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SPECIES VARIABILITY IN THE MODIFICATION OF ERYTHROCYTE SURFACE PROTEINS BY ENZYMATIC PROBES*

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SUMMARY

Bovine and equine erythrocytes have been studied by three different surface modification techniques to investigate the accessibility of the surface components to the external medium. Lactoperoxidase labeling of equine erythrocytes results in a significant labeling of only one membrane component, a 100 000-mol.wt polypeptide corresponding to the membrane-spanning Component III of human erythrocytes. The major sialoglycoprotein of the equine erythrocyte is not labeled. This is in contradistinction to the situation for human and bovine cells, where both components are labeled. The equine membrane sialoglycoprotein is also not markedly affected by pronase, chymotrypsin or trypsin treatment of whole cells under the treatment conditions used, although it can be cleaved by pronase in isolated membranes. Experiments with the isolated glycoprotein show that its cleavage by trypsin is quite selective, whereas cleavage by pronase and chymotrypsin is much more extensive. Labelling of bovine red cells by galactose oxidase treatment followed by reduction with ^{3}H-labeled borohydride yields radioactivity in only one major peak, that corresponding to the glycoprotein. Pretreatment of the cells with neuraminidase causes a dramatic increase in the labeling. Equine erythrocytes do not show significant labeling by this technique unless a neuraminidase pretreatment has been performed. Then only the major glycoprotein is labeled. Thus the equine glycoprotein is apparently inaccessible to the cell surface by standard surface modification methods, although it is clearly a surface component. These experiments point out some of the limitations of surface labeling and proteolysis methods in probing the accessibility of membrane components. The results suggest that the apparent inaccessibility of the equine glycoprotein is due partially to its structure and partially to its localization in the membrane.

Abbreviation: AcNeu, *N*-acetylneuraminic acid.

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INTRODUCTION

Glycoproteins appear to be ubiquitous and important components of the mammalian cell surface [1–5]. The discoveries of glycoprotein-related phenomena during cell transformation have led to development of several methods for monitoring changes in these molecules at cell surfaces [6–11]. In view of the significance of these types of investigations it is clearly important to understand how membrane glycoproteins associate with cell membranes and how they can be detected at cell surfaces. An interesting system for examining certain aspects of this problem involves comparative studies of erythrocytes from different species. The red cell is quite simple morphologically and is metabolically rather inert, but it possesses a complex mosaic of blood group characters at the cell surface, many of whose determinants are based upon carbohydrates [12]. The glycoprotein composition of erythrocyte membrane appears to be simple, with one major glycoprotein component for most species [13]. However, the glycoproteins isolated from the different species show considerable variability in their carbohydrate compositions [14]. In addition proteolysis studies on intact erythrocytes show significant species variations in the cleavage of the major polypeptide component, which is not heavily glycosylated [15]. It is possible that this behavior might relate to glycoprotein differences.

The current study was undertaken to determine what kinds of differences in surface glycoprotein behavior can be detected among the erythrocytes from different species. These differences can then serve as focal points for developing explanations of glycoprotein behavior of more complex cells.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free ^{125}I was purchased as $Na^{125}I$ from New England Nuclear. Lactoperoxidase, isolated from bovine milk [16], was a gift from Dr K. E. Ebner. KB^3H_4 was from New England Nuclear. Enzymes were purchased from the following sources: *Vibrio cholerae* neuraminidase, Calbiochem; galactose oxidase, trypsin and chymotrypsin, Sigma. Red blood cells from different species were obtained as described previously [13] and were washed free of buffy coat in 310 mosM phosphate buffer, pH 7.4, or 5 mM phosphate in isotonic saline, pH 7.0. Membranes were prepared by hypotonic hemolysis [17]. Purified equine glycoprotein was prepared as described previously [14].

Protease treatments of intact cells and membranes

Proteolysis of intact cells was performed as described by Triplett and Carraway [15]. Hematocrits of 10–33 % in 310 mosM phosphate (pH 7.4), phosphate-buffered saline (pH 7.4) or Tris/saline (pH 7.4), were used. Membranes were digested at equivalent membrane concentrations.

Proteolysis of isolated glycoprotein

Digestion of glycoprotein (4 mg/ml) in 310 mosM phosphate (pH 7.4) was performed at 37 °C at a protease concentration of 1 mg/ml. The reaction was stopped by adding sodium dodecylsulfate (2.5 %) and mercaptoethanol (5 %) and heating to 100 °C for 5 min. Samples were used directly for electrophoresis.

Labeling of bovine and equine erythrocytes with lactoperoxidase

Blood was washed as previously described [13, 18]. 1 ml of washed, packed erythrocytes was suspended in 2 ml of labeling solution containing 150 mM NaCl, 10 mM Tris, 10 μM KI, 100 μCi/ml $Na^{125}I$ and 0.5 μM lactoperoxidase (pH 7.4) at room temperature [19]. 25 aliquots of 20 μl each of 2.33 mM H_2O_2 in 0.155 M NaCl/7 mM phosphate (pH 7.4) were added at 15-s intervals to the suspension. After 5 min 400 μl of 100 mM KI and 3 ml of cold 0.155 M NaCl/7 mM phosphate (pH 7.4) were added and the suspension was centrifuged at 4 °C. The labeled erythrocytes were washed twice with 8 ml of 0.155 M NaCl/7 mM phosphate before hemolysis to obtain labeled membranes [19].

Erythrocyte labeling with galactose oxidase and KB^3H_4

The procedure used was similar to that of Gahmberg and Hakomori [20]. After a final wash of cells in phosphate-buffered saline, pH 7.0, the cells were resuspended (33 % suspension) in phosphate buffered saline, pH 7.4, for protease treatment or in phosphate-buffered saline, pH 6.0, containing 1 mM $CaCl_2$ for neuraminidase treatment. Cells were treated with neuraminidase (50 μl or 25 units per 5 ml packed cells) for 30 min at 37 °C. Protease treatment (1 mg/ml) was for 30 min at 37 °C in the pH-7.4 buffer. Control cells were also incubated in the pH-6.0 phosphate-buffered saline. After initial enzymatic treatment, the cells were centrifuged at 1700 × *g* for 10 min, washed twice in phosphate-buffered saline, pH 7.0, and resuspended to 50 % concentration in this buffer. 100 μg of galactose oxidase was added per 5 ml packed cells, and the cells were incubated at 37 °C with gentle shaking for 3 h. The cells were centrifuged, washed and resuspended in an equal volume of phosphate buffered saline, pH 7.4, containing 0.25 mCi KB^3H_4 per 5 ml packed cells. After incubation at room temperature for 30 min with occasional shaking, 5 ml of phosphate-buffered saline containing 1 mg unlabeled $NaBH_4$ was added per 5 ml packed cells and the cells centrifuged. The cells were washed with phosphate-buffered saline, pH 7.4, until the supernatant was essentially free of radioactivity. The cells were then resuspended in phosphate-buffered saline, pH 7.4, with or without protease (1 mg/ml) and incubated an additional 30 min at 37 °C. The cells were centrifuged and washed 3 times in 310 mosM phosphate buffer. Membranes were then prepared in the usual manner.

Analytical procedures

Membranes were dissolved in 2.5 % dodecyl sulfate containing 25 mM phosphate (pH 7.4), 2.5 mM EDTA and 5 % β-mercaptoethanol. The tubes were heated at 100 °C for 3–5 min, then incubated at 37 °C for 3–16 h after adding an additional aliquot of β-mercaptoethanol to 10 %. Electrophoresis was performed on 5 or 6 % acrylamide gels with pyronin Y as tracking dye [19]. Gels were stained with Coomassie blue or with HIO_4-Schiff using the procedure of Fairbanks et al. [21], and omitting the second Coomassie blue-staining step.

Gels were sliced into 2-mm slices [18], and the slices were counted in Bray's solution [22] after solubilization in 1 ml 90 % NCS at 50 °C overnight [18].

Neutral carbohydrate was measured using the phenol-H_2SO_4 method [23] with galactose as standard and sialic acid by the Warren procedure [24] with *N*-acetylneuraminic acid (AcNeu) as standard, after prior hydrolysis in 0.05 M H_2SO_4. Protein was measured by the method of Lowry et al. [25], and cholesterol by the procedure of Zlatkis et al. [26].

RESULTS

Lactoperoxidase labeling

In a previous study we have shown that Component III, the major erythrocyte membrane-spanning polypeptide, shows a different response to protease treatments in erythrocytes from different species of animals [15]. It was suggested that this surface property of the erythrocytes might relate to known differences in erythrocyte membrane glycoproteins from different species. Since Uhlenbruck et al. [27] have suggested that the study of erythrocyte surfaces might be a useful model for understanding surface differences between normal and neoplastic cells, we sought further information about the variability of the accessibility of components. Lactoperoxidase labeling of human erythrocyte surfaces shows the presence of two major membrane components accessible to the cell surface, the sialoglycoprotein and Component III [19, 28]. The surface of bovine and equine erythrocytes can also be labeled by the lactoperoxidase method, as indicated by Table I. When bovine erythrocytes are labeled, a pattern of two bands is seen (Fig. 1). The major band corresponds to the

TABLE I

IODINE INCORPORATION INTO MEMBRANES AND HEMOGLOBIN OF BOVINE AND EQUINE ERYTHROCYTES

Sample	Specific activity (cpm/μg protein)		Iodine distribution (% of total)	
	Membranes	Hemoglobin	Membranes	Hemoglobin
Bovine	4500	3.6	95	5
Equine	930	1.1	94	6

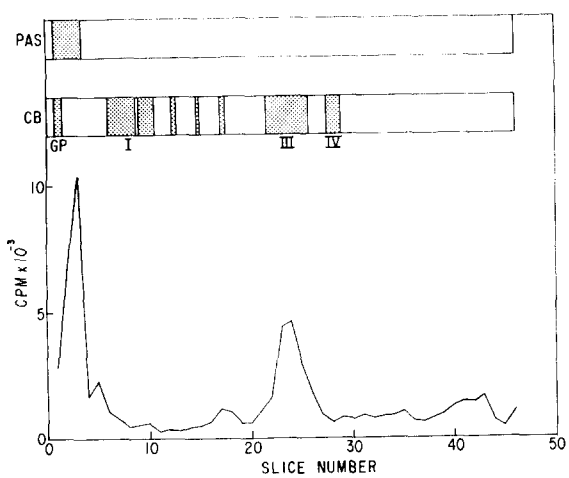

Fig. 1. Lactoperoxidase labeling of bovine erythrocytes. Cells were reacted as described in Experimental Procedures. Membranes were prepared and subjected to dodecylsulfate/acrylamide electrophoresis, and the gels were sliced and counted as previously described. PAS, periodate-Schiff; CB, Coomassie blue; GP, glycoprotein.

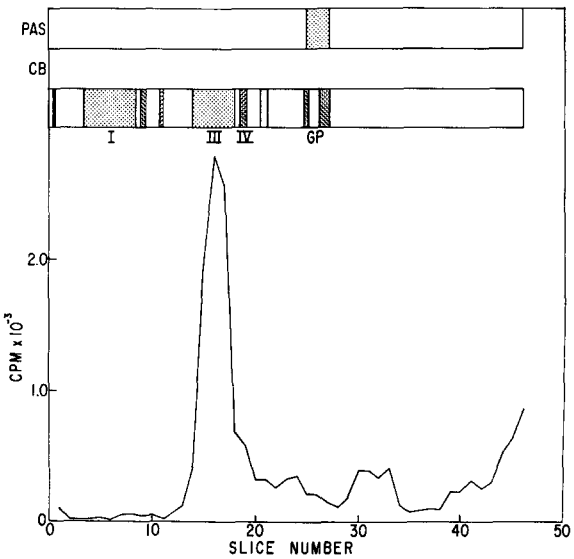

Fig. 2. Lactoperoxidase labeling of equine erythrocytes. PAS, periodate-Schiff; CB, Coomassie blue; GP, glycoprotein.

position of the periodate-Schiff-stained glycoprotein band and the smaller band to Component III. The labeling of Component III indicates that it is accessible to the cell surface, even though it is not cleaved by pronase, trypsin or chymotrypsin treatments of intact bovine cells [15]. Labeling of equine cells with lactoperoxidase showed an entirely different and unexpected result. Component III was iodinated in intact equine cells but no labeling of significance was found in the glycoprotein region of the gels (Fig. 2). These results suggest that there are no iodinatable amino acid residues on the glycoprotein which are accessible from the exterior of the cell.

Proteolysis of cells, ghosts and glycoproteins

Because of the unusual behavior of the equine glycoprotein in lactoperoxidase surface-labeling experiments, the susceptibility to external proteolysis of the cells was tested. Equine and bovine cells were subjected to chymotrypsin and pronase digestions under conditions where both glycoprotein and Component III are cleaved in human cells and glycopeptides are released into the medium. Neutral sugar analyses were performed on the supernatant fractions of the treated cells to monitor glycopeptide release. As shown in Table II, the amount of carbohydrate released from the equine erythrocytes was about 10-fold less than that released from bovine cells under equivalent conditions. In addition examination of dodecylsulfate/acrylamide gels of the membranes from treated equine cells showed no discernible losses or displacements of protein (Coomassie blue) or glycoprotein (periodate-Schiff) bands. By these criteria glycoprotein cleavage could only have occurred to a very limited extent, unless it were near a terminus of the polypeptide chain. Protease treatment of bovine cells caused disappearance of the glycoprotein band from the gel patterns.

Proteolysis was also performed on isolated equine erythrocyte ghosts to assess the accessibility of the glycoprotein. As with human membranes essentially all of the

TABLE II

CARBOHYDRATE RELEASE FROM BOVINE AND EQUINE ERYTHROCYTES BY CHYMOTRYPSIN AND PRONASE

Hematocrit was 10 %.

Species	Treatment	Concentration (mg/ml)	Carbohydrate released (μg/ml packed cells)
Bovine	Chymotrypsin	0.5	168
	Chymotrypsin	2.0	174
	Pronase	0.05	216
	Pronase	0.1	246
Equine	Chymotrypsin	0.5	12
	Chymotrypsin	2.0	18
	Pronase	0.05	12
	Pronase	0.1	30

polypeptide chains detected by Coomassie blue are cleaved when equine ghosts are treated with trypsin or chymotrypsin. However, in contrast to the human case [29], no discernible cleavage of the glycoprotein is found when equine ghosts are treated with trypsin or chymotrypsin concentrations up to 0.5 mg/ml. This is illustrated in Fig. 3 by scanning profiles of periodate-Schiff stained acrylamide gels of trypsin treated and untreated membranes. If pronase, a more nonspecific collection of proteases, is used on equine ghosts, then cleavage of the glycoprotein is observed concomitant with extensive degradation of the other proteins. No lower mol. wt glycopeptides were observed. Therefore it appears that the glycoprotein is not completely resistant to digestion in the membranes, but that its accessibility is limited.

That the equine glycoprotein is not inherently resistant to digestion can be

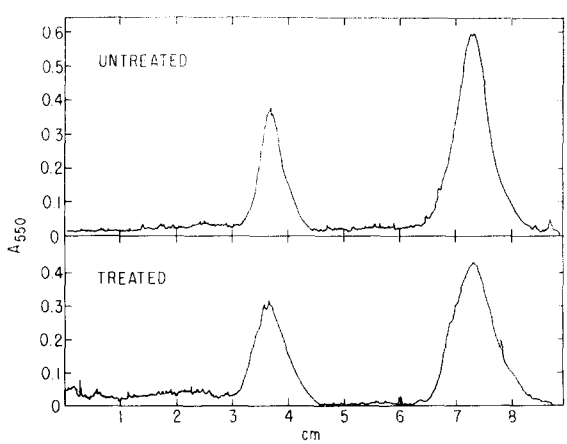

Fig. 3. Effect of trypsin on glycoprotein in equine erythrocyte membranes. Scanning profiles from periodate-Schiff-stained gels are shown. The gels are from duplicate samples treated with 100 μg ml of trypsin, or no trypsin. Additional experiments with chymotrypsin and trypsin showed no discernible cleavage at concentrations up to 0.5 mg/ml. The glycoprotein band is at the center of the profile with a lipid band on the far right.

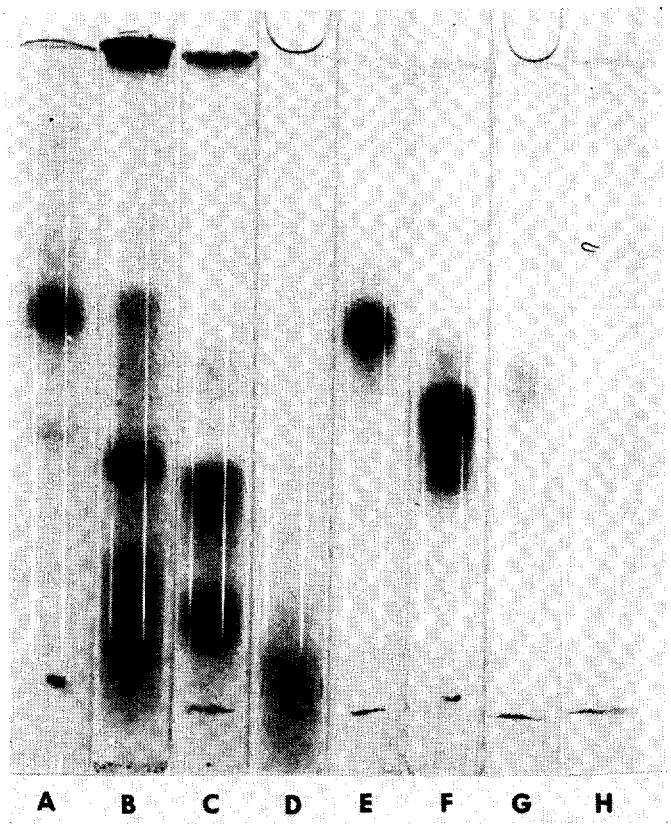

Fig. 4. Proteolytic digestion of isolated equine erythrocyte membrane glycoprotein. Glycoprotein was treated with trypsin, chymotrypsin or pronase as described in Experimental Procedures. Reaction mixture was subjected to acrylamide gel electrophoresis after heating in dodecylsulfate to destroy enzyme. Gels A–D, Coomassie blue stain for protein; gels E–H periodate-Schiff stain for carbohydrate. Gels A and E are from untreated glycoproteins; B and F, trypsin treated; C and G, chymotrypsin treated; and D and H, pronase treated.

shown by proteolysis of the isolated glycoprotein in aqueous solution. Pronase and chymotrypsin cleave the glycoprotein to small fragments which are not detected by periodate-Schiff staining of acrylamide gels run in dodecylsulfate (Fig. 4, gels G and H). Trypsin causes a much more selective cleavage to give primarily one periodate-Schiff-positive product (Fig. 4, gel F). In addition the trypsin-cleavage patterns show a band of higher apparent molecular weight which will not penetrate the gel (Fig. 4, gel B) and does not stain significantly for carbohydrate. It is apparent from these results that most of the carbohydrate of the equine glycoprotein resides in a region of the polypeptide which is resistant to trypsin digestion.

Galactose oxidase labeling

Steck and Dawson [30] and Gahmberg and Hakomori [20] have used treatment with galactose oxidase followed by ^{3}H-labeled borohydride to label cell surface glycosylated components of human erythrocytes. The labeling pattern obtained for

TABLE III

GALACTOSE OXIDASE-BOROHYDRIDE LABELING OF BOVINE ERYTHROCYTES

Cells were treated according to the protocol in the table as described in the Experimental Procedures. Isolated, washed membranes were assayed for radioactivity, neutral sugar, sialic acid, protein and cholesterol. All values are expressed per mg membrane cholesterol. All samples were treated with borohydride.

Sample	Pre-treatment	Galactose oxidase	Post-treatment	cpm	Neutral sugar (μmol)	Protein (mg)	AcNeu (μg)
A				750	1.2	2.0	81
B		+		6 520	1.4	2.3	76
C	Neuraminidase	+		28 500	1.4	2.5	48
D	Neuraminidase	+	Trypsin	6 950	1.2	2.5	27
E		+	Trypsin	2 590	1.1	2.5	34
F	Trypsin	+		2 910	0.9	2.6	35

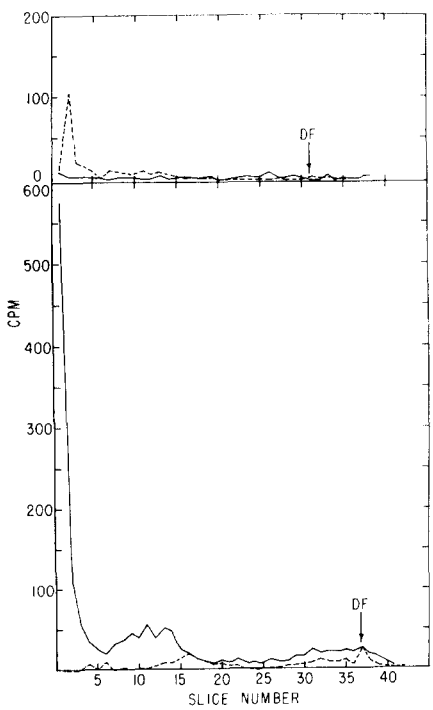

Fig. 5. Radioactivity profiles of membranes from galactose oxidase-borohydride labeled bovine red cells. Membranes from labeled cells were fractionated by electrophoresis on acrylamide gels, which were sliced and counted. The top profile shows the control, which was not treated with galactose oxidase (Expt A, Table III; ——) and the cells labeled without pre- or post-treatment (Expt B; - - -). The bottom profile shows the samples pretreated with neuraminidase before labeling (Expt C, ——) and posttreated with trypsin after labeling (Expt D, - - -). DF, dye front.

intact cells is considerably more complex than patterns observed for periodate-Schiff staining of membrane components, suggesting the presence of minor glycosylated components, high in accessible galactose or galactosamine [30]. Neuraminidase treatment of the erythrocytes causes a several-fold enhancement of label incorporation, with most of the label now residing in the peak for the major glycoprotein [20]. When bovine erythrocytes are treated according to a protocol similar to that of Gahmberg and Hakomori [20], an even simpler pattern is observed. Table III shows the labeling conditions, incorporation of label and analytical data on isolated membranes for cells labeled under different conditions. Galactose oxidase treatment causes an 8–10-fold enhancement of borohydride label incorporation over that of untreated cells. An additional 4-fold enhancement of labeling occurs if the cells are treated with neuraminidase before the galactose oxidase reaction. Trypsinization of the labeled cells causes a substantial decrease in the radioactivity attached to the membranes, even though electrophoretic analysis of the membranes shows significant degradation of only the glycoprotein, of all the major polypeptides in the membrane. The labeling patterns for the samples separated by dodecylsulfate/acrylamide electrophoresis are shown in Fig. 5. A single major peak is evident for membranes from both neuraminidase-treated and untreated cells. The peak shows a decreased mobility after neuraminidase treatment, as previously observed by Kobylka et al. [13] for the glycoprotein in this electrophoresis system. Membranes from cells which were trypsinized after labeling show a rather disperse labeling pattern, indicating that there are no highly labeled discrete trypsin fragments.

Galactose oxidase-borohydride labeling was also performed on equine erythrocytes. Because a higher concentration of borohydride was used in the reduction step, the radioactivity incorporated into the equine membranes in the absence of galactose oxidase treatment is higher than was observed with the bovine (Table IV). The most interesting feature of this experiment is that labeling is negligible unless the cells have been pretreated with neuraminidase. The neuraminidase-treated and labeled cells show a 10-fold enhancement of radioactivity, even through a rather small fraction of the sialic acid was removed under the conditions used. The radioactivity profiles are shown for membranes of the labeled equine cells in Fig. 6. The radioactivity band from neuraminidase-pretreated, labeled cells corresponds to the position of the glycoprotein. In addition pronase digestion of labeled membranes causes concomitant loss of the periodate-Schiff staining band and the radioactive

TABLE IV

GALACTOSE OXIDASE-BOROHYDRIDE LABELING OF EQUINE ERYTHROCYTES

Conditions are essentially equivalent to comparable experiments in Table III except for higher borohydride concentrations.

Sample	Pre-treatment	Galactose oxidase	cpm	Neutral sugar (μmol)	AcNeu (μg)	Protein (μg)
A	–	–	4 730	1.9	140	2.4
B	–	+	5 300	1.9	150	2.6
C	Neuraminidase	+	50 200	1.9	130	2.6

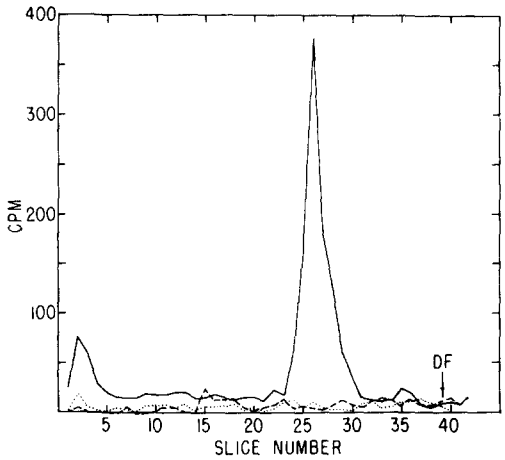

Fig. 6. Radioactivity profile of galactose oxidase-borohydride labeled equine cells. Patterns correspond to Expts A (untreated, · · ·), B (galactose oxidase treated, - - -), and C (neuraminidase and galactose oxidase treated,) of Table IV. The label at the top of the gel appears to result from glycoprotein which did not disaggregate during solubilization.

band, together with the degradation of most of the other membrane polypeptides. This is good evidence for the glycoprotein nature of the labeled material.

An additional interesting feature of this work is the negligible labeling of glycolipid in the bovine and equine cells. This contrasts with previous studies on human erythrocytes, in which extensive glycolipid labeling was shown [20, 30]. It is not known whether this difference is a function of the labeling conditions or whether it reflects actual glycolipid structural or organizational differences in these cell membranes which make galactose in the lipids unavailable for reaction.

DISCUSSION

When enzymatic surface modification procedures are applied to the study of bovine and equine erythrocytes, they show rather unexpected differences. Bovine erythrocytes exhibit a behavior similar to human cells, with two surface components demonstrated by lactoperoxidase labeling and the release of glycopeptides observed during proteolysis. The galactose oxidase labeling pattern is simpler than for human cells, probably due to dominance of the major sialoglycoprotein with its high galactose content. In contrast the equine glycoprotein in the intact cell is not modified significantly by lactoperoxidase, trypsin, chymotrypsin, pronase or galactose oxidase. The presence of the glycoprotein at the cell surface can be demonstrated by galactose oxidase labeling after neuraminidase treatment of the cells. The results indicate some of the limitations of these procedures for detection of surface components. Each of the methods is dependent on substrate availability, i.e. the accessibility of particular regions of the protein which can serve as substrates for the modifier enzyme. It is evident that the substrate regions of the equine glycoprotein are less accessible in the membrane than for the glycoproteins of the other two species which have been studied.

Because of the interest in factors affecting the expression of glycoproteins at cell surfaces, it is instructive to ask why the equine glycoprotein appears so inaccessible. This can be partially answered by examining the proteolytic digestion of cells, isolated membranes and the isolated glycoprotein. The glycoprotein is not cleaved by trypsin, chymotrypsin or pronase in the intact cell under the conditions used. It is readily cleaved by pronase, but not by trypsin or chymotrypsin in the membrane, and it is selectively cleaved by trypsin in aqueous solution. The latter suggests an inherent resistance to trypsin. This probably results from the distribution of carbohydrate along the polypeptide chain and the presence of a lower number of basic amino acids than have the human and bovine glycoproteins [14], since the equine is lower in total carbohydrate [14] and has the same predominantly random conformation exhibited by the other two [31]. The resistance to chymotrypsin and pronase in the membrane and cell, respectively, appear to result from the association of the glycoprotein with the membrane, which renders the glycoprotein substrate groups unapproachable. One feature of the equine erythrocyte which may make its surface different from the others examined is the higher membrane concentration of glycolipid [14], particularly sialoglycolipid [32]. Yu et al. [33] have suggested a possible specific association of glycoproteins and glycolipids in the human erythrocyte membrane. This type of association might also be important in determining the accessibility of the glycoproteins in transformed cells in which both glycoproteins and glycolipids appear to be altered.

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