote 2, p. 173).

studies were performed with the human erythrocyte in order to directly compare the mouse and human erythrocyte membranes and to relate our data to the extensive literature on the human erythrocyte membrane. The results are summarized in Table 5. It must be stressed that one-dimensional SDS-polyacrylamide gel electrophoresis gives incomplete res-

olution of individual species. The multiplicity of functions associated with Band 3 of the human erythrocyte membrane [reviewed in 77], and the recent results of separation of erythrocyte membrane proteins by two-dimensional methods [70] attest to this point. The association of protein bands identified using different techniques to single proteins must therefore be regarded as provisional.

Our results with the human erythrocyte generally agreed closely with previous studies on the Coomassie Blue [21, 45, 82, 89], PAS [4, 21, 45, 82, 85] and SA [45] stained proteins of the erythrocyte ghost, and the results of external protein labeling by lactoperoxidase catalyzed radioiodination [8, 29, 40, 58, 62, 65, 68, 79, 85, 88] periodate/NaB^3H_4 treatment [4, 26, 29, 50, 61] and treatment with galactose oxidase ($\pm$ neuraminidase)/NaB^3H_4 [25, 27, 83]. The M_r obtained for human erythrocyte proteins shown in Table 5 are very similar to those obtained by Fairbanks *et al.* [21]. One quantitative difference between our results for the human erythrocyte using the galactose oxidase/NaB^3H_4 technique and those of previous authors was the extent of Band 3 labeling. Much greater labeling of Band 3 compared to PAS I was observed in an earlier study [29], whereas Band 3 was poorly labeled by this method in our experiments. It may be significant that the experiments reported here utilized cells within 2 hr of bleeding, rather than blood bank supplies.

The patterns of Coomassie Blue stained proteins for mouse and human erythrocyte membranes showed many regions of homology. The results of labeling external membrane proteins by lactoperoxidase catalyzed radioiodination were also similar, with the major labeled proteins having almost identical molecular weights (89,000 for Band 3 and 92,000 for Band *b* of human and mouse erythrocytes, respectively) and with only a small number of additional labeled bands. The major radioiodinated bands were also affected similarly by protease treatment. Band 3 is converted into a 70,000 M_r fragment which remains in the erythrocyte membrane [3, 9, 15, 21, 66, 87], and treatment of radioiodinated mouse erythrocytes with *S. griseus* protease converts Band *b* to a radiolabeled fragment (M_r 60,000) which remains associated with the labeled cells and is presumably also embedded in the membrane. Band *b* and Band 3 also exhibit similarities in their carbohydrate components. Band *b* is weakly stained for carbohydrate (PAS stain) (Fig. 2) and is radiolabeled by the carbohydrate labeling techniques, while Band 3 is PAS-negative or very weakly positive [10, 21, 82] and is also radiolabeled, although poorly, by the same techniques [26]. Further evidence that Band *b* is a glycoprotein was provided by the binding of ^{125}I-WGA and ^{125}I-Con

A. Of note is the fact that it did not bind ^{125}I-SBA which has its major specificity for terminal N-acetylgalactosaminyl residues [51]. These residues were also shown to be absent by the very poor labeling of Band *b* using galactose oxidase/NaB^3H_4 in the absence of neuraminidase. Although Band *b* stained weakly with PAS reagent, showed enhanced galactose oxidase/NaB^3H_4 labeling upon neuraminidase addition and was specifically labeled by periodate/NaB^3H_4 treatment, it did not appear to contain sialic acid under the conditions used for analysis of N-acetylneuraminic acid in slices of electrophoresed gels.

In contrast to Band *b* and Band *3*, the other glycoproteins of these membranes identified by carbohydrate stains, lectin binding, sialic acid analysis of gel slices and radiolabeling methods specific for carbohydrates, were markedly different. These differences will be considered in detail.

As a group, the glycoproteins of the mouse erythrocyte labeled by the periodate/NaB^3H_4 or galactose oxidase/NaB^3H_4 techniques are much less heavily labeled than the human erythrocyte glycoproteins. This difference could reflect different susceptibilities of the glycoproteins of mouse and human erythrocytes to the labeling techniques (i.e., differences in the number and/or nature of susceptible carbohydrate groups per molecule) and/or different numbers of molecules of similar structure in these membranes. Mouse and human erythrocyte glycoproteins both exhibit enhancement of labeling by the galactose oxidase/NaB^3H_4 technique when neuraminidase is added, indicating more galactosyl/N-acetylgalactosaminyl residues penultimate to terminal sialic acid than at the terminal position of the glycoprotein oligosaccharides [25, 27, 83].

It is clear that the mouse erythrocyte membrane lacks an analogue of PAS I, the major sialoglycoprotein of the human membrane. There were no bands in the mouse membrane in the comparable molecular weight region (M_r 60,000–100,000) with the following properties characteristic of PAS I (Table 5): (i) strong staining with PAS reagent [21, 45, 85] and SA [45], (ii) heavy radiolabeling by periodate/NaB^3H_4 treatment [4, 26, 29, 50, 61, 83], (iii) radiolabeling by galactose oxidase/NaB^3H_4 treatment which is enhanced by incubation with neuraminidase [25, 27, 83], (iv) a sialic acid containing glycoprotein detected by N-acetylneuraminic acid analysis of gel slices after electrophoresis [3, 10], and (v) strong binding of ^{125}I-WGA in slices of polyacrylamide gel [69 and Fig. 9]. Although PAS I and Band 3 of the human erythrocyte membrane are not resolved under some conditions of polyacrylamide gel electrophoresis [10, 40, 78 and Fig. 4], we were able to resolve these bands using 8% acrylamide gels with a ratio of 20:1 acrylamide to bis-acryl-

amide, as others here reported for similar gel systems [40]. PAS I and Band 3 of the human erythrocyte both label after lactoperoxidase catalyzed radioiodination [8, 29, 40, 58, 62, 65, 68, 79, 88] and could be resolved using these gel conditions; however, the major radioiodinated band of the mouse erythrocyte (Band *b*, M_r 92,000) migrated as a single symmetrical peak on these gels (Fig. 4), providing additional evidence for the absence of a PAS I analogue in the mouse membrane.

Band *d* of the mouse membrane has several properties characteristic of PAS IV of the human erythrocyte membrane. It has a similar apparent mol wt on polyacrylamide gels to PAS IV (M_r 65,000 and 68,000, respectively, Table 5). Band *d* is also labeled by the lactoperoxidase reaction, it is a glycoprotein in its staining properties and susceptibility to labeling by carbohydrate specific radiolabeling probes (Table 5), and it binds ^{125}I-WGA but not ^{125}I-Con A (Fig. 9); all properties shared by the PAS IV band. However, Band *d* is not labeled as heavily as PAS IV by the carbohydrate labeling techniques [83], nor does it stain as strongly with PAS and SA. In common with Band *b*, Band *d* appears to lack sialic acid when gel slices are analyzed, even though it is specifically labeled by periodate/NaB^3H_4, indicating it to be a sialoglycoprotein.

The glycoproteins from the mouse erythrocyte membrane with apparent molecular weights of 30,000 to 60,000 are markedly different from the human erythrocyte glycoproteins. Basically similar distributions in polyacrylamide gels were observed for mouse erythrocyte glycoproteins identified with the following techniques specific for carbohydrates: (i) sialic acid analysis of gel slices, (ii) external glycoprotein radiolabeling using periodate/NaB^3H_4 treatment, (iii) external glycoprotein radiolabeling with galactose oxidase/NaB^3H_4 treatment plus neuraminidase treatment, and (iv) binding of ^{125}I-WGA to polyacrylamide gels. In each case there were no clearly resolved protein bands; instead, there was a broad distribution with the maximum amount of carbohydrate at Bands *g* or *h*. These glycoproteins are therefore sialoglycoproteins with terminal sialic acid on oligosaccharide chains, few galactose/N-acetylgalactosaminyl termini, but a high content of these residues penultimate to sialic acid. It is noteworthy that these glycoproteins did not stain with PAS or SA techniques. Although the other glycoproteins which stained with PAS and SA stained weakly, they did migrate on electrophoresis as discrete bands. The broad distribution of glycoproteins in the 60,000 to 30,000 mol wt region was also different from the distribution of proteins labeled by lactoperoxidase catalyzed radioiodination and from the distribution of membrane proteins stained with Coomassie Blue. Both

of these sets of proteins were clearly resolved into distinct protein bands by polyacrylamide gel electrophoresis. This broad band of glycoproteins (M_r 30,000–60,000) therefore lacks tyrosyl residues accessible to lactoperoxidase. The higher carbohydrate content of these proteins may lead to increased steric hindrance to the approach of the enzyme. These glycoproteins also appear to stain poorly, if at all, with Coomassie Blue, a feature in common with the reaction of other membrane glycoproteins with this stain [21]. These results highlight the necessity to use several different radiolabeling probes, based on different specificities, in order to identify the maximum number of surface membrane proteins. Bands *e*, *f* and *c* of the mouse erythrocyte membrane, for example, were only labeled by one of the four labeling techniques used.

There are at least three possible explanations for the broad distribution of apparent molecular weights of mouse erythrocyte glycoprotein: (i) the number of distinct glycoproteins, each with a unique amino acid sequence, with closely spaced molecular weights between 30,000 and 60,000 could be very large. Although there are no reasons to expect the number of surface glycoproteins on the mouse erythrocyte to greatly exceed the human erythrocyte (the functions of these cells being presumed to be comparable), this possibility cannot be discounted. One difference between the mouse and human erythrocyte surface which could be relevant to this point is the presence of antigens of the major histocompatibility complex on the surface of mouse cells in addition to blood group antigens [81]. The human erythrocyte expresses blood group antigens on its surface, including antigenic determinants of the MN system on the major sialoglycoprotein [44, 53], but it has only minor antigens of the major histocompatibility complex [59, 60, 64]. We have observed differences in the minor labeled proteins after lactoperoxidase catalyzed radioiodinization of mouse erythrocytes from different inbred strains[6], and these differences may be paralleled by differences in the surface glycoproteins, (ii) there is a relatively small number of glycoproteins incorporated into the mouse erythrocyte membrane, and artifactual or *in vivo* modification of these molecules produces a spectrum of products at various stages of modification. Although artifactual degradation of glycoproteins has not been ruled out, we have shown that the inclusion of 2 mM phenylmethylsulfonylfluoride in the sample buffer used for solubilization of labeled cells, has no effect on the profile of glycoproteins, suggesting that serine-proteases are not involved. The hu-

[6] *See* footnote 2, p. 173.

man erythrocyte membrane is reported to contain various glycosidase activities [6] and neuraminidase activity [7], and it is possible that activation of similar enzymes in the mouse membrane during cell solubilization results in various degrees of digestion of glycoprotein oligosaccharide chains. These changes might in turn result in slight changes in electrophoretic mobility. Such enzymes, however, would have to withstand immediate heating to 100 °C and high concentrations of SDS and 2-mercaptoethanol. Furthermore, the same broad band of glycoproteins has been observed in over ten labeling experiments by the periodate/NaB^3H_4 and galactose oxidase/B^3H_4 methods. *In vivo* modification of glycoproteins is an interesting possibility that has not been discounted. The correlations which have been shown to exist between experimental modification of the terminal sialic acid or galactose/N-acetylgalactosaminyl residues of erythrocytes from several species and changes in blood clearance times [1, 2, 20, 30, 31, 34, 42] indicate the potential importance of such a modification. The broad distribution of glycoprotein mobilities on polyacrylamide gel electrophoresis would therefore reflect the various degrees of modification of erythrocyte glycoproteins from cells of different ages, glycoproteins from older cells having the highest degree of modification. A significant difference in electrophoretic mobility was observed for the broad band of mouse erythrocyte glycoproteins between Bands *d* and *i* when cells were treated with neuraminidase in the galactose oxidase/NaB^3H_4 labeling technique, compared with the periodate/NaB^3H_4 method (Fig. 8). A similar difference has been observed in the mobility of the PAS II band of the human erythrocyte after labeling by these two techniques, neuraminidase treatment causing an anomalous low mobility [29]. Enzymic modification of mouse erythrocyte glycoproteins at their carbohydrate portion is capable therefore of causing shifts in electrophoretic mobility. It remains to be demonstrated whether an accumulated series of *in vivo* modifications are responsible for the broad distribution of glycoproteins observed, and (iii) there are a relatively small number of glycoproteins on the mouse erythrocyte membrane, and noncovalent association of these molecules in various ways during cell solubilization in SDS buffer a high temperature produces a wide range of species which are not dissociated during electrophoresis, and which therefore migrate with a wide range of electrophoretic mobilities. This possibility is suggested by the association of human erythrocyte sialoglycoproteins observed during various conditions of membrane solubilization [24, 54, 67, 76, 80]. Although a similar phenomenon may account for some of the glycoprotein bands seen with electrophoresis

of mouse erythrocyte proteins, it appears unlikely that a small number of glycoproteins could associate in a sufficient number of different ways to produce a continuum of species with small differences in electrophoretic mobility in such a wide band.

Analyses of the sialic acids of the mouse erythrocyte membrane have indicated the presence of O-acetylated sialic acids in addition to N-acetylneuraminic acid which is found in the human membrane (R. Schauer and R.J. Howard, *unpublished results*). Several results of this report also indicate differences in the sialic acids of mouse and human erythrocyte sialoglycoproteins. The sialoglycoproteins on the human membrane are stained strongly with PAS and SA and contain terminal sialic acid residues readily oxidized by periodate which are also rapidly cleaved by *V. cholerae* neuraminidase. In contrast, the PAS and SA positive sialoglycoproteins of the mouse erythrocyte membrane are only a minor group of the total sialoglycoproteins, and the broad band of mouse sialglycoproteins seen on electrophoresis did not stain with these reagents, even though sialic acid analysis of gel slices in this region of the gels indicated a sialic acid content similar to the human sialoglycoproteins. Furthermore, these sialoglycoproteins labeled poorly with the periodate/NaB^3H_4 technique considering their total sialic content. We propose that there are at least two groups of sialoglycoproteins on the mouse erythrocyte membrane: one group contains a low content of unmodified terminal sialic acid compared to the human PAS I–IV proteins and is weakly stained with PAS and SA. A second group contains sialic acids modified by O-acetylation of hydroxyls on carbons 7, 8 or 9, as has been described for a wide range of natural sialoglycoproteins [reviewed in 72]. This group would stain poorly with PAS and SA reagents and would be slowly oxidized by periodate, or be resistant to oxidation, depending on the position of acetylation [73]. Provided that O-acetylation occurs at these positions, and not at the hydroxyl group on carbon 4, these modified sialic acids would still be susceptible to cleavage by *V. cholerae* neuraminidase, although at reduced rates [73, 74], to expose penultimate gal/galNAc residues for the galactose oxidase reaction. We are at present characterizing the sialoglycoprotein sialic acids of the mouse membrane in order to test these possibilities.

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