Cross-Linking of Glycolipids in Erythrocyte Ghost Membrane*

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SUMMARY

Human erythrocyte ghosts were chemically cross-linked with a family of bifunctional cross-linking reagents, imidates. Since the reagents are identical in their chemical structures, except for the number of methylene groups, they differ by that distance between the functional groups. The maximum distance is 4.9 Å in dimethyl malonimidate, 6.1 Å in dimethyl succinimidate, 8.6 Å in dimethyl adipimidate, and 11 Å in dimethyl suberimidate. Cross-linked membrane glycoproteins and glycolipids were solubilized in 1% sodium dodecyl sulfate and analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. After cross-linking, two prominent new bands of cross-linked products, GP-A and GP-B, appeared. Cross-linking of the new bands was dependent on the length of the reagents. GP-A could be produced by all of the reagents, but GP-B only by dimethyl adipimidate (8.6 Å) and dimethyl suberimidate (11 Å). In our previous report, GP-A was shown to be a complex of two species of the glycoproteins. In this study, it is shown that the glycolipids and proteins are components of GP-B. The conclusion is based on the following observations. (a) The GP-B formation was dependent on the distance between the functional groups of the reagents. (b) GP-B could not be produced by reacting the ghosts with methyl butyromaldilidate, a monofunctional analogue of dimethyl adipimidate. (c) GP-B could be stained by periodate-Schiff's reagent and by Coomassie brilliant blue. (d) Cleavage of the cross-links in GP-B by ammonolysis produced three protein bands and a lipid band which was positive to periodate-Schiff's reagent. (e) The cleaved protein bands could be stained by Coomassie brilliant blue and not by periodate-Schiff's reagent. (f) Synthetic aminophospholipids and trilinolein could not be stained by periodate-Schiff's reagent. (g) When ghosts were prepared from erythrocytes which had been labeled with tritium (catalyzed by galactose oxidase), GP-B also appeared to be labeled. (h) Upon cleavage of labeled GP-B, most of the label was found in the lipids and not in the proteins. (i) GP-B was not labeled when erythrocytes were labeled without galactose oxidase treatment.

The cross-links of the glycolipids and the proteins suggest possible specific association between them. The following observations substantiate the premise. (a) The glycolipids and the proteins always exhibited an identical electrophoretic mobility, regardless of the acrylamide concentration in the gel. Two different molecules having an identical electrophoretic mobility on a gel can be separated on another gel of a different acrylamide concentration. In particular, this has been successful in the case of one molecule which has a significantly higher carbohydrate content than the other. (b) Purified bovine glycolipids could be cross-linked with one another but the apparent particle size was significantly smaller than that of GP-B. (c) Diphenyl succinimidate (6.1 Å) could cross-link the glycolipids but not the proteins in the membrane. (d) The relative surface charge density of the glycolipids and the proteins in GP-B was the same, which was distinctively different from that of the cleaved proteins. Such specific associations may be a possible way to control lateral movement of the glycolipids which carry the blood group-specific antigens and are receptors for other extrinsic molecules.

Complex carbohydrates in cell membranes have been shown to be vital in determining the surface structure of plasma membranes (1-4) and there is evidence indicating their important role in cell-to-cell contact, cell cycle, adhesion, hormone interaction, and viral transformation (5-11). Among other aspects, the position of complex carbohydrates in membranes appears to be related to the cellular processes and functions (12-14).

In human erythrocyte membranes, most carbohydrates have been found to be exposed to the outer membrane surface and to be associated with glycoproteins and glycolipids (15-19). Receptor glycoproteins in the membrane have been extensively studied (2-4, 15, 17, 20). Little is known about the organization of receptor glycolipids in the membrane.

It has been demonstrated that lipids in cell membranes are in a state similar to lipid bilayer and that they diffuse rapidly in the membrane plane (21-23). If the glycolipids in the membrane are similarly mobile, the position of their carbohydrate moiety on the membrane surface may readily change at random. Considering the significance of the antigenic activities of the glycolipids (24), control over their possible lateral diffusion may be critical in determining the surface membrane structure and related cellular functions. Interactions with other membrane components may be important in determining their possible lateral movement. Chemical cross-linking of the glycolipids to adjacent molecules is one approach to studying their organization and interactions in the membrane. Recently, it was reported that aminophospholipids in the membrane could be cross-linked to unknown proteins (25).

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Previously, we reported cross-links of glycoproteins in the membrane (26–28). In this communication, I report cross-links between the glycolipids and proteins in the membrane.

EXPERIMENTAL PROCEDURES

Chemicals—Chemicals were purchased from the following companies: mononitrate, succinonitride, adiponitride, and butynitrile from Aldrich Chemical Co.; sodium dodecyl sulfate from Sigma; galactose oxidase from Worthington Biochemicals; tritiated sodium borohydride from New England Nuclear; other chemicals for gel electrophoresis from Eastman Kodak Co.; all lipids from Applied Science Laboratories Inc.

Ghost Preparation—Out-dated human blood was obtained from the Wyoming Blood Service, Cheyenne, Wyoming and kept at 5° for 5 to 10 hours. When erythrocytes were separated in the lower layer from the upper layer of leukocytes, the erythrocytes were collected carefully and ghosts were prepared as described previously (24).

Cross-Linking—Cross-linking reagents, DMM, DMS-1, DMA, DMS-2, and methyl butyromimidate were synthesized according to the method of McMclvain and Schroeder (29). The reagents were dissolved in a saline buffer containing 0.9% NaCl and 5 mM Na2HPO4, and pH was adjusted to 7.8. Erythrocyte ghosts and the reagents were mixed in the saline buffer to the concentration of glycoproteins, 0.15 mg per ml, and the reagents, 0.5 mg per ml. Cross-linking was performed for 60 min at 25° and was stopped by centrifugation of the ghosts at 12,000 × g for 15 min and resuspension of the ghosts twice in the saline buffer. Protein concentration was determined by the modified Lowry method (30).

Electrophoresis—The ghost membranes were solubilized in 1% sodium dodecyl sulfate at 25°, subjected to electrophoresis on 5.6% polyacrylamide gels, and stained either for carbohydrates with periodate-Schiff's reagent or for proteins with Coomassie brilliant blue as described previously (31). Stained gels were photographed and scanned at 550 nm on an ISCO gel scanner equipped with a Varian A-3 recorder.

Cleavage of Cross-Links—Unstained duplicate gels were sliced into pieces of about 2 mm corresponding to specific bands. The slices were ammonolyzed to cleave the cross-links by incubating them in a solution containing 35% ammonium hydroxide, 10% sodium dodecyl sulfate, and glacial acetic acid in a volume ratio of 82:10:7. An identical gel slice from another unstained duplicate gel was used as a control for the cleaving experiments. The gel slices were placed on the top of fresh gels and re-electrophoresed.

Labeling—Intact erythrocytes were labeled with tritiated sodium borohydride, following the method of Gahmberg and Hakomori (18). Erythrocytes were isolated from blood, washed once with the saline buffer, and were incubated with galactose oxidase (20 μg per ml) for 20 min at 37°. The treated cells were washed in the saline buffer and centrifuged at 7000 × g for 20 min. The packed cells were incubated with 250 μCi per ml of tritiated sodium borohydride (specific activity 700 mCi per mmol) in the saline buffer for 30 min at 25°. Labeling was stopped by mixing the cells with unlabeled sodium borohydride (250 μg per ml) and the cells were washed five times with the saline buffer. Ghosts were prepared from the labeled cells as before. After electrophoresis, gels were sliced in 3-mm pieces and treated with hyamine solution. Radioactivity was counted in a scintillation fluid containing 5% naphthalene, 0.5% 2,5-diphenyloxazole (POPO), 0.01% p-bis[2-(5-phenyloxazolyl)]benzenene (POPPOP) in a mixture of toluene, dioxane, and ethyl alcohol (5:5:3, v/v). Radioactivity was counted on a Nuclear Chicago liquid scintillation counter. The counting efficiency for tritium was 45%.

Microscopic Examination—After erythrocyte ghosts were reacted with the cross-linking reagents, they were examined without staining under a microscope equipped with Nomarski optics to see if there were any intercellular cross-links.

RESULTS

Cross-Linking—The erythrocyte membranes were reacted with a family of bifunctional cross-linking reagents, imidoesters, NH2+ NH2+ \[ \text{R'}-O-C-R-C-O-R' \]. In the imidoesters, the size of the connecting group R can be varied (29). In this study, we synthesized and used four types of the reagents, R being (CH2)n for DMM, (CH2)3 for DMS-1, (CH2)4 for DMA, and (CH2)4 for DMS-2. The maximum distance between their functional groups is 4.9 A, 6.1 A, 8.6 A, and 11 A, respectively.

After cross-linking for 60 min at 25°, the ghost membranes were solubilized in 1% sodium dodecyl sulfate, and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. The human erythrocyte membrane contains three distinctive glycoprotein bands (Fig. 1a: GP-1, GP-2, and GP-3) and a band of glycolipid (Fig. 1a, GL) (18, 32–35). As the ghost membranes were reacted with either DMM or with DMS-1, a new glycoprotein band (GP-A in Fig. 1, b and c) appeared and the intensity of two original glycoprotein bands, GP-1 and GP-2, diminished. GP-A is a cross-linked product of the glycoproteins GP-1 and GP-2 (27, 28). Changes were more dramatic when ghosts were reacted with DMA or DMS-2 (Fig. 1, d and e). Besides GP-A, at least one more band of possibly cross-linked products (GP-B) was noticeable and the intensity of the glycolipid band decreased. As the ghosts were reacted with DMS-2 for 3 hours, the original bands of glycoproteins and glycolipids disappeared along with GP-A. At the same time, the band near the top of the gel increased. The data indicate that GP-B may be an intermediate cross-linked product. However, the electrophoretogram remained the same as that of the unreacted control sample (Fig. 1a), when the ghosts were reacted with methyl butyromimidate (a monofunctional analogue of the reagents) or with inactivated reagents by keeping the reagents in aqueous solution over 24 hours (28, 36).

The data suggest that the appearance of GP-B is dependent on covalent cross-links and on the distance between the functional groups of the cross-linking reagents. The results exclude a possibility that GP-B is formed simply by the presence of the reagents. The apparent molecular weights of GP-B, GP-A, GP-1, and GP-2 are about 180,000, 130,000, 90,000, and 50,000, with respect to the known molecular weights of the erythrocyte ghost proteins (31, 33). Considering the anomalous electrophoretic behavior of the glycoproteins in sodium dodecyl sulfate-polyacrylamide gels (37, 38), these molecular weights should not be taken too seriously.

Cleavage of Cross-Links: The zone of GP-B in two unstained gels (identical with gel of Figs. 1e and 2a) was cut into three consecutive slices of about 1.5 mm. One set of the slices was ammonolyzed for 60 min to cleave the cross-links and washed in an excess amount of 1% sodium dodecyl sulfate to remove the cleaving reagents. The other set was incubated in 1% sodium dodecyl sulfate solution and used as a control for the cleaving experiment. When each of the slices was re-electrophoresed on fresh gel and stained with periodate-Schiff's reagent, the control samples (unleaved) revealed one conspicuous band which corresponded to GP-B (Fig. 2, b through d). The cleaved samples, however, produced a minor band at GP-B and a major band which corresponded to GL (Fig. 2, e through g).

When the re-electrophoresed gels were restained with Coomassie brilliant blue to visualize protein bands, the control gel slices exhibited a protein band at the GP-B position (Fig. 3, 1 The abbreviations used are: DMM, dimethyl malonimidate; DMS-1, dimethyl succinimidate; DMA, dimethyl adipimidate; DMS-2, dimethyl suberimidate.

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FIG. 1 (left). Cross-linking. Erythrocyte ghosts (0.15 mg of protein per ml) were reacted with a family of cross-linking reagents (0.5 mg per ml) for 60 min. After removing unreacted reagents, the ghosts were solubilized in 1% sodium dodecyl sulfate at 25° and subjected to electrophoresis on 5.6% polyacrylamide gels. The gels were stained with periodate-Schiff's reagent and scanned. a, uncross-linked control; b, cross-linked by DMM (4.9 Å); c, cross-linked by DMS-1 (6.1 Å); d, cross-linked by DMA (8.6 Å); e, cross-linked by DMS-2 (11 Å). Note the appearance of new bands, GP-A and GP-B, after cross-linking.

FIG. 2 (center). Cleavage of the cross-linked products. All gels were stained for carbohydrates. a, identical with the gel of Fig. 1a. The zone of GP-B in two duplicate unstained gels was cut in three consecutive slices, A, B, and C for cleaving experiment. Note the absorbance scale is different from the rest in the figure. b through d, a set of gel slices, A, B, and C, was re-electrophoresed on fresh gels without ammonolysis (uncleaved). b through d, another set of gel slices, A, B, and C, was ammonolyzed to cleave cross-links and re-electrophoresed (cleaved). Note the appearance of the glycolipid band in the cleaved samples. The band intensity of the cleaved samples is higher than that of the uncleaved control samples.

FIG. 3 (right). Protein staining of the gels in the cleaving experiments. The gels in Fig. 2 (b through g) were stained in a second time with Coomassie brilliant blue to visualize protein bands. a, uncleaved control sample which was stained only with Coomassie brilliant blue. This gel corresponds to the gel stained with periodate-Schiff's reagent in Fig. 1a. b through d, uncleaved samples identical with gels in Fig. 2 (b through d). e through g, cleaved samples identical with gels in Fig. 2 (e through g). The uncleaved controls exhibited one protein band at the GP-B position. The cleaved samples exhibited three protein bands and the lipid band, in addition to the protein band at the GP-B position.

The carbohydrate stain, periodate-Schiff's reagent, has been used to detect aldehyde. Periodate oxidizes carbohydrates to produce aldehydes. Basic fuchsin in the Schiff's reagent reacts with aldehydes to visualize proteins. a, uncleaved control sample which was stained only with Coomassie brilliant blue. This gel corresponds to the gel stained with periodate-Schiff's reagent in Fig. 1a. b through d, uncleaved samples identical with gels in Fig. 2 (b through d). e through g, cleaved samples identical with gels in Fig. 2 (e through g). The uncleaved controls exhibited one protein band at the GP-B position. The cleaved samples exhibited three protein bands and the lipid band, in addition to the protein band at the GP-B position.

Identity of Lipids—Since the lipids cleaved from GP-B were stained with periodate-Schiff's reagent, the lipids are expected to be glycolipids. However, there are several points to be cautious about in the interpretation.
and was approximately proportional to the amount of the sample applied. Such a band was also present on gels of other lipids which could not be stained. The result suggests that the staining of the lipid band by periodate-Schiff's reagent is not due to the presence of phospholipids or unsaturated lipids.

The conclusion is in accord with other data. First, when the gels were not treated in a periodate solution during the staining process, the lipids which were cleaved from GP-B were not stained significantly. Second, the free amino group in the aminophospholipids is the only group capable of reacting with the cross-linking reagents. Since the aminophospholipids contain only one free amino group per molecule, cross-linked aminophospholipids do not have any free amino groups which may react with the Schiff's reagent. The results suggest that the lipids stained with periodate-Schiff's reagent in GP-B are not the aminophospholipids. However, the possible involvement of the aminophospholipids in the GP-B formation cannot be ruled out.

The lipid band stained by periodate-Schiff's reagent and Coomassie brilliant blue is almost twice as large as that stained only by periodate-Schiff's reagent (Fig. 3). Is the increase in the band intensity after restaining with Coomassie brilliant blue due to phospholipids? A type of glycolipid, purified bovine cerebrosides could not be stained significantly by Coomassie brilliant blue after gel electrophoresis (Fig. 4). The glycolipids, however, were stained by periodate-Schiff's reagent. Furthermore, the periodate-Schiff-stained glycolipids were stained extensively by Coomassie brilliant blue. The band intensity was nearly twice that stained only by periodate-Schiff's reagent. It is concluded that the staining of the lipid band cleaved from GP-B by periodate-Schiff's reagent and Coomassie brilliant blue is mainly due to the glycolipids.

Surface Labeling of Glycolipids—It has been reported that glycolipids, which could not be stained in the presence of phospholipids or unsaturated lipids.

**Table I**

**Staining of lipids in gels**

<table>
<thead>
<tr>
<th>Stain</th>
<th>PC</th>
<th>PE</th>
<th>TP</th>
<th>TL</th>
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<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
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<td>+</td>
<td>-</td>
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**Table II**

**Electrophoresis of synthetic phosphatidylcholine**

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<th>50</th>
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<tr>
<td>Relative band area</td>
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<td>2.2</td>
<td>4.1</td>
<td>8.5</td>
</tr>
</tbody>
</table>

**Fig. 4. Staining of glycolipids.** Purified bovine cerebrosides were solubilized in 1% sodium dodecyl sulfate (1 mg of the lipids in 1.5 ml). The sample was subjected to electrophoresis on three gels (30 µg of the lipids per gel). One gel was stained only with Coomassie brilliant blue (CBB) and another gel was stained only with periodate-Schiff's reagent (PAS). The third gel was first stained with periodate-Schiff's reagent and then restained with Coomassie brilliant blue. The band area of the gel scans was cut from chart paper and weighed. The number represents the relative band area. Arrow indicates the dye front.

**Fig. 5. Tritium labeling of intact erythrocytes.** a, intact erythrocytes were treated with galactose oxidase, labeled with tritiated sodium borohydride, and ghosts were prepared. After solubilization the sample was subjected to electrophoresis and the gel was sliced in pieces of about 3 mm. Radioactivity of each slice was counted. The labels are supposed to be specific for galactose and galactosamine (18). b, the same as c except that the labeled ghosts were reacted with DMS-2. c, intact erythrocytes were labeled without the enzyme treatment and ghosts were prepared. The labels are nonspecific, compared with the labels in a and b. d, the same as c, except that the labeled ghosts were reacted with DMS-2.

galactose and galactosamine of intact erythrocytes can be labeled specifically by tritiated sodium borohydride after the treatment with galactose oxidase (18, 19). Ghosts were prepared from the labeled erythrocytes, solubilized in 1% sodium dodecyl sulfate, and subjected to electrophoresis (Fig. 5). When the labeled ghosts were reacted with DMS-2, the label in glycoproteins and
As the ghosts were reacted with DMS-2, the nonspecific labels of the lipids decreased and the labels near the top of the gels not apparent. Increased (Fig. 5, c and d). Label at the GP-B position was near the top of the gel. Also, there is an indication of label in glycolipids decreased and some of the labeled materials appeared cleaved (Fig. 6b). Phospholipids are only nonspecifically labeled and their sample (uncleaved) showed labels in the GP-B position (Fig. 5a). The zone of GP-B in two unstained duplicates of the gel of Fig. 5b (the membranes treated with galactose oxidase, labeled, and reacted with DMS-2) was cut into a 3-mm slice. One slice was ammonolyzed and the other was incubated in 1% sodium dodecyl sulfate. On the re-electrophoresis, the cleaved samples exhibited labels at the lipid zone (Fig. 6a), whereas the control sample (unammonolyzed) showed labels in the GP-B position (Fig. 6b). Phospholipids are only nonspecifically labeled and their label is insignificant compared with specific labels in glycolipids (19). The data demonstrate that the labels of the lipids cleaved from GP-B are specific labels. It is concluded that glycolipids are present in GP-B. Also, the data substantiate the conclusion that the three protein bands cleaved from GP-B are not the glycoprotein bands.

It has been reported that the globosides and ceramide trihexosides, among the membrane glycolipids, are mainly labeled by the method (18, 19). The glycolipids carry free hydroxyl groups in their carbohydrate moiety. It is possible that a part of the hydroxyl groups could have reacted with the cross-linking reagents, although the reagents primarily react with free amino groups. The premise is consistent with the results of the cleaving experiment. In Fig. 2, the cleaved lipids of GP-B showed more staining than the uncleaved control samples of GP-B. How could a cleaved product be stained more intensely than the original material? If a part of the free hydroxyl groups in the carbohydrates of the glycolipids are reacted with the reagents, periodate can not oxidize them. Consequently, some of the carbohydrates do not react with the dye (basic fuchsin) and fail to develop the color. On the ammonolysis of the cross-links, the hydroxyl groups become free and available for the staining reaction to increase the band intensity. Alternatively, the reagents may react with free amino groups of the glycolipids which may be present in a small quantity but are not detected by ordinary methods. Another possibility is that the carbohydrates of the glycolipids in GP-B may not be available for the staining reaction, possibly because of steric factors. However, these explanations are tentative. Currently, we are investigating the possibility of such links between the reagents and the potential reactive groups of the carbohydrates.

Cross-Links between Glycolipids and Proteins—The staining behavior and the position of the protein bands which were cleaved from GP-B indicate that they are not the membrane glycoproteins and probably are proteins. Then it is obvious that periodate staining of GP-B is due to the cross-linked glycolipids and Coomassie brilliant blue staining is mostly due to the cross-linked proteins (Figs. 3 and 4). Several tests were then performed to determine whether the glycolipids and proteins are cross-linked to form a complex in GP-B, or whether they form different species of complexes which incidentally show an identical electrophoretic mobility.

Purified cerebroside were cross-linked by DMS-2 (Fig. 7). The electrophoretic mobility of the complex was a little slower than that of the control cerebroside but was significantly faster than that of GP-B.

Two different molecules of an identical electrophoretic mobility on a gel can be separated on another gel of a different acrylamide concentration. This has been particularly successful when one of the molecules has a significantly higher carbohydrate content than the other (40, 41). The zone of GP-B was cut from two identical unstained gels and was re-electrophoresed on fresh gels of different acrylamide concentration. The gels were cut in halves longitudinally. One set was stained with Coomassie brilliant blue and the other was stained with periodate-Schiff's reagent. The protein band and the glycoprotein band appeared at identical positions on the different types of the gels (Fig. 8).
4.5y0, 5.6%, 6.5y0, an67.5%. The RF values were determined by indicating little or no disruption or intercellular cross-links.

A trackin; dye, pyronin Y. A--A, membrane glycolipids and proteins which migrates close to the protein bands cleaved from comnarina the electronhoretic mobilitv of each band with that of bovine cerebrosides (stained with periodate-Schiff's reagent); 7846 that the proteins and glycoproteins are cross-linked together to GP-B are significantly different from the original materials as data indicate that the proteins and glycolipids cross-linked to GP-B in the third group is the proteins cleaved from GP-B and band V. This is identical relative surface charge density. Two major glyco-

DMS-2 (stained with periodate-Schiff's reagent). A--A, complex of cross-linked cerebrosides by DMS-2 (stained with periodate-Schiff's reagent); □—□, band V of membrane proteins which migrates close to the protein bands cleaved from GP-B (stained with Coomassie brilliant blue); □—□, the first of the protein bands cleaved from GP-B (stained with Coomassie brilliant blue). The gel slice of GP-B zone was re-electrophoresed. The gel was cut in halves longitudinally; one-half was stained with Coomassie brilliant blue (○—○), and the other half with periodate-Schiff's reagent (O—O).

Use of Ferguson plots (41, 42) is another way to distinguish two different materials which show an identical electrophoretic mobility on a gel. The plots can reveal the relative surface charge density of molecules solubilized by sodium dodecyl sulfate. When the $R_f$ value of a molecule is plotted against the acrylamide concentration, the extrapolated mobility at 0% acrylamide is primarily dependent on the surface charge density of the molecules. Most protein species in the membrane show an identical relative surface charge density. Two major glycoprotein species, GP-1 and GP-2, also have a same relative surface charge density which is different from that of the proteins. The relative surface charge density of proteins and glycoproteins in the membrane is not affected by their chemical cross-links as long as the cross-links occur between homologous species (28, 43, 44). In Fig. 8, several molecules show three different relative surface charge densities. The first group consists of the proteins and the glycolipids cross-linked to GP-B. This is similar to that of GP-A, GP-1, and GP-2 (28). The second group composed of the proteins cleaved from GP B and band V. This is identical with that of most membrane proteins (41). The third group is the glycolipids and the glycolipids cross-linked by DMS-2. The data indicate that the proteins and glycolipids cross-linked to GP-B are significantly different from the original materials as well as from the homologously cross-linked complex, suggesting that the proteins and glycoproteins are cross-linked together to form a complex, GP-B.

No Intercellular Cross-Links—After cross-linking, the erythrocyte ghosts were washed twice and diluted in the buffer. When the ghosts were examined without staining under a microscope equipped with Nomarski optics, individual ghosts were seen, indicating little or no disruption or intercellular cross-links. Therefore, the components of GP-B are not likely to be cross-linked between the ghosts.

**DISCUSSION**

The results in this study show the cross-links between lipids and several species of proteins in the erythrocyte ghost mem-

brane. Some of the lipids are glycolipids. The cross-linked complex, GP-B, has been produced under various conditions: a pH range of 6 to 9, different concentrations of the cross-linking reagents, and a wide range of the ionic strength of the solvents. The present data suggest a possibility that the association between the glycolipids and proteins in GP-B is specific and stable. GP-B appears to be an intermediate of cross-linked products and the glycolipids of GP-B are a minor fraction of total membrane glycolipids.

One may argue that the reactions between the reagents and the reactive groups of the GP-B components may have changed the nature of the associations, suggesting that GP-B is an artifact of the cross-linking reactions. This study alone cannot exclude a possible distortion of the associations in GP-B. However, previous cross-linking studies of the erythrocyte membranes and other proteins indicate that the reactions are mild and preserve some of the original properties of the molecules, such as immunological activity, enzymatic activity, and surface charge density (36, 45, 46). GP-A (the cross-linked product of GP-1 and GP-2 by DMM, DMS-1, and DMA) has the same surface charge density as that of the original glycoproteins, GP-1 and GP-2 (28).

Since the distance between the two functional groups of DMS-1 and DMA is 6.1 A and 8.6 A, respectively, it is possible that the reactive groups of the components of GP-B are separated from one another by a distance between 6.1 A and 8.6 A, at least in the cross-linked complex (GP-B) and possibly in vivo. If the carbohydrate moiety of the glycolipids is cross-linked to the proteins, the cross-links between them may span from the outer membrane surface to the hydrophobic domain of the membrane. The possibility is advanced on the basis of the facts that the carbohydrates of the glycolipids are exposed to the outer membrane surface and that only one species of membrane proteins has been proven to be exposed to the outer membrane surface (47-50). The proteins cleaved from GP-B are not the exposed protein. Then it is possible that the glycolipids may have been associated with the proteins much closer than 8.6 A. Since the imidoesters decompose instead of polymerizing by themselves, unlike some other bifunctional cross-linking reagents like glutaraldehyde, the estimate of the distance between the reactive groups in the cross-linked complex in this study should be more accurate than that by using the other reagents.

Since Frye and Edidin (1) have found that cell surface antigens move rapidly in the membrane plane, there have been numerous reports on lateral movements of surface receptors in membranes and on their role in a variety of cellular functions and processes (9, 12, 13, 51, 52). In the human erythrocyte membrane, most carbohydrates have been found to be exposed to the outer membrane surface and to be associated with glycoproteins and glycolipids. Some of the receptor molecules have been demonstrated to change their position in the membrane under certain conditions, suggesting that there is a control on the change of the receptor position and the lateral movement. It has been suggested that some receptors contain polypeptides (2, 3, 53). The lateral movement of glycoproteins and proteins can be easily manipulated by their associations with other membrane proteins. For example, the major glycoproteins (GP-1 and GP-2) in the erythrocyte membrane span across the membrane (37, 54). They could readily interact with other proteins within the membrane as well as on the membrane surfaces. In fact, there is an indication of such interactions (26, 55).

We have evidence suggesting that there is a linkage between lectin receptors and spectrin (55), which is exclusively located on
the inner membrane surface (56). Nicolson and his colleagues (56) have found that aggregation of spectrin by anti spectrin immunoglobulins at the inner surface perturbs the topography of anionic binding sites at the outer surface of the ghost membrane (57, 58). These are consistent with the findings that the lateral movement of the receptors in the membrane can be prevented by chemical cross-linking of proteins with glutaraldehyde (2, 3, 59).

However, little is known about the distribution and mobility of glycolipids in the membrane. It has been demonstrated that membrane lipids can rapidly diffuse in random motions (21-23). Whether glycolipids are similarly mobile is unknown. With respect to the immunological activity of the glycolipids (24), movement of the receptors in the membrane can be prevented by chemical cross-linking of proteins with glutaraldehyde (2, 3, 59).

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Cross-Linking of Glycolipids in Erythrocyte Ghost Membrane
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