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Functional incorporation of synthetic glycolipids into cells

(concanavalin A/agglutination/erythrocytes/liposome exchange/hydrophilic spacer arm)

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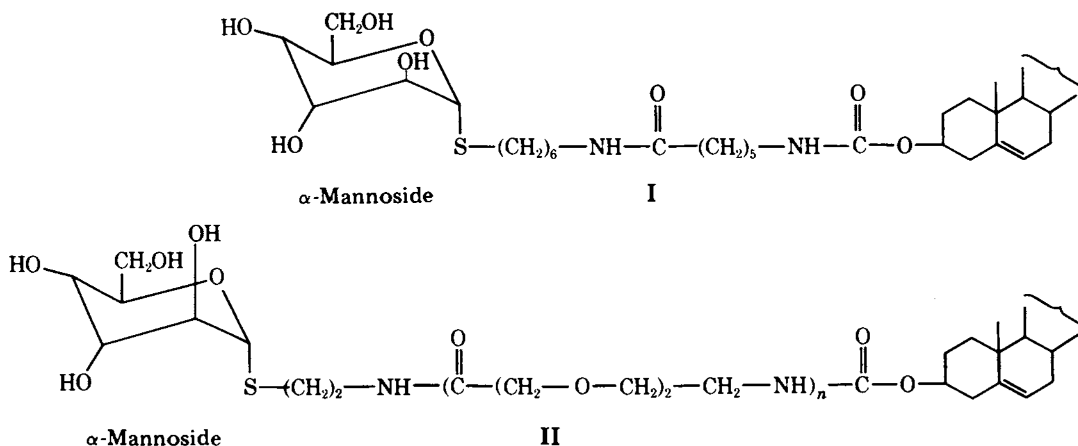
ABSTRACT Synthetic glycolipids containing an α-mannoside group linked by a hydrophilic spacer arm to cholesterol were incorporated into bovine erythrocytes by exchange from glycolipid-containing liposomes. When the distance between the sugar and the cholesterol moieties was approximately 26 Å, functional incorporation of these glycolipids could be easily detected, as revealed by the concanavalin A-mediated agglutination of these cells. Bovine erythrocytes are not themselves susceptible to concanavalin A-mediated agglutination. The minimal concentration of concanavalin A required for agglutination of modified erythrocytes, containing 9.15×10^6 glycolipid molecules per cell, was 4 μg/ml. Under these conditions, only approximately 4% of the membrane-bound cholesterol had been exchanged for the synthetic glycolipid. The observed aggregation was reversible in the presence of α-methyl mannoside and did not occur when β-galactosyl-containing glycolipids were used in place of their α-mannoside isomers. These studies demonstrate a technique of sugar incorporation into cell membranes which should be of great advantage in studies on the roles of cell surface sugars in biological recognition. Furthermore, they demonstrate that the sugars need only be a short distance (26 Å) from the membrane in order to functionally bind concanavalin A.

Cell surface sugars have been implicated in various cellular recognition phenomena. On the surface of mammalian cells they function as virus, bacterial, and toxin receptors and are implicated as mediators in cell–cell adhesion (1). An approach to understanding the roles that cell surface sugars play in these phenomena involves the incorporation of chemically defined sugars onto the cell surface. The reconstitution or modulation of a recognition response as a consequence of the incorporation of a specific sugar or sequence of sugars can be taken as evidence for the functional role(s) of these sugars in the response. A crucial step here is to devise specific ways to introduce sugars onto the cellular membrane. A chemical method has been formulated which involves the periodate oxidation of the sialic acid side chain of membrane-bound glycoproteins, followed by the condensation of sugar-containing acyl hydrazides with the liberated aldehyde groups (2, 3). This method can be less than optimal because of the required chemical alteration of the membrane and the fact that the sugars are introduced in a random, nonuniform array. A second possible method that could potentially circumvent these difficulties, and hence potentially generate more information, is one involving the noncovalent incorporation of amphipathic sugar-containing compounds into the cell membrane. Synthetic glycolipids containing cholesterol as an anchor, linked by a spacer group to a sugar (structure **I**), can be incorporated into small unilamellar liposomes, and these derivatized liposomes are rendered susceptible to aggregation by the appropriate lectins (4). The cholesterol-containing synthetic glycolipids distribute evenly on both sides of the bilayer and exhibit a condensing effect above the phase transition of both lipids (5). By these and other criteria the cholesterol analogs appear to be bound in the membrane much like cholesterol itself, with the polar oxygen being near the phosphate head group (6).

Synthetic glycolipids analogous to structure **I** cannot immediately be applied to cells because of the hydrophobic nature of the spacer groups. It could be anticipated that due to the cell surface glycoproteins, relatively large distances between the sugar residue and cholesterol anchor would have to be achieved before the sugar could functionally interact with a binding protein. Simply polymerizing hydrophobic spacers of the aminocaproyl type would not be expected to yield fruitful results. The hydrophobic chains would either "ball up" and interact with themselves in the aqueous environment or they would dissolve in the lipid bilayer. Neither situation would be fruitful for functional incorporation studies because the sugar residue would remain sequestered too close to the membrane to interact with binding proteins. In order to circumvent this problem we have developed a hydrophilic spacer group that combines the water solubility of ethers with the ease of polymerization of amino acids. In this communication we show that synthetic glycolipids containing 3,6-dioxa-8-aminooctanoic acid-based spacer groups can be functionally incorporated into cells.



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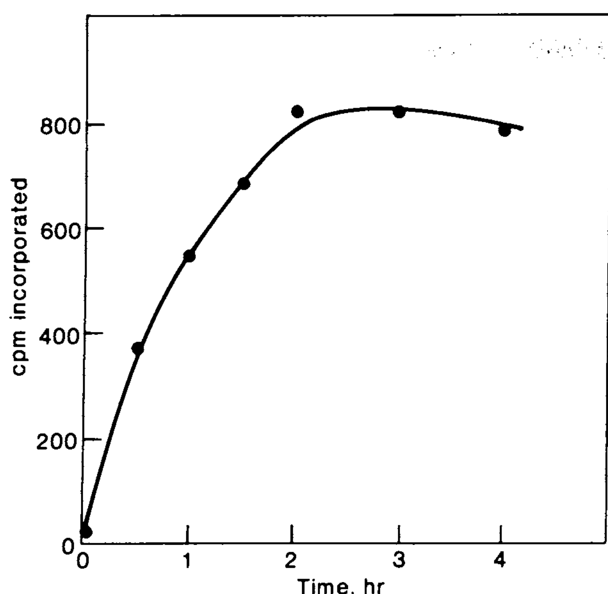

FIG. 1. Incorporation of glycolipids into bovine erythrocytes. Liposomes were prepared containing 5 mol % [6-^{3}H]galactosyl-containing glycolipid (M_r = 796), β-[6-^{3}H]Gal-S-$(CH_2)_2$-NH-CO-$(CH_2$-$OCH_2)_2$-CH_2-NH-CO_2Chol (specific activity, 2.35 × 10^5 cpm/μmol). The stock solution of the liposomes was 10 μmol of phospholipid per ml as determined by phosphate assay. To test tubes containing 6.9 × 10^8 cells in 1 ml of phosphate-buffered saline was added 40 μl (4770 cpm) of the transparent liposomal preparation. The erythrocytes were incubated for various periods of time at 37°C with shaking in a Dubinoff shaking bath. At the indicated times the cells were centrifuged at 3000 × *g* and washed three times with 10 ml of phosphate-buffered saline. The final pellet was lysed in 10 ml of 10 mM potassium phosphate (pH 7.5). The membrane pellet was centrifuged at 27,000 × *g* for 10 min and washed twice with 10 ml of the phosphate buffer. The final pellet was dissolved in Aquasol (New England Nuclear) and radioactivity was measured.

Specifically, we show that bovine erythrocytes, which cannot be agglutinated by the α-mannosyl binding lectin concanavalin A (Con A), can be rendered so as a consequence of the incorporation of synthetic glycolipids of the type shown in structure **II**. Furthermore, we show that the distance requirement from the cholesterol anchor to the sugar in order for Con A-mediated aggregation to occur is relatively modest (n = 2).

MATERIALS AND METHODS

Con A (three times recrystallized) was purchased from Miles. Solutions of Con A in 50 mM Tris·HCl/140 mM $CaCl_2$ were centrifuged at 5000 × *g* for 5 min before use. The concentrations of Con A solutions were determined by absorbance readings at 280 nm ($A^{1\ mg/ml}_{1\ cm}$ = 1.3). Egg phosphatidylcholine was prepared and purified by the method of Litman (7). The phospholipid concentrations were determined as inorganic phosphate after ashing and acid hydrolysis (8). The phospholipid was dissolved in benzene at a concentration of 40 μmol/ml and stored at −70°C under an atmosphere of nitrogen. The purity of the preparation was checked routinely by thin-layer chromatography (silicic acid; $CHCl_3$/CH_3OH/H_2O, 65:25:4). The glycolipids were prepared by a procedure similar to that already published (4, 5). The main differences were that the thiosugar was alkylated with *N*-trifluoroacetamido iodoethane instead of with *N*-trifluoroacetamido iodohexane and the coupling reaction was done with *N*-trifluoro-3,6-dioxaoctanoic acid instead of with *N*-trifluoroacetyl aminocaproic acid. These changes eliminated the hydrophobicity of the earlier spacer groups. All of the compounds used here gave satisfactory glycolipids and the hydrophilic spacer groups will be published in detail elsewhere. The [6-^{3}H]galactosyl-containing glycolipid used here was synthesized by treating the galactosyl-containing glycolipid with galactose oxidase followed by NaB^3H_4 reduction according to a published procedure (5).

The concentrations of the sugar cholesterol compounds were determined by enzymatic assay of the free mannose after mercuric ion-catalyzed hydrolysis of the 1-thioglycoside linkage (9). To a solution of the cholesterol-mannose derivative (0.1–2 μmol) in ethanol (1 ml) was added 0.2 M mercuric acetate in 0.1 M acetic acid (0.1 ml). After 2 hr at 60°C, the incubation mixture was treated with 2-mercaptoethanol (10 μl) and the solvent was removed under reduced pressure. The residue was suspended in H_2O (0.5 ml), shaken vigorously, and centrifuged in a clinical centrifuge. The liberated mannose was assayed by coupling the ADP produced by the hexokinase/ATP-catalyzed phosphorylation of the hexose to the enzyme system of pyruvate kinase/phospho*enol*pyruvate and lactate dehydrogenase/NADH (4).

The liposomes containing the synthetic glycolipids were prepared as described (4, 10). After sonication, the glycolipid-bearing liposomes were centrifuged at 12,000 × *g* to remove insoluble material. The clear supernatant containing the unilamellar and multilamellar liposomes was then used in the exchange reactions with bovine erythrocytes. The erythrocytes were collected in heparin/phosphate-buffered saline (pH 7.4) from a local slaughterhouse on the day that they were to be used. The erythrocytes were repeatedly washed with ice-cold phosphate-buffered saline (pH 7.4) and centrifuged (2000 × *g*) three or more times. The buffy coat was removed by aspiration. The cells were continuously washed until no more white layer appeared. The cells were then incubated with the glycolipid-containing liposomes at 37°C for varying periods of time. The cells were centrifuged at 5000 × *g* and washed three times with phosphate-buffered saline. These cells were then used in the aggregation studies.

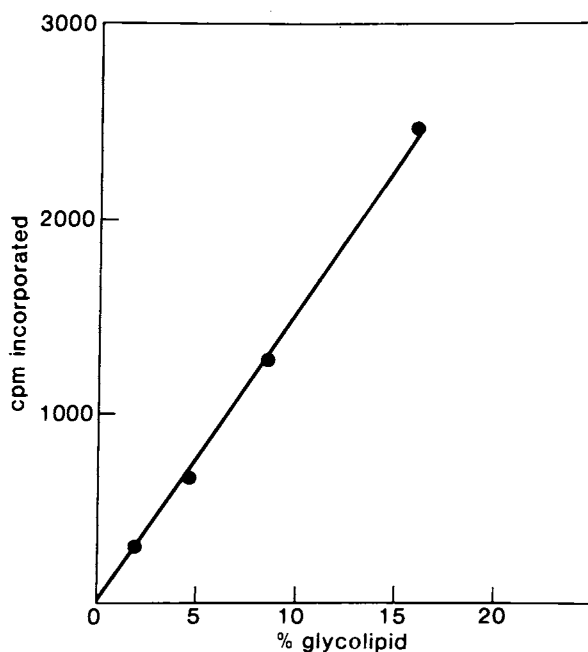

FIG. 2. Incorporation of glycolipids at varying concentrations into bovine erythrocytes. Liposomes were prepared containing 2, 5, 8, and 15% of the [6-^{3}H]galactosyl-containing glycolipid. The concentration of phospholipid was adjusted to 10 μmol/ml in each case.

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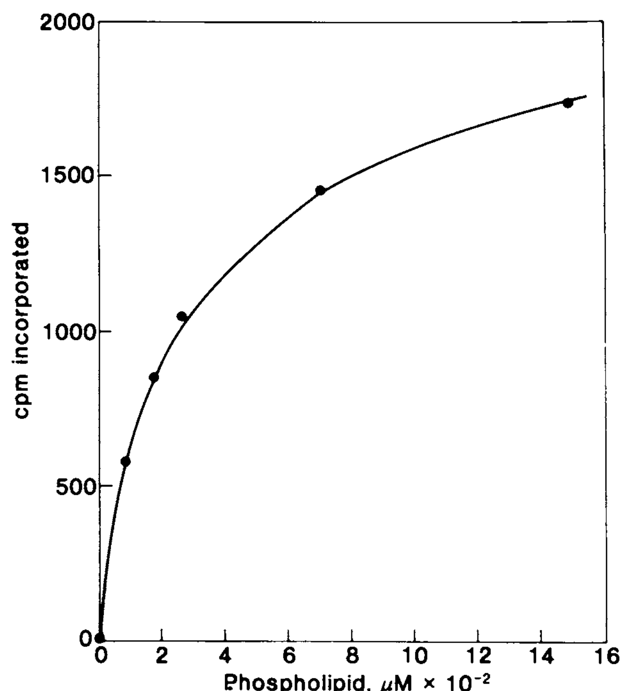

FIG. 3. Incorporation of glycolipids at varying liposome concentrations into bovine erythrocytes. Liposomes containing 10% of the [^{3}H]galactosyl-containing glycolipid at a final phospholipid concentration of 10 μmol/ml were made. To 1 ml of 7 × 10^8 cells in phosphate-buffered saline was added 10, 20, 40, 80, and 160 μl of the liposomal preparation. The total volumes were kept constant by addition of the appropriate amount of buffer to each sample. The cells were incubated at 37°C with shaking for 2 hr and then washed and lysed. Radioactivity was then measured.

RESULTS AND DISCUSSION

The synthetic glycolipids were incorporated into the bovine erythrocytes by a liposome-exchange technique used for cholesterol exchange (11). Briefly, egg yolk phosphatidylcholine-based liposomes were prepared containing the synthetic glycolipids. Simply incubating these liposomes with bovine erythrocytes allows an exchange process to occur between the synthetic glycolipids in the liposomes and the cholesterol in the erythrocytes. In Fig. 1 a time course for the incorporation of radioactive glycolipids into erythrocytes is shown under defined conditions. After about 1–2 hr, the exchange appeared to reach equilibrium. Under these conditions, the erythrocytes contained 3.05 × 10^6 synthetic glycolipid molecules per cell. Given the average surface area of a bovine erythrocyte to be 10^9 μm^2, the average density of sites can be calculated to be 2.79 × 10^4 sites/μm^2, assuming that the synthetic glycolipids do not transverse the bilayer. The glycolipids used here cannot be incorporated into the erythrocytes by direct addition. Incubation of the erythrocytes with a dispersion of glycolipid gives approximately 5% of the amount bound to cells relative to the exchange technique. Under the conditions of the liposomal exchange technique, 17.2% of the total glycolipid incorporated in the liposomes exchanges with the erythrocytes.

If a one-to-one exchange process is assumed, only approximately 1–2% of the total cholesterol in the bovine erythrocyte has been exchanged with the synthetic glycolipids. In Fig. 2, the relationship between the percentage of glycolipid in the liposomes and the amount incorporated in the erythrocytes is shown. The total phospholipid concentration and time of incubation (2 hr) were kept constant in these experiments. In Fig. 3 the relationship between the amount of phospholipid added shown. Rough linearity was observed in both cases at low phospholipid concentrations.

The α-D-mannosyl-containing synthetic glycolipids of the type shown in Structure II were prepared, incorporated into liposomes, and exchanged into the bovine erythrocytes. The modified erythrocytes were then treated with varying concentrations of Con A and the extent of agglutination was assessed with microtiter plates (2). The results of these experiments are shown in Table 1. These experiments show that functional incorporation of the synthetic glycolipids into the bovine erythrocytes was achieved. The effect was specific for the α-mannosyl residue because β-galactosyl-containing glycolipids were not agglutinable and the agglutination response in the presence of II was specifically blocked by added α-mannosides but not by β-galactosides. In addition, cells treated with pure phosphatidylcholine-based liposomes were also not rendered agglutinable by Con A. Furthermore, the incorporation was reversible. Bovine erythrocytes containing II (n = 2 or 4) were rendered nonagglutinable by Con A by simply incubating them with cholesterol-containing liposomes. The synthetic glycolipids exchanged out of the cells as the cholesterol exchanged in. By microscopic reversibility, then, the synthetic glycolipid must have been incorporated into the erythrocyte by an exchange process rather than by another process such as fusion. The synthetic glycolipids are, however, bound in the erythrocyte membrane in a stable form in the absence of exchange-mediating liposomes. It is of interest to compare the effects of the spacer group length on the susceptibility of the modified erythrocytes to aggregate. When n = 0 or 1 (II), aggregation either did not proceed or it proceeded poorly, requiring relatively large amounts of Con A. When n = 2 or 4 (II), aggregation proceeded rapidly at 4 μg of Con A per ml. The distance from the sulfur group to the carbamate carboxyl group is approximately 16 Å in II (n = 1) and 26 Å in II (n = 2). We have shown that within experimental error glycolipids with one or two spacer groups are incorporated to the

Table 1. Lowest lectin concentration that causes agglutination of native and modified bovine erythrocytes

Glycolipid	Concentration, %	α-Methyl mannoside, mM	Con A, μg ml^{-1}*
	—†		>500
I	10	—	>500
II (n = 0)	10	—	>500
II (monomeric spacer, n = 1)	5	—	>500
II (monomeric spacer, n = 1)	15	—	50
II (dimeric spacer, n = 2)	5	—	4
II (dimeric spacer, n = 2)	5	40	>500
II (tetrameric spacer, n = 4)	5	—	4
II (tetrameric spacer, n = 4)	5	40	>500
III (β-Gal-dimeric spacer, n = 2)	10	—	>500

To 1 ml of washed erythrocytes in phosphate-buffered saline (5 × 10^8 cells) was added 0.1 ml of liposome (10 μmol/phospholipid molecule per ml of phosphate-buffered saline) containing the synthetic glycolipid at the indicated concentrations. The samples were incubated with shaking at 37°C for 2 hr; the cells were then thoroughly washed and resuspended in 1 ml of phosphate-buffered saline. Fifty microliters of the prepared cells was placed in microtiter wells along with 50 μl of various concentrations of Con A in phosphate-buffered saline. The cells were allowed to remain at room temperature for 2 hr and then agglutination was scored. In the absence of liposomes, no agglutination occurred. In the presence of α-methyl mannoside at the indicated concentration, agglutination did not occur. When agglutination was positive by this assay, the results were checked by microscopic examination. In all instances, the results from both determi-

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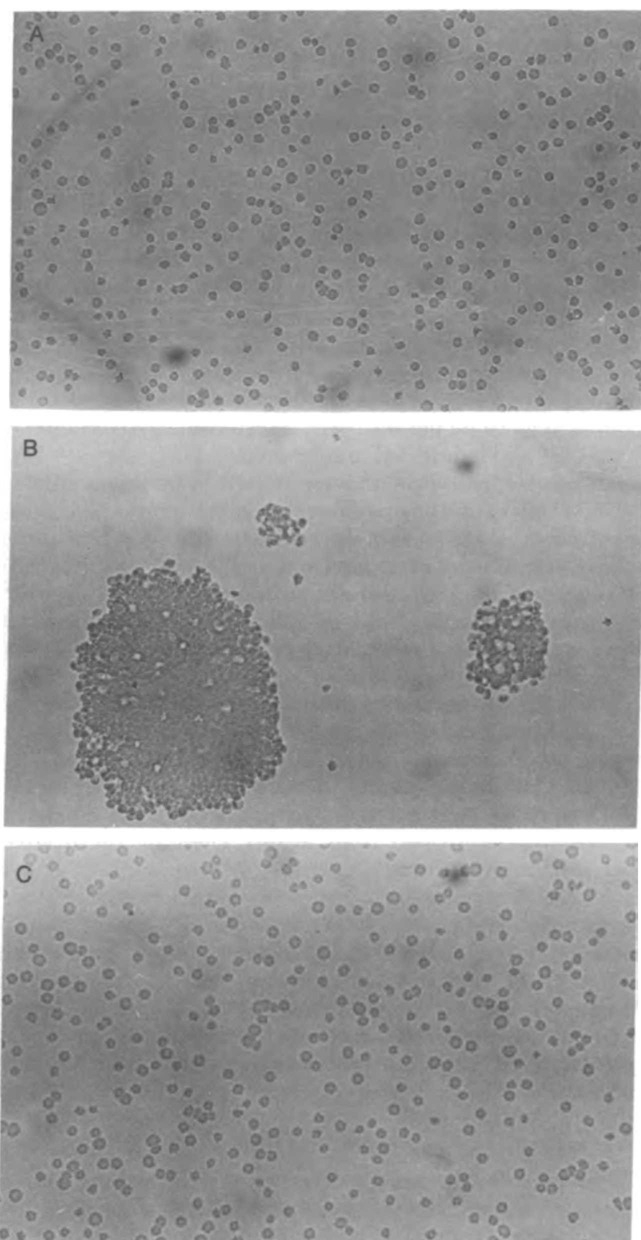

FIG. 4. Aggregating cells: 1 ml of bovine erythrocytes (5×10^8 cells) in phosphate-buffered saline was modified with liposomes containing 5% II ($n = 2$) as in Table 1. Aliquots (0.1 ml) of the cells were placed in three test tubes: (*A*) control; (*B*) Con A (50 μg/ml) was added; (*C*) 40 mM α-methyl mannoside was added followed by Con A (50 μg/ml). The cells were incubated for 10 min at room temperature; aliquots were removed and examined by light microscopy with bright-field objectives at a magnification of ×125. When α-methyl mannoside was added to agglutinated cells, the aggregates were dispersed (not shown).

same extent into the erythrocyte. Therefore, the differences observed here cannot be due to differential incorporation of the glycolipid. Further increases beyond 26 Å do not appear to enhance the aggregation response. When the hydrophobic spacer groups were used (I), no aggregation of the cells occurred in the presence of Con A, supporting our contention that hydrophilic spacer groups are required. Under conditions where cells modified with II ($n = 2$) were aggregated with 4 μg of Con A per ml, 9.15×10^6 sites per cell were introduced and the average density of sites equaled 8.4×10^4 sites per μm^2. Incorporated into the bovine erythrocytes by the periodate/α-mannosyl hydrazide method, the cells were agglutinated at a minimum concentration of 7.5 μg of Con A per ml (2). Therefore, both techniques give roughly equivalent results.

In order to be certain that the aggregates formed upon the Con A-mediated agglutination of the modified erythrocytes were typical of agglutinated cells, photomicrographs were taken of control cells, modified and agglutinated cells, and modified and agglutinated cells with α-methyl mannoside. As can be judged from Fig. 4, the agglutinated cells look typical of aggregating cells and can be easily dispersed by added α-methyl mannoside. The latter results show that the aggregates were not the result of an irreversible process such as cell–cell fusion. When the sugar was added before the lectin, agglutination was prevented; when the sugar was added after, it was reversed.

In this communication we have shown that synthetic cholesterol-containing glycolipids can be exchanged from liposomes into bovine erythrocytes. These cells, which normally cannot be agglutinated by Con A, can be rendered so by incorporating α-thiomannosyl-containing glycolipids into their outer membrane. One of the more surprising aspects of this work was the relatively modest distance requirement between the cholesterol anchor and the sugar needed for agglutination. This distance requirement is not dissimilar to that seen with these synthetic glycolipids in small unilamellar liposomes (4). Assuming that the cholesterol anchor is bound in the erythrocyte membrane in a way similar to the way it is bound in liposomes, this suggests that the glycoprotein coat of the erythrocyte does not prevent access of so large a protein as Con A close to the bilayer. This technique of sugar incorporation is, of course, not limited to erythrocytes. We have recently found that synthetic glycolipids of the kind discussed here can be functionally incorporated into such diverse cells as embryonic skeletal muscle cells and liver hepatocytes. Furthermore, the use of water-soluble spacer groups should allow the determination of the minimal distance a sugar (or other receptor) must be from the membrane before it becomes a functional receptor. This dimension of the membrane is not obtainable by electron microscopy, and its determination should be of interest for a molecular understanding of what terms such as crypticity mean when applied to receptors.

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