Lectin-Mediated Aggregation of Liposomes Containing Glycolipids with Variable Hydrophilic Spacer Arms†

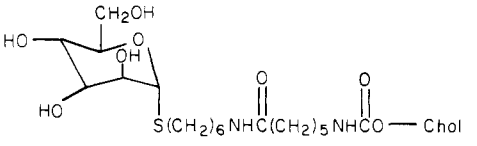
James S. Slama‡ and Robert R. Rando*

ABSTRACT: Synthetic glycolipids containing a cholesterol anchor group attached via a spacer group to a sugar moiety can be incorporated into small unilamellar liposomes, rendering them susceptible to agglutination by the appropriate multivalent lectin [Rando, R. R., Orr, G. A., & Bangerter, F. W. (1979) *J. Biol. Chem. 254*, 8318–8323; Rando, R. R., & Bangerter, F. W. (1979) *J. Supramol. Struct. 11*, 295–309]. We report here on the role of spacer arm length in rendering these liposomes susceptible to agglutination. In order to eliminate the ambiguities inherent in using hydrophobic or charged spacer groups, we have synthesized a hydrophilic, ethylene glycol based amino acid (8-amino-3,6-dioxaoctanoic acid) for these studies. The *Ricinus communis* agglutinin (ricin) mediated agglutination of these β-galactoside-containing glycolipids was studied. A spacer arm length of four atoms will not support agglutination under any conditions. A seven-atom spacer will support agglutination, but only at high phospholipid concentrations (0.24 μmol/mL) with a pseudo-first-order rate of agglutination of 0.0079 min^{-1}. With 13 and 22 atom spacer groups, the pseudo-first-order rate constants were 0.4 min^{-1} and 1.3 min^{-1}, respectively, at a phospholipid concentration of 0.06 μmol/mL. Under conditions where the liposomes containing the glycolipid with the 13-atom hydrophilic spacer arm were completely agglutinated by ricin, 9.3% of the total available sugar moieties of the liposome were bound to ricin. This means that, on the average, 38 interliposomal bonds were formed in an aggregrate.

Contemporary research into the areas of biological membranes and cell surfaces has witnessed an increasing appreciation of the role of membrane-bound carbohydrate in diverse and crucially important biological processes (Hughes, 1976). Saccharide determinants have been postulated to play a role in cellular adhesion (Gartner & Podleski, 1975; Huang, 1978) and in the agglutination of normal and transformed cells (Sharon & Lis, 1972) and are thought to function as receptors for binding of such agents as toxins (Sandvig et al., 1976), hormones (Sandvig et al., 1976), and viruses (Haywood, 1974) to the plasma membrane.

Despite the above advances, the specific mechanism(s) through which saccharide groups interact with saccharide binding proteins has (have) not been elucidated. That glycolipids may be involved in such a process has been suggested by the ability of gangliosides, when incorporated into liposomes, to serve as receptors for various lectins (Surolia et al., 1975; Redwood & Poefka, 1976; Rendi et al., 1976; Boldt et al., 1977; Curatolo et al., 1978; Surolia & Bachhawat, 1978). The specificity of such an interaction is due, in part, to the structure of the saccharide units. In addition, and particularly in the case of receptors associated with glycolipids, the topological distribution of receptors in the membrane may be of importance. This would include such factors as the density of receptor in the membrane, the mobility of the receptor, and the distance of the saccharide determinant from the lipid bilayer.

Investigation into the role of glycolipids as mobile cell surface receptors has been hampered by the fact that the naturally occurring glycolipids (gangliosides) are difficult to obtain in a highly purified form and contain only a limited range of saccharide determinants. Recently, attempts have been made to produce synthetic glycolipids in which a single well-defined saccharide residue is linked to a membrane-incorporated steroid nucleus by a hydrophobic spacer group (Chabala & Shen, 1978; Rando et al., 1979; Rando & Bangerter, 1979). In addition, a new class of synthetic glycolipids containing alkylamines covalently linked to aldonic acid has also been reported (Williams et al., 1979). We have found that synthetic sugar-containing glycolipids, like compound I,



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can be incorporated into small unilamellar liposomes which are then rendered susceptible to agglutination by the appropriate lectin (Rando et al., 1979; Rando & Bangerter, 1979). In order to evaluate the role of the length of the spacer arm on the ability of such compounds to participate in protein binding at membrane surfaces, we sought a new set of derivatives wherein the spacer arm could be modified with ease. More importantly, for long spacers, a structure was required in which the anticipated hydrophobic interactions of long hydrocarbon chains would be minimized.

The objective of the present work is to produce a series of neutral water-soluble spacer arms for such synthetic glycolipids and to incorporate these into a series of derivatives in which the saccharide can be placed at various known distances from the lipid bilayer. In this way a systematic study can be made of the effect of the length of the spacer arm on the ability of the derivative to serve as a receptor for lectins such as the *Ricinus communis* agglutinin (ricin). These studies indicate, in a very simple well-characterized system, the likely minimum distance from the membrane that a sugar must be in order to function as a receptor. The relevant hydrophilic spacer group is the amino acid 8-amino-3,6-dioxaoctanoic acid [$NH_2CH_2(CH_2OCH_2)_2CO_2H$]. Being an amino acid, the

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‡ Present address: Department of Chemistry, University of Chicago, Chicago, IL 60637.

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compound is easily polymerized to generate discrete lengths and is easily incorporated into the synthetic glycolipids.

Experimental Procedures

Materials. *R. communis* agglutinin (ricin) was obtained from Boehringer Manneheim GmbH and used without further purification. The concentration was determined by using the published extinction coefficient at 280 nm of $E_{ICM}^{10\%} = 117$ (Olsnes et al., 1974). [^{125}I]Ricin was produced from the above material by using the iodine monochloride procedure of Helmkamp et al. (1960) as modified for application to RCA by Nicolson et al. (1974). The ricin (1 mg) was treated with a stoichiometric quantity of ^{125}ICl, in a 50 mM Tris buffer, pH 8.0, for 2 min. The reaction was stopped by adjusting the pH to 7.0 and adding excess sodium thiosulfate. The protein was separated from inorganics on a column of Sephadex G-25 (0.10 mL), followed by exhaustive dialysis. To ensure that the ^{125}I-labeled ricin was active prior to its use in binding assays, we diluted portions of this labeled material (10–25 μg, $\sim 10^{-7}$ count/min) with $\sim$0.8–1 mg of carrier ricin and subjected them to an affinity purification on a column of agarose (5.3-mL total volume; Sepharose 4B, using 0.2 M NaCl and 5 mM sodium phosphate, pH 7.2, as the developing solvent) as described by Nicolson et al. (1974). Specifically absorbed [^{125}I]ricin was eluted by using 0.1 M lactose. L-α-Lecithin (egg) was purchased from Avanti Biochemicals as a solution in chloroform and used without further purification. 2-[2-(Chloroethoxy)ethoxy]ethanol, potassium phthalimide, and trifluoroacetic anhydride were purchased from the Aldrich Chemical Co.

Preparation of Liposomes. The synthetic glycolipids were incorporated into egg lecithin based small unilamellar liposomes of $\sim$400-Å diameter by the method previously published (Barenholz et al., 1977; Rando et al., 1979; Rando & Bangerter, 1979). This method involves sonicating the dry mixture of lecithin and glycolipid in 140 mM NaCl plus 50 mM Tris, pH 7.4, until dissolution occurs. The liposomes were then fractionated by centrifugation for 16 h at 110000*g* (35000 rpm in a Beckman preparative ultracentrifuge). Phospholipid concentrations were determined by the standard phosphate assay (Ames, 1976). The amount of incorporated glycolipid was determined by using 6-^{3}H-labeled galactosyl compounds. We had previously demonstrated that the synthetic glycolipids partition equally on both halves of the bilayer (Rando et al., 1979; Rando & Bangerter, 1979). In addition, the synthetic glycolipids fluidize the membranes and decrease their permeability to small polar molecules and thus behave much like cholesterol itself (Rando et al., 1979; Rando & Bangerter, 1979).

Agglutination Assays. Treatment of the clear sonicated and fractionated preparation of the glycolipid-containing small unilamellar liposomes with a solution of *R. communis* agglutinin (ricin) leads to a time-dependent increase in turbidity as measured by absorbance at 360 nm. Approximately 90% of the total phospholipid and glycolipid could be agglutinated at added ricin concentrations $\geq$40 μg/mL. The liposomal aggregates were easily dispersed by adding lactose but not mannose. Fusion of the liposomes does not occur even after remaining aggregated overnight. Details of this assay procedure have been previously published (Rando et al., 1979; Rando & Bangerter, 1979).

[^{125}I]Ricin Binding Assays. Plastic test tubes (13 × 100 mm) were treated with 2 mL of a solution of bovine serum albumin (1 mg/mL) and lecithin vesicles (0.06 μmol of phosphorus/mL) in a buffer of 50 mM Tris, pH 7.4, containing 140 mM NaCl at 4 °C prior to use in the binding assays. Polycarbonate centrifuge tubes were similarly treated.

After removing this solution and rinsing the tubes with buffer, we prepared the assays. Each assay contained vesicles, ricin, and buffer in a total volume of 1 mL. The concentration of vesicles was adjusted so that the concentration of the galactose-containing glycolipid was maintained as 6.7 nmol/mL (i.e., 6.7×10^{-9} mol/mL). For vesicles containing 10 mol % glycolipid, this resulted in a phosphorus concentration of 0.06 μmol of phosphorus/mL. Next, the desired amount of unlabeled ricin was added, followed by a sufficient quantity of ^{125}I-labeled ricin to give $\sim$10000–20000 counts per min per assay (the specific activity of this preparation was sufficiently high so that the amount of protein added was insignificant). The mixture of vesicles and ricin was incubated at 4 °C overnight with mechanical shaking. The next day, the aggregates were resuspended on a vortex mixer, transferred to 10-mL polycarbonate centrifuge tubes, and centrifuged for 2 h at 110000*g* (Beckman Ti 50 rotor; 35000 rpm). A sample of the supernatant was removed after the centrifugation (0.5 mL), and the activity present in this supernatant as well as that remaining with the pellet was determined by using a γ counter. The amount of ricin which was precipitated was calculated from the known amount of ricin present in each sample once the fraction of label bound vs. unbound was known.

Syntheses. (*1*) *Hydrophilic Spacer Group: 8-Trifluoroacetylamino-3,6-dioxaoctanoic Acid.* This compound was prepared from the commercially available 2-[2-(2-chloroethoxy)ethoxy]ethanol in the four steps shown below.

(*a*) *3,6-Dioxa-1-hydroxy-8-iodooctane* (*Triethylene Glycol Moniodide*). Sodium iodide (48.72 g, 0.325 mol) was dissolved in 350 mL of reagent-grade methyl ethyl ketone, by stirring at room temperature. The 2-[2-(chloroethoxy)ethoxy]ethanol (42.16 g, 0.25 mol) was added, and the solution was brought to a boil and refluxed for 19 h.

The mixture was filtered to remove salts, and the precipitate was washed twice with 50-mL portions of methyl ethyl ketone. The filtrates were combined, the solvent was removed under reduced pressure, and the residue was dissolved in 150 mL of methylene chloride. The resulting solution was extracted with a solution of sodium bisulfite (20 g of $NaHSO_3$ in 50 mL of water), followed by a mixture of sodium bicarbonate (20 mL of 0.5 M) and saturated NaCl (50 mL), and finally washed 3 times with 100-mL portions of saturated NaCl solution.

The organic extract was dried above 10 g of magnesium sulfate, and the solvent was removed under reduced pressure to leave a slightly yellowish oil weighing 61.4 g. This product could be further purified by Kugelrohr distillation (50 μm; 105–125 °C) to yield a liquid weighing 58.3 g.

(*b*) *3,6-Dioxa-1-hydroxy-8-phthalimidooctane* (*Phthalimidotriethylene Glycol*). A 500-mL three-neck flask, fitted with a thermometer and a nitrogen blanket, was charged with monoiodotriethylene glycol (39 g, 0.15 mol), dry dimethylformamide (150 mL, dried by distillation from above CaH_2 and stored above 3-A molecular sieves), and potassium phthalimide (30 g, 0.165 mol). This mixture was stirred at 95 °C under nitrogen for 18 h, at which time the mixture was allowed to cool and solvent was removed in vacuo. The residue was dissolved in 60 mL of water, and this aqueous solution was extracted with methylene chloride in a continuous extraction apparatus for 18 h. Organic extracts were dried above magnesium sulfate and filtered, and the solvent was removed under reduced pressure to leave the phthalimido compound as an amber-colored oil weighing 54.3 g, which was submitted to Kugelrohr distillation (155–195 °C; 50–100 μm), yielding an oil weighing 38.2 g.

It was necessary to further purify this material by chromatography on silica gel. The product was divided in half, and ~20 g of the impure phthalimido compound was applied to a column of silica gel (300 g, wet packed in methylene chloride in a column 42 cm high and 4.5 cm in diameter) as a solution in 75 mL of methylene chloride. Column was developed with 2 L of methylene chloride, 1.5 L of $CHCl_3$, and 2 L of 5% CH_3OH in $CHCl_3$. The phthalimidoethylene glycol was eluted in the methanolic chloroform. Evaporation of solvent left a colorless oil weighing 16.7 g which solidified on standing. Drying under high vacuum left a white solid: mp 55–57 °C; NMR ($CDCl_3$) δ 3.0 (br, 1 H, OH), 3.6 and 3.65 (s, 10 H, CH_2O), 3.87 (t, J = 4 Hz, 2 H, N–CH_2), 7.8 (d, J = 2 Hz, 4 H, Ar–H). Anal. Calcd for $C_{14}H_{17}O_5N$: C, 60.20; H, 6.15; N, 5.02. Found: C, 60.57; H, 6.13; N, 5.06.

(*c*) *3,6-Dioxa-8-phthalimidooctanoic Acid.* Phthalimidotriethylene glycol (1.4 g, 5 mmol) was dissolved in 75 mL of acetone and stirred as 3.0 mL of the 2.67 M Jones reagent (8 mmol; prepared by dissolving 26.72 g of chromic trioxide in 23 mL of concentrated sulfuric acid and diluting to a volume of 100 mL) was added dropwise over ~15 min. The solution was allowed to stir for ~30 min at which time a few drops of 2-propanol was added.

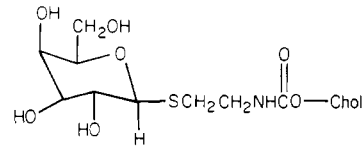
Sufficient water was added to dissolve the precipitated chromium salts (50 mL), and the acetone was removed by evaporation under reduced pressure. The aqueous mixture was transferred to a separatory funnel, 25 mL of saturated NaCl solution was added, and the aqueous mixture was extracted 7 times with 25-mL portions of CH_2Cl_2. The combined CH_2Cl_2 extracts were dried above magnesium sulfate, filtered, and evaporated to dryness, leaving a solid weighing 1.4 g, which was crystallized from ethyl acetate and hexane to leave 1.1 g of white crystals: mp 105–109 °C; NMR ($CDCl_3$) δ 3.72 (s, 6 H, CH_2O), 3.87 (t, J = 3 Hz, 2 H, CH_2–N), 7.77 (d, J = 2 Hz, 4 H, Ar–H), 8.50 (s, 1 H, COOH).

(*d*) *8-Trifluoroacetylamino-3,6-dioxaoctanoic Acid.* 3,6-Dioxa-8-phthalimidooctanoic acid (4.0 g, 13.64 nmol) was dissolved in a solution of hydrazine hydrate (1.82 g, 68.4 mmol) in methanol (50 mL). The solution was brought to a boil and refluxed for 3 h at which time TLC analysis revealed no starting material.

The solvent was removed in vacuo and high vacuum was applied to remove excess hydrazine. The residue was dissolved in 25 mL of water, the pH was adjusted to 2.8 with concentrated HCl, and the resultant suspension was allowed to stand overnight in a refrigerator. The suspension was filtered, and the precipitate was washed and discarded. The combined filtrates were evaporated under reduced pressure, leaving a residue (the amine hydrochloride salt) weighing 3.74 g.

In order to obtain the free base, we applied the salt to a column of Bio-Rex 1-X8 ion-exchange resin (–OH form; 50-mL bed volume; 1.4 mequiv/mL resin) as a solution in 20 mL of H_2O and eluted the product with 400 mL of 2 M CH_3C-OOH. The solvent was removed under reduced pressure to leave 2.15 g of an amorphous solid. The solid was treated with 20 mL of trifluoroacetic anhydride. It dissolved over a period of ~15 min with stirring, and the resultant solution was allowed to stand at ambient temperature for 2 h. The excess anhydride was removed under reduced pressure, and the residue was taken up in water and allowed to stand for 1 h. The solvent was evaporated, leaving a liquid weighing 3.18 g. The trifluoroacetylamino acid was purified by Kugelrohr distillation (150–155 °C; 50 μm), resulting in 2.8 g of a colorless viscous liquid: NMR ($CDCl_3$) δ 3.67 (s, 2 H, $-CH_2$–N), 3.73 (s, 4 H, $-CH_2O$), 4.20 (s, 2 H, $-CH_2COO$–), 8.33 (br, 1 H, NH), 10.3 (s, 1 H, –COOH). Anal. Calcd for $C_8H_{12}O_5NF_3$: C, 37.07; H, 4.68; N, 5.40. Found: C, 37.27; H, 4.73; N, 5.47.

(*2*) *Glycolipids.* The following glycolipids were synthesized for the studies reported here.



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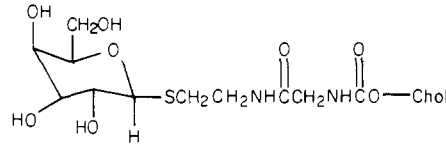

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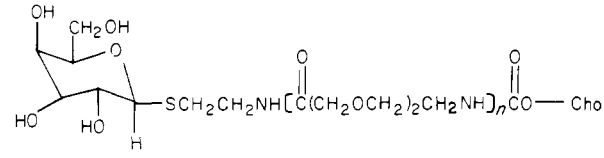

IV, n = 1
V, n = 2

These compounds were prepared by the methodology already reported for the synthesis of the glycolipids save for two modifications (Rando et al., 1979; Rando & Bangerter, 1979). In the reductive alkylation of the galactosyl thiopseudourea, 1-iodo-2-(trifluoroacetylamino)ethane was substituted for 1-iodo-2-(trifluoroacetylamino)hexane, and in the peptide bond forming step, the hydrophilic spacer was substituted for (trifluoroacetylamino)hexanoic acid. Detailed syntheses of these compounds will be published elsewhere; however, some of their physical characteristics are as follows.

Glycolipid II: Field desorption mass spectroscopy, M^+ = 651. Anal. Calcd for $C_{36}H_{61}O_7NS$: C, 66.32; H, 9.43, N, 2.15. Found: C, 66.09; H, 9.61, N, 2.05. Glycolipid III: Field desorption mass spectroscopy, M^+ = 708. Anal. Calcd for $C_{38}H_{64}O_8N_2S{\cdot}0.5H_2O$: C, 63.57; H, 9.12; N, 3.90. Found: C, 63.41; H, 9.29; N, 3.86. Glycolipid IV: Field desorption mass spectroscopy, M^+ = 708. Anal. Calcd for $C_{42}H_{72}O_{10}N_2S$: C, 63.29; H, 9.10; N, 3.41. Found: C, 62.97; H, 9.51; N, 3.43. Glycolipid V: Field desorption mass spectroscopy, M^+ = 796. Anal. Calcd for $C_{48}H_{83}O_{13}N_3S$: C, 61.19; H, 8.87; N, 4.46. Found: C, 61.36; H, 8.71; N, 4.44.

The tritium labeled glycolipids were prepared by a method previously published (Rando et al., 1979; Rando & Bangerter, 1979).

Results

Ricin-Mediated Aggregation of the Glycolipid-Containing Liposome. We have already found that β-D-galactosyl-containing glycolipids with hydrocarbon spacer groups can be agglutinated by ricin when incorporated into small unilamellar liposomes (Rando et al., 1979; Rando & Bangerter, 1979). Similar experiments were performed with liposomes containing glycolipid IV. In Figure 1A the OD at 360 nm is measured as a function of the ricin concentration added. At 40 μg and above, the same extent of reaction is achieved although the rates of the agglutination reactions differ. Pseudo-first-order plots for the agglutination reaction are shown in Figure 1B. A lag phase, determined as the X intercept, was found in all of the experiments. The temperature at which maximal rates

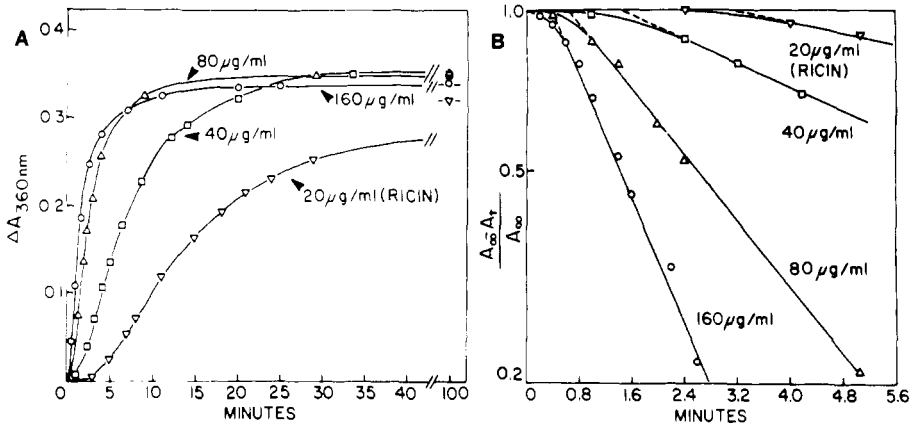

FIGURE 1: (A) Turbidity increase observed upon the addition of the stated amounts of ricin to a preparation of lecithin vesicles (0.06 μmol of phosphorus/mL) containing 10 mol % glycolipid IV. Assays were conducted at 4 °C. (B) Replots of turbidity increase vs. time as pseudo-first-order processes: $(A_\infty - A_t)/A_\infty$ vs. time.

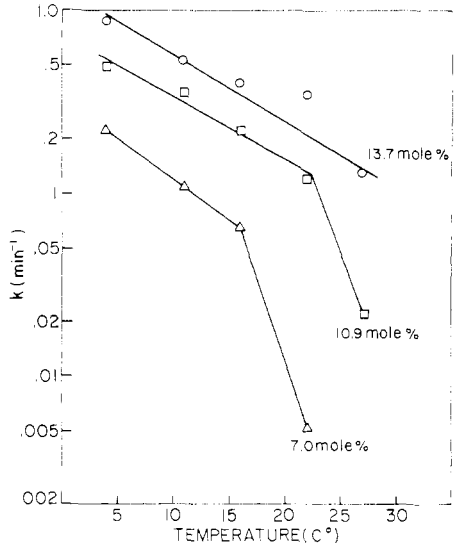

FIGURE 2: Temperature dependence on the rates of the pseudo-first-order portion of the agglutination of vesicles containing stated amounts of IV.

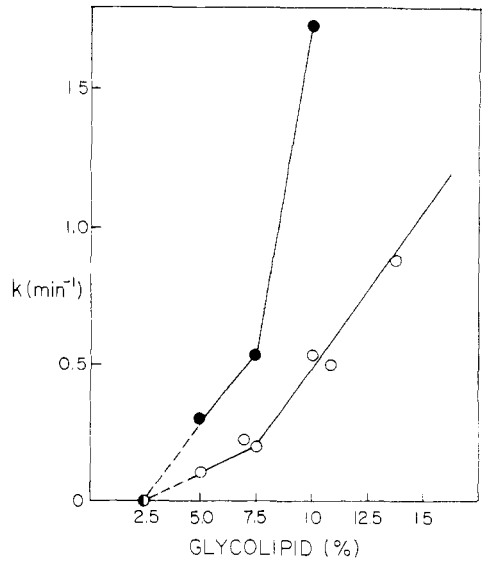

FIGURE 3: Pseudo-first-order rate constants for aggregation of liposomes containing variable percentages of synthetic glycolipids IV (○) and V (●). The lipid concentrations were 0.06 μmol of phosphorus/mL, and the ricin concentration was 160 μ/mL, with a interval temperature of 4 °C.

of aggregation was achieved was first determined with liposomes containing IV. Figure 2 shows that, at all glycolipid concentrations tested, the maximal rate occurred at 4 °C. This kind of inverse temperature dependence on aggregation rates has been observed before with ricin (Curatolo et al., 1978; Rando et al., 1979; Rando & Bangerter, 1979). The remaining experiments reported here were all performed at 4 °C.

In Figure 3 a plot of the pseudo-first-order rate constants for the aggregation of liposomes containing glycolipids IV and V is shown. The experiments were performed at 4 °C. The rates seem to be biphasic with a break at ~7.5% glycolipid. No aggregation occurred here when the glycolipid concentrations were below 5%. These threshold effects have been noted previously in similar systems and are related to the density of glycolipid in the membrane as well as the total glycolipid concentration per unit volume. This can be shown by comparing two preparations of lipid in which the mole percent of glycolipid is changed but in which the total concentration of glycolipid, measured as micromoles per milliliter, remains the same. Such as comparison was carried out and the results are presented in Table I. The preparation in which the density of receptors in the membrane decreased showed a marked decrease in the rate of agglutination despite the fact that the concentration of receptor per unit volume was unchanged.

Table I: Rates of Aggregation of Liposome Containing IV and V[a]

	$t_{1/2}$ (min)	k (min^{-1})
10 mol % IV and 0.06 μmol of P/mL	1.3	0.53
5 mol % IV and 0.12 μmol of P/mL	2.4	0.29
10 mol % V and 0.06 μmol of P/mL	0.4	1.73
5 mol % V and 0.12 μmol of P/mL	1.0	0.69

[a] Liposomes at the indicated concentrations were treated with ricin at 160 μg/mL and the rates of aggregation were measured.

Although there was an effect of spacer arm length on pseudo-first-order rate constants (two- to threefold) for the aggregation of the glycolipid-bearing vesicles in Figure 3, it was not dramatic. This suggests that a critical minimum distance had been passed. To investigate this further, aggregation of the liposomes bearing the shorter spacers was studied. It was found that when liposomes were prepared containing 10% glycolipid III, they were not aggregated at the low phospholipid concentrations previously studied (0.06 μmol/mL). In fact, measurable aggregation only occurred at 0.24 μmol/mL phospholipid (Figure 4). The measure first-order rate constant is 0.0079 min^{-1}, some 214-fold slo than the rate obtained with 10% IV. In addition, a lon

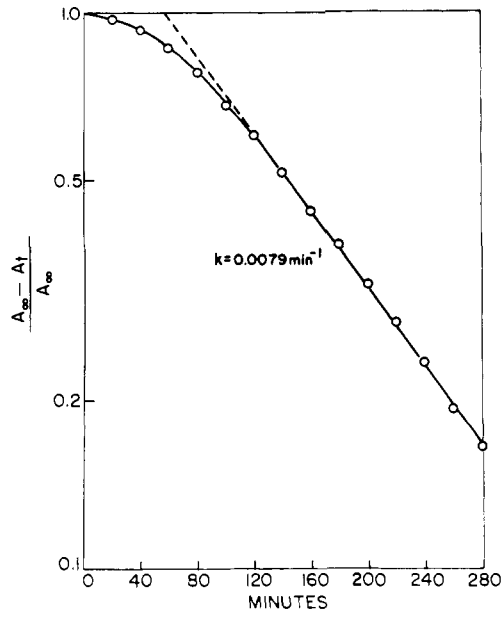

FIGURE 4: Aggregation of liposomes containing 10 mol % of III, at a concentration of 0.25 μmol of phosphorus/mL at 4 °C using 160 μg/mL ricin.

phase of 56 min is observed here prior to the onset of the pseudo-first-order aggregation process. Decreasing the spacer arm length further led to an abolition of aggregation. For example, ricin-mediated aggregation of liposomes containing glycolipid I could not be obtained under any conditions, suggesting that a critical minimal distance to support aggregation had been passed in going from glycolipid II to III.

[^{125}I]Ricin Binding. A question unanswered is the relationship between the amount of ricin bound and the facility with which the liposomes aggregate. This was studied by adding the radioactive lectin to a preparation of liposomes containing a constant amount of synthetic glycolipid (6.7 nmol/mL) in the buffer of 50 mM Tris, pH 7.4, and 140 mM NaCl. After shaking this mixture at 4 °C for 18 h, the suspension was centrifuged for 2 h at 110000*g*. The amount of ricin remaining in the supernatant and the amount of ricin in the pellet were determined. Since the liposomal particles of the type used in this study are known to have a sedimentation coefficient of $s_{20,w} = 2.15$, and since ricin has a M_r of 120 000, neither the liposomes nor the protein is pelleted under the conditions of the centrifugation step unless part of a high molecular weight aggregate (Huang, 1969).

The relationship between ricin binding and agglutination is shown in Figure 5. Liposomes containing 10 and 5 mol % of compounds III, II, and V were treated with [^{125}I]ricin at the indicated concentrations. The amount of ricin bound to the aggregates was determined. The nonspecific binding of ricin to control liposomes (−glycolipid) has been subtracted. In a qualitative way at least, these data conform to expectation. Little or no binding is found to liposomes prepared from III and progressively more is bound to liposomes prepared from IV and V. Furthermore, it is clear that much more ricin can be bound to the liposomes than is required for agglutination. For example, liposomes containing 10% IV are maximally aggregated at 40 μg/mL ricin. At this concentration of added ricin, only 23 μg/mL is bound, although saturation is not reached until 48 μg/mL is bound.

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...investigated the ricin-mediated aggregation of ...ontaining liposomes having spacer arms of 4, 7, ...atoms. The spacer arms of 13 and 22 units were

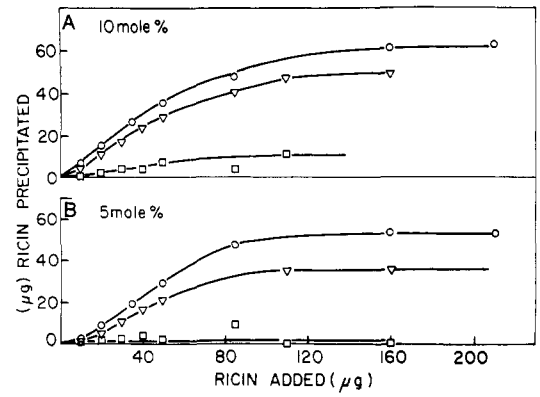

FIGURE 5: Agglutination of EYL vesicles containing 10 mol % of III (□), IV (▽), and V (○) (A) and 5 mol % of the same synthetic glycolipids (B), measured by precipitation of [^{125}I]ricin. Binding data have been corrected for nonspecific [^{125}I]ricin precipitation. Each assay (1 mL) contains 6.7 nmol of gal/mL.

based on the neutral highly water soluble 8-amino-3,6-dioxaoctanoic acid to ensure that these longer chains would not fold back on themselves as a consequence of hydrophobic interaction. Since the cholesterol moiety of these glycolipids is probably bound in the membrane much in the same way as cholesterol itself, the spacer arm lengths achieved measure the distance between the membrane and the sugar moiety. The shortest chain length glycolipid (II) would not support ricin-mediated agglutination under any of the conditions tried. Synthetic glycolipid (III), which contains a seven-atom spacer, failed to support agglutination even at 10% glycolipid in the liposomal preparation. However, a slow agglutination could be observed when the concentration of liposomes was increased from 0.06 to 0.24 μmol of phosphorus/mL. The course of this reaction followed the pattern previously described with a lag phase (56 min), followed by a pseudo-first-order turbidity increase ($t_{1/2}$ = 88 min; k = 0.0079 min^{-1}). This slow agglutination was surprising in light of the fact that lactosylceramide has been observed to clearly support agglutination of egg yolk lecithin based liposomes at 10% glycolipid and a phosphorus concentration of 0.24 μmol/mL, where maximum turbidity was reportedly reached within 5–10 min at saturating ricin concentrations (Curatolo et al., 1978). The spacer arm of the synthetic glycolipid III is sufficiently long that if fully extended, the galactose residue of III and the galactose residue of lactosyl ceramide should be at roughly similar distances from the lipid bilayer. However, since lactosylceramide is a complex glycolipid, the observed results may indicate that the ricin binding site has an increased affinity for a β-D-galactoside moiety when linked to a second glycoside. Alternatively, it is also possible that the lactosylceramide is bound in the membrane in such a fashion that the sugar moiety is held out further from the membrane than it is in the cholesterol analogues.

Lengthening of the spacer arm to a total of 13 atoms as in IV and further to 22 atoms as in V results in the preparation of derivatives which serve very effectively as ricin receptors. As expected, the compound with the largest chain, V, was the best receptor by the criterion of rate of agglutination as well as by the criterion of ricin precipitability. The difference between IV and V was not as great as that between III and IV, however, indicating that a "critical distance" had been passed somewhere between the length of the 7-atom spacer and that of the 13-atom spacer. Rates of agglutination were much more sensitive to spacer arm length and receptor density than was the amount of lectin precipitated. For example, rates of agglutination at 4 °C for 10 mol % of preparations of

synthetic glycolipids IV and V differed by a factor of ~3.5, whereas the maximum amount of ricin bound by these two preparations differed only by a factor of 1.3 (Figure 5).

The temperature dependence of rates of agglutination is clearly unusual in that it is maximal at 4 °C and declines steadily as the temperature increases. This constrasts with the temperature dependence observed for agglutination of lecithin–lactosylceramide liposomes with ricin, where a maximal rate of aggregation at 25 °C was observed (Curatolo et al., 1978). Since the same lectin as well as the same phospholipid was used in both studies, the differential temperature effects may be due to differences in behavior of the respective lipid anchors in the bilayer.

It is also important to note that the threshold concentrations of glycolipid are temperature-dependent phenomena. The 7 mol % of preparation of IV which agglutinates well at 4 °C fails to agglutinate at all at 27 °C. Likewise, the rate of agglutination of the preparation containing 10.9 mol % of IV decreases rapidly above 22 °C. Changes in the threshold concentration with temperature could influence the ability of a given receptor type to play a role in various biological processes.

From the amount of [^{125}I]ricin precipitated during agglutination, we can calculate an approximate value for the average number of ricin molecules bound per liposome. In a 10 mol % preparation of synthetic glycolipid IV, saturation of ricin precipitation occurs at 63 μg of ricin bound. However, as reported before, the liposomes are completely agglutinated at 40 μg/mL added ricin. Under these conditions, 24 μg/mL ricin is bound. This corresponds to 0.20 nmol of ricin bound. The preparation of liposomes contains 6.7 nmol of synthetic glycolipid/mL. In liposomes of 400-Å diameter, the relative surface area is 64% on the outside to 36% in the inside. Therefore, we would expect 4.3 nmol of glycolipid to be on the outside of the liposomes available for binding. Hence, the observed maximal ricin binding represents ~4.7% of the theoretical maximum possible, assuming a stoichiometry of 1, and 9.4%, assuming a stoichiometry of 2. We can also calculate that there are ~403 outside glycolipid molecules in the outside of the liposome, which means that under conditions where complete agglutination occurs, 38 sites per liposome are bound, assuming the stoichiometry of ricin binding is 2. Under conditions where saturation of binding sites occurs with ricin, ~100 sites per liposome are bound. A rough comparison can also be made between the binding of ricin by liposomes containing IV or V and the precipitation of ricin by liposomes containing ganglioside GM_1 as described by Surolia et al. (1975). Under identical conditions Surolia reports precipitation of roughly twice as much ricin with GM_1 as was observed for IV and V. Considering the fact that GM_1 is a complex polysaccharide, these values are remarkably close. The increased precipitation would possibly reflect a lower K_D for GM_1, although meaningful binding constants cannot be easily calculated from precipitation data on liposomes.

A novel aspect of the work reported here is the use of the neutral water-soluble ethylene glycol based amino acid spacer groups introduced. The reasoning behind their introduction is simply that large distances between the cholesterol anchor group and sugar moiety are likely to be required for the function incorporation of these synthetic glycolipids into cells. Spacer chemistry based on hydrophobic amino acids, such as the routinely used 6-aminohexanoic acid, is clearly not indicated here because of hydrophobic interactions which will cause the hydrocarbon chains to fold back on one another. This is also the reason why these amino acids are useless for determining distances. In fact, we have found that synthetic glycolipids containing the amino hexanoate spacers cannot be functionally incorporated into cells. On the other hand, synthetic glycolipids bearing the hydrophilic spacer group can be functionally incorporated into cells (Rando et al., 1980).

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