Topographical Distribution of Complex Carbohydrates in the Erythrocyte Membrane*

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SUMMARY

Human red blood cell membranes treated with galactose oxidase were specifically labeled in galactose and N-acetyl-galactosamine residues by reduction with tritiated borohydride. Ceramide tri- and tetrahexosides were the most intensely reactive lipid species. Several labeled polypeptide peaks were resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. While some of the radioactivity appeared to correspond to known, principal glycoproteins, a major part of the label was distributed in regions of the gel bearing only weakly stained protein and glycoprotein components.

The pattern of labeling was essentially the same for intact erythrocytes and unsealed ghosts. Preparations of sealed, inside-out vesicles showed very little labeling, all of which could be attributed to small amounts of contamination from accessible outer surface. On this basis, all of the glycoprotein and glycolipid sugars reactive with galactose oxidase plus tritiated borohydride can be assigned to the external surface of the membrane.

Isolated human erythrocyte membranes ("ghosts") contain approximately 8% carbohydrate (1), both as glycoprotein and glycolipid (2). The glycoproteins show a complex periodic acid-Schiff staining pattern on polyacrylamide gels subjected to electrophoresis in sodium dodecyl sulfate (3). The most conspicuous component is the dialoglobin glycoprotein, termed PAS-1 (3) or glycocephalin (4). Another major membrane polypeptide, Band 3 (3), has recently been isolated and identified as a glycoprotein (5, 6), although it reacts poorly with periodic acid-Schiff. Both Components 3 and PAS-1 are thought to extend asymetrically across the membrane thickness (7-10). The total number of distinct glycoprotein species, their chemical character, and their disposition within the membrane remain to be established.

The intent of this study was to utilize a specific modification technique to enumerate and probe the orientation of sugar-bearing macromolecules in the erythrocyte membrane. Galactose oxidase has been found to oxidize the C-6 position of a-galactose, N-acetyl-D-galactosamine, and related sugars present in oligosaccharides (11). The resultant aldehyde groups have been labeled by reduction with tritiated borohydride in both isolated glycolipids (12) and soluble glycoproteins (13). We reasoned that while PH₃ might readily penetrate even well sealed membranes, galactose oxidase would not, and could therefore be used to probe the two membrane surfaces selectively for reactive sugars. For this purpose, intact erythrocytes or resealed ghosts were used to examine the external membrane face, while sealed inside-out vesicles presented the cytoplasmic membrane surface for unilateral reaction. Our findings indicated a surprising multiplicity of glycoprotein species not clearly demonstrated by conventional stains. Furthermore, all of the reactive glycoprotein and glycolipid sugars appeared to be available only at the external membrane surface. Since our first communication of these findings (9), Gahmberg and Hakomori (14) have used this technique to confirm certain features of the labeling reaction.

EXPERIMENTAL PROCEDURES

Materials—Galactose oxidase of Polyporus circinatus was Lot No. DpB 138 from Kabi (Stockholm). Its specific activity was given as 180 i.u. or 5 x 10⁴ Kabi units per mg of protein. Galactose oxidase preparations from General Biochemicals, Inc. and Worthington were used in early experiments. Proteolytic digestion of the membrane proteins, as reflected by degradation of their gel-banding pattern, was observed following incubation of ghosts with all of these enzymes. The protease could be inactivated without significant loss of galactose oxidase activity by a preincubation of the diluted enzyme at 50°C for 30 min.

Sodium and potassium PH₃ were purchased from Amersham-Searle; the nominal specific activities of the three batches used were 304, 102, and 590 mCi per mmole. NaBH₄ was obtained from Sigma Chemical Co. Reagents for gel electrophoresis and glycolipid analysis were essentially as in Refs. 3 and 15, respectively. All inorganic chemicals and solvents were reagent grade or better, from Fisher, Mallinckrodt, or Baker.

Erythrocyte Membrane Preparations—Human erythrocytes from fresh or outdated bank blood were washed three times in Buffer A (5 mm sodium phosphate, pH 8.0), made 150 mM in NaCl. Hemoglobin-free, unsealed ghosts were prepared by hemolysis and washing in Buffer A (3, 16-18). Resealed ghosts were prepared by including 1 mM MgSO₄ in Buffer A during hemolysis...
and subsequent washing (17, 18). Sealed inside-out vesicles were prepared as described previously (16-18).

Assay—Membrane sidedness and sealing were estimated by the latency of the external surface acetylcholinesterase (AChE) of membrane preparations and the latency of inner (i.e. cytoplasmic) surface glyceraldehyde 3-P-dehydrogenase in resealed ghost preparations (17, 18). Complete accessibility of the cryptic, sequestered surface was obtained by incubating the membranes in 0.1% Triton X-100. Since unsealed ghosts and inside-out vesicles contain varying amounts of loosely associated protein, they were compared on the basis of their total acetylcholinesterase activity (17, 18).

Isolation of Lipids and Glycosphingolipids—The lipid fraction of membrane pellets was obtained by extraction with 2:1 chloroform-methanol (19). Glycolipids were isolated from tritiated membrane fractions (to which 1 ml of unlabeled carrier ghosts had been added) by a modification (15) of the method of Vance and Sweeley (20). All five major glycolipids were well resolved on thin layer plates of Silica Gel G (Analtech, Inc.) developed in chloroform-methanol-water, 100:40:6 (15). Individual glycolipids were visualized with iodine, scraped from the plates and eluted from the gel with chloroform-methanol-water (100:40:6) prior to determination of radioactivity by liquid scintillation counting and quantitative analysis by gas-liquid chromatography (15).

Polyacrylamide Gel Electrophoresis—Gels containing 5.0% acrylamide, 0.19% N,N'-methylenebisacrylamide, and 0.2% sodium dodecyl sulfate (w/v) were prepared, run, and stained according to the method of Fairbanks et al. (3) as modified by Steck and Yu (21). Each gel was loaded with the equivalent of 10 μl of packed ghosts (which normally contain 34 to 40 μg of protein). Gels were stained and scanned, then cut transversely into 0.3 to 1.0-mm sections. Each slice was dried and then dissolved in 0.1 ml of 30% H2O2 (22) in scintillation vials. NCS solubilizer (0.7 ml) was added, followed by 8 ml of scintillation fluid (4 g of 2,5-diphenylxazole (PPO) and 0.2 g of 1,4-bis(5-phenylloxazolyl)benzene (POPOP) per liter of toluene). Samples were counted in a Packard scintillation spectrometer.

Labeling Reaction—In a typical experiment, 100-μl aliquots of pelletized ghost membranes (540 to 400 μg of protein or 6 to 7 X 109 ghosts) or their equivalent in inside-out vesicles were diluted with 100 μl of Buffer A in centrifuge tubes. Two hundred microliters of 50 mM sodium phosphate buffer, pH 8.5, containing 0 or 0.28 mM sodium dodecyl sulfate (all w/v) were prepared, run, and stained according to the method of Fairbanks et al. (3) as modified by Steck and Yu (21). Each gel was loaded with the equivalent of 10 μl of packed ghosts (which normally contain 34 to 40 μg of protein). Gels were stained and scanned, then cut transversely into 0.3 to 1.0-mm sections. Each slice was dried and then dissolved in 0.1 ml of 30% H2O2 (22) in scintillation vials. NCS solubilizer (0.7 ml) was added, followed by 8 ml of scintillation fluid (4 g of 2,5-diphenylxazole (PPO) and 0.2 g of 1,4-bis(5-phenylloxazolyl)benzene (POPOP) per liter of toluene). Samples were counted in a Packard scintillation spectrometer.

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sialic acid moieties, thus exposing the penultimate galactose.

Fig. 5 (right). Oligosaccharide oxidation by galactose oxidase: 200 \( \mu \)g each of lactose, \( \alpha \)-lacto-\( \alpha \)-lactosamine (Gal-GlcNAc-Gal-Glc). O, lacto-\( \alpha \)-fucopentaose II. (Gal-GlcNAc-[Fuc]-Gal-Glc). \( \Delta \) -- \( \Delta \), were incubated with 0.5 mg of galactose oxidase (Worthington) in 3 ml of 1 x Tris-HCl (pH 7.0). Oxidation was followed by measuring the generation of \( \mathrm{H}_2\mathrm{O}_2 \) with a peroxidase plus 3,3'-dimethoxybenzidine system by the increase in optical absorbance at 420 nm (cf. 11). Raffinose, which is completely oxidized within one hour, was used as a reference standard.

Fig. 4 (left). Galactose oxidase concentration dependence of ghost labeling. Unsealed ghosts were incubated with the designated amount of galactose oxidase plus \( \mathrm{KB}^3\mathrm{H}_4 \), (11.6 \( \times \) 10\(^6\) cpm) as described under “Experimental Procedures,” except that the reaction mixture was scaled to one-half volume.

Fig. 5 (right). Oligosaccharide oxidation by galactose oxidase:

- 1 mg each of lactose, \( \alpha \)-lacto-\( \alpha \)-lactosamine (Gal-GlcNAc-Gal-Glc), O, and lacto-\( \alpha \)-fucopentaose II. (Gal-GlcNAc-[Fuc]-Gal-Glc). \( \Delta \) -- \( \Delta \), were incubated with 0.5 mg of galactose oxidase (Worthington) in 3 ml of 1 x Tris-HCl (pH 7.0). Oxidation was followed by measuring the generation of \( \mathrm{H}_2\mathrm{O}_2 \) with a peroxidase plus 3,3'-dimethoxybenzidine system by the increase in optical absorbance at 420 nm (cf. 11). Raffinose, which is completely oxidized within one hour, was used as a reference standard.

\[ \text{Labeling of Galactose Oxidase} \]

1. **Preincubation of ghost membranes with sialidase from Clostridium perfringens** (16) led to a mild (\(<\)2-fold) stimulation of labeling by galactose oxidase plus tritiated borohydride (cf. also Ref. 14). Presumably, the effect of sialidase was to release terminal sialic acid moieties, thus exposing the penultimate galactose residues of the membrane sialoglycoproteins (1, 23, 24), as was found for ceruloplasmin by Morell et al. (13). This effect lends support to the premise that only galactose and N-acetylgalactosamine in the nonreducing terminal position are attacked by galactose oxidase. In contrast to sialidase, pretreatment with trypsin led to no significant change in the incorporation of tritium into the red cell membrane or the derived glycolipid fraction. Preincubation with pronase caused a mild reduction in both total and glycolipid labeling (but cf. Ref. 14).

**Sidedness of Membrane Labeling**—A principal aim of this study was to use galactose oxidase as a nonpenetrating probe which would label in turn the carbohydrate present at each membrane surface. The outer membrane face can be reacted by using intact erythrocytes or resealed ghosts. Conversely, the inner (cytoplasmic) membrane surface is unilaterally accessible in sealed inside-out vesicles. Our standard, unsealed ghosts appear freely permeable to proteins and would permit both surfaces to react (cf. Refs. 9, 17, 18).

Intact erythrocyte membranes, resealed and unsealed ghosts were all well labeled by galactose oxidase plus \( \mathrm{KB}^3\mathrm{H}_4 \). While intact cell membranes were less well labeled than the isolated ghosts, it will be demonstrated later that the labeling pattern of membranes in the intact cell and in isolation were qualitatively the same. In contrast, the reactivity of inside-out vesicles was invariably greatly reduced compared to an equivalent amount of unsealed or resealed ghosts, as illustrated in Table I. Here, the labeling of resealed ghosts was stimulated more than 10-fold by galactose oxidase, while less than a 2-fold increase over background was observed in the inside-out vesicles. The correlation between the relative amount of inside-out vesicle labeling (6.3% that of ghosts) and the fraction of total outer surface acteylcholinesterase accessible (6.5%) has been observed repeatedly, although such precise agreement does not always occur.

Several possible causes for the diminished labeling of inside out vesicles were considered. The presence of an inhibitor in these preparations appeared unlikely, because 1:1 mixtures of reactive ghosts and unreactive vesicles gave essentially additive labeling (Table I, Line 3). The loss of reactive carbohydrate from the membrane during vesicle preparation was ruled out by these observations. (a) Disrupting the membrane permeability barrier with Triton X 100 enhanced inside-out vesicle labeling 10-fold, to a value nearly equal to that of the ghosts (which were not stimulated by detergent) (Table I, Lines 4 and 5). (b) Inside-out vesicles are not diminished in sialic acid (16), periodic acid-Schiff-reactive glycoproteins (e.g., Fig. 6), glycolipids, or neutral sugars. (c) Vesicles prepared from membranes which had been reacted with galactose oxidase and tritiated borohydride while in the intact cell showed little or no loss of specific activity compared to the parent ghosts. Finally, the possibility that the inverted vesicles react very slowly was excluded by the demonstration that overnight incubation with galactose oxidase led to no increase in tritium incorporation (Table I, Line 6). The most likely explanation for the diminished labeling of inside-out vesicle preparations, therefore, was that few, if any, reactive sugars are localized at the cytoplasmic surface of the membrane. It remained to be established, however, whether all of the small amount of label incorporated into inside-out vesicle preparations could be attributed to contamination by exposed outer surface or whether unique minor constituents were present at the inner surface.

**Characterization of Labeled Proteins**—Fig. 6 demonstrates the electrophoretic pattern of the membrane polypeptides and glycoproteins, and the profile of tritium incorporation following treatment of membranes with galactose oxidase + \( \mathrm{KB}^3\mathrm{H}_4 \). Since
Table I
Reactivities of right side out and inside-out membranes

| Incubation time | Stimulation | Percent of No. 1 |
|-----------------|-------------|-----------------|
|                 | cpm         |                 |
| 1. Resealed ghosts | 256,537    | 100            |
| 2. Inside-out vesicles | 16,150     | 6.3            |
| 3. 1/2 resealed + 1/2 inside-out vesicles | 151,429    | 59.0           |
| 4. Resealed ghosts + Triton X-100 | 206,290    | 80.4           |
| 5. Inside-out vesicles + Triton X-100 | 150,417    | 62.1           |
| 6. Inside-out vesicles | 15,050     | 8.5            |

* Incorporation in the presence of galactose oxidase minus incorporation in its absence. Minus enzyme values ranged from 20,100 to 29,321 cpm.

** Predicted cpm = (256,537 + 16,150)/2 = 131,348 or 52.3%.

Coomassie blue does not stain the glycoproteins sufficiently (3), they are considered separately in terms of their periodic acid-Schiff profile, and no correspondence is drawn between the two scans. The only peak of label observed in the absence of galactose oxidase occurred in the lipid region, which trails just behind the tracking dye (Fig. 6, Panel A-9). Incubation with galactose oxidase brought about no alteration in the Coomassie blue or periodic acid-Schiff-staining profiles (compare Panels A-1 and A-2 with B-1 and B-2). These scans indicate that no significant proteolysis or cross-linking occurred. Galactose oxidase caused several closely spaced peaks of radioactivity in the midzone of the gel (Fig. 6, Panel B-3). The slow moving peaks (Slices 27 to 37) corresponded in relative mobility to Bands 3 and PAS-1, both of which have been demonstrated to be major glycoproteins (cf. Refs. 3 to 6). Most of the radioactivity, however, fell in a series of peaks which ran between Bands 4.2 and 5, a region of the gel where only minor components are detected by the protein and glycoprotein stains. The most intense labeling seemed to coincide with the minor glycoprotein zone, termed PAS-4 (5), and the ill-defined Coomassie blue-stained complex, designated 4.5 (Fig. 6, B-1 to B-3). It should be noted that the pattern of labeled peaks was not precisely duplicated in relative intensity, position, or number from experiment to experiment. We suspect that this variation resides in the multiplicity of overlapping component species which may vary slightly in relative mobility and thus sum differently in each experiment (3).

A peak of radioactivity running just behind the tracking dye was also created by galactose oxidase treatment; this is attributable to glycolipids, as discussed below. In addition, there is frequently a low profile of radioactivity between the gel origin and Band 3. Since the labeling pattern in this region is not consistent and does not correspond to any demonstrable glycoprotein components, we suspect that this low mobility material may represent traces of glycoproteins which have become covalently cross-linked through Schiff bases formed from galactooligosaccharides and reduced by BH4.

The identical labeling procedure was applied to inside-out vesicle preparations (Fig. 6C). The protein profile of these vesicles (Panel C-1) differs characteristically from that of ghosts, in that Bands 1, 2, and 5 are depleted. The elution of these three components is an inevitable concomitant of the vesication process (16, 18), but does not affect the results here since the missing polypeptides bear no label (vide infra). The Coomassie blue and periodic acid-Schiff staining profiles of galactose oxidase plus BH4-treated vesicles were indistinguishable from untreated controls (not shown). These vesicles, which showed a 90% accessibility of their outer surface acetylcholinesterase, incorporated 9.2% as much tritium as the equivalent amount of unsealed ghosts (shown in Fig. 6B). The radioactivity was not confined to any distinctive electrophoretic peak, but was distributed much as in the unsealed ghosts, only at one-tenth the intensity (Fig. 6, Panel C-5). It is noteworthy that the glycolipid region was no better labeled than the glycoproteins.

Additional experiments were performed to substantiate that the tritium was being incorporated only into glycosylated constituents of the outer surface of the red cell membrane.

1. We knew from previous studies (21) that all of the membrane sialic acid, neutral sugar, and glycoproteins remain membrane-bound following treatment with 0.1 N NaOH and other protein perturbers, while the nonglycosylated polypeptide species are eluted. If the membrane-bound radioactivity was in fact confined only to glycoproteins and glycolipids, it should be recovered quantitatively in the NaOH residue. We therefore performed such an extraction on ghosts labeled by galactose oxidase plus BH4 treatment. As predicted, the radioactivity released by NaOH was no greater than that recovered in the "minus enzyme" control. Gel electrophoresis confirmed that Bands 1, 2, 4.1, 4.2, 5, 6, and several minor components were quantitatively eluted by the NaOH (cf. 21), but bore no detectable radioactivity. The NaOH residue contained Bands 3, 4.5, and 7, all of the periodic acid-Schiff peaks, and the complete radioactivity profile.

2. To verify that the complex, high molecular weight labeling pattern seen in Fig. 6 represented only glycoproteins, ghosts (200 µg of protein) labeled by galactose oxidase plus BH4 treatment were incubated with 2 µg of pronase (Calbiochem) in 0.1% sodium dodecyl sulfate for 1 hour at 37°. Gel electrophoresis showed that the Coomassie blue- and periodic acid-Schiff-stained proteins and the corresponding radioactive peaks were extensively degraded, while the periodic acid-Schiff stain and tritium counts in the lipid region remained unchanged.

3. Since no distinctive gel component was labeled at the cytoplasmic surface of the membrane (Fig. 6C), it was postulated that the unilateral action of galactose oxidase on the outer surface of the membrane (i.e., on intact erythrocytes) should generate the same radioactivity profile seen with unsealed ghosts, where both surfaces are accessible. A comparison of Fig. 6B with Fig. 7 affirms this prediction.

4. Finally, we sought to determine whether the galactose oxi-
Fig. 6. Labeling of ghost and inside-out vesicle polypeptides. Packed unsealed ghosts were mixed with an equal volume of Buffer A; an inside-out vesicle preparation (with 9.9% of total acetylcholinesterase accessible) was diluted with Buffer A to the same membrane concentration in terms of total acetylcholinesterase activity. 200-μl aliquots of these suspensions were mixed with 200 μl of 100 mM sodium phosphate, pH 8.5, containing 0 or 2 i.u. of heat-treated galactose oxidase plus 20 μl of KB3H4 (48.1 X 10⁶ cpm in 0.01 N NaOH). After 1 hour at room temperature, the membranes were washed, and aliquots equivalent to 10 μl of packed ghosts were electrophoresed. The gels were stained with periodic acid-Schiff and scanned at 560 nm, stained with Coomassie blue, and scanned again at 515 nm, then sliced for counting as described under "Experimental Procedures." A, ghosts minus ghosts; B, ghosts plus galactose oxidase; C, inside-out vesicles plus galactose oxidase. 1, upper panels: scans of Coomassie blue-stained gels; 2, middle panels: scans of periodic acid-Schiff-stained gels; 3, lower panels: radioactivity profiles.

Characterization of Labeled Lipids—The pattern of BaH₄ labeling of ghost and inside-out vesicle lipid classes following galactose oxidase treatment is shown in Table II. Approximately 15% of the total radioactivity was recovered in the lipid fractions. In the absence of galactose oxidase, most of the lipid radioactivity was found in the phospholipid and cholesterol fractions, presumably as a result of the reduction of their double bonds. Incubation with galactose oxidase stimulated the labeling of glycolipids 1.5-fold in the ghosts and 5.2-fold in the inside-out vesicles. The glycolipid fraction of the inside-out vesicles was thus labeled 17% as well as in the ghosts, i.e. about at the same level as the unfraccionated sample. (While the acetylcholinesterase accessibility was initially 9.9%, it is conceivable that the overnight incubation increased the accessibility to 17%).

The apparent mild stimulation of labeling in the phospholipid fractions by galactose oxidase (Line 2) was probably caused by the presence of some contaminating substances such as glycoproteins, since only 29% of this radioactivity could be recovered in the principal phospholipid classes following thin layer chromatography (i.e. phosphatidylethanolamine, 9.8%; phosphatidylserine, 4.8%; phosphatidylcholine, 7.8%; and sphingomyelin, 11.4%). The remainder was confined to the base-line (30%) and solvent front regions (40%). In any case, the galactose oxidase labeling pattern corresponded to the membrane sialoglycoprotein or represented a set of different polypeptides. Ghosts were treated with NaIO₄ so as to oxidize only sialic acid (25), then reduced with B⁢H₄. The periodate treatment led to a 10-fold stimulation of labeling. That this incorporation of radioactivity was attributable to sialic acid was shown by the fact that 78% of tritium incorporation was abrogated by prior digestion of the membranes with sialidase and that 90% of the label was released from the ghosts by digestion with 0.1 N H₂SO₄ at 80°C for 1 hour. The pattern of radioactivity upon polyacrylamide gel electrophoresis is depicted in Fig. 8. The profile of label closely resembles that of the normal periodic acid-Schiff-staining pattern (Fig. 6, R-1 to B-3). That little radioactivity was retained in the lipid region is consistent with the fact that human erythrocyte glycolipids contain very little sialic acid (2). Similar results have been reported by Blumenfeld et al. (26). A comparison of Figs. 6B-3, 7, and 8 indicates that species of membrane glycoproteins other than the major sialoglycoproteins are labeled by the action of galactose oxidase plus B⁢H₄.

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Fig. 7. Labeling of polypeptides in the intact erythrocyte membrane: 200 μl of pelleted, washed erythrocytes were mixed with 200 μl of 0.15 M NaCl-5 mM sodium phosphate buffer (pH 7.0) containing or lacking 20 pg of galactose oxidase (General Biochemicals, Inc.). After a 2-hour incubation at room temperature, the cells were washed once in 40 ml of saline, then lysed in 40 ml of Buffer A. The pelleted membranes were resuspended in 100 μl of 100 mM sodium phosphate, pH 8.0, and 10 μl of NaB₃H₄ (2.0 × 10⁶ cpm in 0.01 N NaOH) were added. After 10 min at room temperature, the membranes were washed and an aliquot equivalent to 10 μl of packed ghosts was electrophoresed. Top, scan of the Coomassie blue-stained gel of the galactose oxidase-reacted membranes at 530 nm. (This wavelength was chosen so that the absorbance scale was comparable to that in Fig. 6.) Middle, radioactivity profile of galactose oxidase-treated sample. Bottom, radioactivity profile of minus galactose oxidase control.

The specificity of glycolipid labeling was further defined by isolating the principal ceramide fractions and analyzing their sugar, fatty acid, and sphingosine moieties (Table II). In the absence of galactose oxidase there was consistently more label in the sphingosine than in the fatty acid fractions. This feature may reflect the low degree of unsaturation in the glycosphingolipid fatty acids (2) and the presumed localization of the fatty acid double bonds deep in the membrane hydrophobic core, as compared to the more superficial location of the A⁺ unsaturation in sphingosine. This hypothesis is consistent with the aforementioned observation that sphingomyelin was the phospholipid most extensively labeled by B³H₄, despite the relative abundance of unsaturated fatty acids in the other phospholipids.

As demonstrated in Fig. 2, the labeling of the sugars in the ghost glucosyl ceramide was negligible, that in lactosyl ceramide was small but significant, whereas the tri- and tetrahexosyl ceramide fractions showed a large stimulation by galactose oxidase.

**Table II**

| Fraction                        | Ghosts | Inside-out vesicles |
|---------------------------------|--------|---------------------|
|                                 | -Galactose oxidase | +Galactose oxidase | -Galactose oxidase | +Galactose oxidase |
| **pm**                          | cpm    | cpm                 | cpm                 |
| Total sample                    | 87,000 | 763,740             | 75,840              | 220,040             |
| Phospholipids                   | 10,729 | 34,800              | 6,800               | 11,800              |
| Cholesterol                     | 1,874  | 1,949               | 1,654               | 2,229               |
| Total glycolipids               | 2,483  | 78,147              | 3,186               | 10,418              |
| Glucosyl ceramide               |        |                     |                     |
| Hexose                          | 200    | 300                 | 431                 | 117                 |
| Fatty acid                      | 97     | 136                 | 145                 | 85                  |
| Sphingosine                     | 687    | 1,141               | 864                 | 709                 |
| Lactosyl ceramide               |        |                     |                     |
| Hexose                          | 39     | 241                 | 183                 | 69                  |
| Fatty acid                      | 21     | 57                  | 40                  | 35                  |
| Sphingosine                     | 138    | 355                 | 208                 | 206                 |
| Trihexosyl ceramide             |        |                     |                     |
| Hexose                          | 109    | 3,900               | 215                 | 683                 |
| Fatty acid                      | 57     | 237                 | 50                  | 91                  |
| Sphingosine                     | 670    | 1,289               | 420                 | 606                 |
| Globoside                       |        |                     |                     |
| Hexose                          | 151    | 67,000              | 275                 | 12,557              |
| Fatty acid                      | 11     | 1,356               | 24                  | 189                 |
| Sphingosine                     | 42     | 843                 | 252                 | 269                 |
| Hematoside                      |        |                     |                     |
| Hexose                          | 47     | 1,383               | 31                  | 537                 |
| Fatty acid                      | 147    | 69                  | 6                   | 36                  |
| Sphingosine                     | 87     | 226                 | 42                  | 199                 |

Unsealed ghosts and inside-out vesicles (with 9.9% acetylcholinesterase accessibility) equivalent to 0.25 ml of packed ghosts were incubated overnight at room temperature with or without 5 i.u. of heat-treated galactose oxidase in 1 ml of 52.5 mM sodium phosphate, pH 8.5; 0.05 ml of KB₃H₄ (1.8 × 10⁸ cpm) in 0.01 N NaOH was added. After 15 min at room temperature, the membranes were washed twice and the lipids extracted and analyzed as described under "Experimental Procedures."
Because of a potentially high frequency of collisions, proteins in irreversible intra- and intermolecular linkages. We believe this is the source of the anomalous high molecular weight radioactive by preincubating this enzyme at 50°C for 30 min when necessary. We minimized this hazard by using the purer Kabi enzyme and pattern in sodium dodecyl sulfate is exquisitely sensitive (3).

Undergoes progressive degradation typical of proteolysis. That architecture, which would have destroyed the discrimination of sidedness (27, 28); we therefore kept borohydride can cleave peptide bonds (albeit under more drastic conditions) was recognized long ago (27, 28); we therefore kept the concentration of this reagent below 1 mM.

Häkämori (14). Although in some studies a significant level of labeling of lactosyl ceramide was seen after 24 and 48 hours, we elected to restrict the duration and intensity of the reaction so as to avoid perturbing the membrane permeability barrier or architecture, which would have destroyed the discrimination of sidedness. We have also observed that as BH₄ concentrations are raised above 1 mM, the gel pattern of the membrane polypeptides undergoes progressive degradation typical of proteolysis. That borohydride can cleave peptide bonds (albeit under more drastic conditions) was recognized long ago (27, 28); we therefore kept the concentration of this reagent below 1 mM.

(d) All of the various galactose oxidase preparations tested showed protease activity, to which the protein electrophoretic pattern in sodium dodecyl sulfate is exquisitely sensitive (3). We minimized this hazard by using the purer Kabi enzyme and by preincubating this enzyme at 50°C for 30 min when necessary.

(e) The galactoaldehyde enzyme product can conceivably generate Schiff bases, which can be reduced by BH₄ to yield labeled, irreversible intra- and intermolecular linkages. We believe this is the source of the anomalous high molecular weight radioactive material occasionally seen on gels (cf. Figs. 6 and 7 and Ref. 14). Because of a potentially high frequency of collisions, proteins in fluid membranes may be more susceptible to such reactions than soluble proteins. Parenthetically, it is possible that galactose oxidase could be used to advantage in covalently coupling surface glycoproteins to exogenous ligands.

(f) Anomalies were noted in the labeling reaction. First, poor recovery of radioactive sugars was achieved following acid hydrolysis. Second, the labeling of glycoproteins diminished as the time interval between the additions of enzyme and BH₄ to the membranes increased beyond 1 hour (e.g. Fig. 1). Third, a second incubation of prelabeled membranes with galactose oxidase did not release the radioactivity placed there by the initial enzyme plus BH₄ treatment.

These problems notwithstanding, the reaction of galactose oxidase plus BH₄ with human erythrocyte membranes provides support for two important hypotheses: (a) that there exists in the membrane glycoprotein species which are not well recognized by conventional protein and sugar stains; and (b) that the carbohydrate portions of both the glycoproteins and the glycolipids are, without known exception, fixed in orientation toward the external membrane surface.

Which sugar-bearing membrane components are detected by galactose oxidase + BH₄? The glycosphingolipids are clearly labeled (Fig. 2 and Table II). Since the sialoglycoproteins are known to contain galactose in terminal as well as penultimate positions (23, 24) they are susceptible to attack by galactose oxidase. The presence of radioactivity in the region of PAS-1 to 3 on gels is consistent with their being tagged, but we cannot rule out the possibility that minor co-migrating, nonsialylated glycoproteins in fact bear the label. Band 3 also contains galactosyl and in certain cases, N-acetylgalactosamnine (5). We have demonstrated that this component, purified from labeled ghosts following selective Triton X-100 solubilization (29), is, in fact, radioactive.

However, the aforementioned components do not contain all of the membrane carbohydrate and do not account for the entire profile of radioactivity. It can be calculated from Winzler’s data (1) that the sialoglycoproteins (assuming a uniform sugar composition) contribute 61% of the total membrane saccharide. Similarly, Band 3 appears to bear roughly 10% of the membrane sugar (5) and the glycosphingolipids another 7% (2). This leaves approximately 22% of the membrane carbohydrate (and an even higher proportion of the galactose plus galactosamine) associated with other glycoproteins.

One such species might be PAS-4. This small component of the glycoprotein profile coincides with the most prominent peak of tritium label (Fig. 6). A minor glycoprotein of its approximate mobility has been isolated by Tanner and Boxer (5). They equated this component with their Coomassie blue-stained Band F (4.1 and 4.2). This leaves approximately 22% of the membrane carbohydrate and an even higher proportion of the galactose plus galactosamine (5) associated with other glycoproteins.

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species could contribute to the periodic acid-Schiff-staining profile migrating ahead of PAS-4 and to the ill-defined Coomassie blue-stained zone designated 4.5. That such glycoproteins are weakly stained by periodic acid-Schiff is not surprising, considering that Band 3, containing about 10% of the membrane sugar, is so poorly stained by this technique (3, 5). The periodic acid-Schiff profile appears primarily to reflect the sialoproteins, as judged by its resemblance to the mild periodate + B3H4 labeling weakly stained by periodic acid-Schiff is not surprising, considering the thickness of the membrane remains to be established.

The unilateral, outer surface localization of sugars supports the widely held hypothesis that plasma membrane carbohydrate bear information important to intercellular interactions.

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