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Threshold Effects on the Lectin-Mediated Aggregation of Synthetic Glycolipid-Containing Liposomes

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Cholesterol analogs containing sugar residues linked by spacer groups to the cholesterol O can be incorporated into egg yolk lecithin small unilamellar liposomes. The synthetic glycolipid analogs distribute evenly on both sides of the bilayer. These liposomes are aggregated by the appropriate lectin. For example, when the sugar residue is a β-galactoside the liposomes are aggregated by ricin and when it is an α-mannoside they are aggregated by Con A. The lectin-mediated aggregation of these liposomes is reversed by the addition of the appropriate sugar. The rates but not the extents of aggregation of these liposomes are highly sensitive to the amount of glycolipid incorporated. Below approximately 5% glycolipid incorporation the rate of the lectin-mediated aggregation of these liposomes is exceedingly slow, whereas above this level rapid aggregation proceeds. At all concentrations studied the synthetic glycolipids are incorporated in a unimodal fashion so that the observed threshold effects cannot be based on possible differences in the manner in which the glycolipids are incorporated at different concentrations. This conclusion is based on 1) studies with galactose oxidase that show that the percentage of galactose oxidation in a liposome prepared from a galactosyl-containing glycolipid is independent of glycolipid concentration, and 2) studies on the aggregation of liposomes containing mixed glycolipids in which the glycolipids are shown to behave independently. The importance of a critical density of membrane-bound receptors in order for aggregation to occur is discussed.

Key words: **threshold effects, liposomes, aggregation, ricin, concanavalin A, synthetic glycolipids**

Glycolipids can be incorporated into small unilamellar liposomes that are then rendered agglutinable by lectins. Among the glycolipids that have been incorporated are lactosyl ceramide, ganglioside GM_1, and synthetic cholesterol-containing glycolipids I [1–3]. When a mannosyl-containing cholesterol analog (I), AHMC:

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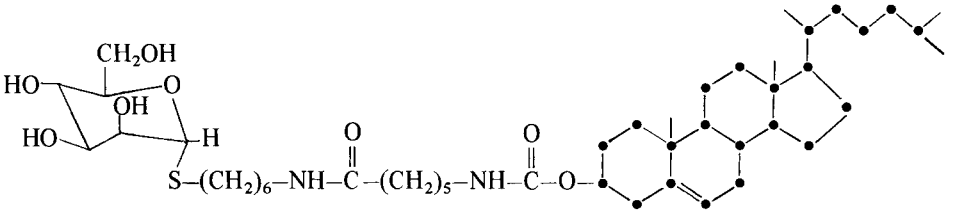


AHMC (I)

is incorporated into egg lecithin-based liposomes, these liposomes become readily agglutinable by concanavalin A (Con A) [3]. However, the initial rates of aggregation, as judged by light scattering, proved to be strongly nonlinear with concentrations of incorporated (I). For example, at concentrations of glycolipid less than 5 mole %, the initial rates of aggregation by added Con A were exceedingly slow or not measurable, whereas at concentration in the region of 7% the rates were rapid [3]. These threshold effects seemed to be shared by other liposome-bound glycolipid-lectin combinations studied [1, 2, 4].

The observed nonlinear effects could result from a variety of causes. If the glycolipids happened to be incorporated in different configurational arrays at the different concentrations, the cause of these effects would be apparent. On the other hand, if the glycolipids were incorporated in a unimodal way, a rather different explanation would have to be considered. Since these derivatized liposomes could be important model systems for understanding some of the ground rules that govern membrane-membrane interaction and recognition, it was considered important to study further the mechanisms underlying the observed threshold phenomena. In this communication we show that the cholesterol-containing glycolipids are indeed incorporated in a unimodal fashion at the concentrations studied and hence that the observed threshold effects are dependent only on the densities of receptors introduced.

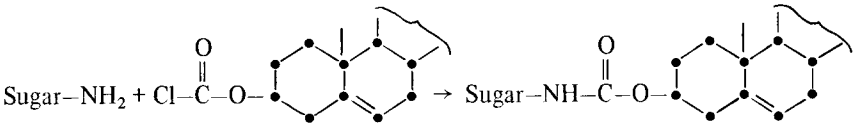
EXPERIMENTAL PROCEDURE

Materials and Methods

Con A (three times recrystallized) was purchased from Miles Laboratories. Solutions of Con A in 50 mM Tris-HCl, containing 140 mM NaCl, were centrifuged at 5000g for 5 min before use. The concentration of Con A solutions was determined by absorbance readings at 280 nm (1 mg/ml/cm = 1.3). Ricin, the β-galactosyl-binding agglutinin from Ricinus communis was obtained from Boehringer Mannheim, Inc. Egg phosphatidylcholine was prepared and purified according to the method of Litman [5]. The phospholipid concentration was determined as inorganic phosphate after ashing and acid hydrolysis [6]. The phospholipid was dissolved in benzene at a concentration of 40 μmoles/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). Cholesterol chloroformate, galactose oxidase, galactose dehydrogenase, hexokinase, nicotinamide adenine dinucleotide, galactose, and mannose were products of the Sigma Chemical Co. α-^{3}H-sodium acetate, Na^3BH_4, and ^{35}S-thiourea were products of New England Nuclear, Inc.

Syntheses

Glycolipids. The carbohydrate ligands used in this paper were either 6-(6-aminohexanamido)hexyl-1-thio-α-D-mannopyranoside or 6-(6-aminohexanamido)hexyl-1-thio-β-D-galactopyranoside. These compounds were synthesized by published methods [7]. These amino sugars were coupled to cholesterol chloroformate by the following reaction:



The detailed protocol for this synthesis has been published [3]. Analytical data on the two synthetic glycolipids used in these studies are as follows:

(I): 6-(6-aminohexanamido)hexyl-1-thio-α-D-mannopyranoside cholesterol ester (AHMC) $C_{46}H_{80}O_8N_2S$

Calc: C, 67.28; H, 9.82; N, 3.41
Found: C, 67.11; H, 9.31; N, 3.56

(II): 6-(6-aminohexanamido)hexyl-1-thio-β-D-galactopyranoside cholesterol ester (AHGC) $C_{46}H_{80}O_8N_2S$

Calc: C, 67.28; H, 9.82; N, 3.41
Found: C, 67.22; H, 9.93; N, 3.37

Unfortunately none of the compounds gave clean melting points, as they all decomposed upon heating prior to melting.

^{35}S-AHGC. ^{35}S-AHGC was prepared identically to the unlabeled material. The ^{35}S was introduced by reacting ^{35}S-thiourea with α-Br-D-tetraacetylgalactose to form the ^{35}S-thiouronium hydrobromide which was then converted into the required amino sugar [8]. The specific activity of the ^{35}S-AHGC used in these experiments was 0.22 mCi/mmole.

6-^{3}H-Galactosyl AHGC. 6-(6-trifluoroacetaminohexanamido)hexyl-1-thio-β-D-galactopyranoside was prepared by the published method [7]. This compound was oxidized by galactose oxidase and reduced with NaB^3H_4 by the published procedure [9]. The labeled compound was then deprotected with anion exchange resin (AG-1X8) and condensed with cholesterol chloroformate. The final purified glycolipid had a specific activity of 8.9 mCi/mmole.

α-^{3}H-Acetylhydrazide-trifluoroacetate salt. This compound (of specific activity 1.35 mCi/mole) was prepared from α-^{3}H-sodium acetate (New England Nuclear, Inc.) by the published method [10].

Liposomes. The small unlamellar liposomes used in these studies were prepared from the synthetic glycolipids and pure phosphatidylcholine by a method identical to that published by Barenholz et al [11]. The sonication steps were done at ambient temperature with lecithin and at 55°C with dipalmitoyl phosphatidyl choline (DPPC). The concentrations of the glycolipid compounds were accurately determined before incorporation by enzymatic assay of the free mannose or galactose after mercuric ion-catalyzed hydrolysis of the 1-thioglycosidic linkage [12]. To a solution of the cholesterol-mannose derivative (0.1–2 μmole) in ethanol (1 ml) was added 0.2 M mercuric acetate in 0.1 M acetic acid

(0.1 ml). After 2 h at 60°C, the incubation mixture was treated with 2-mercaptoethanol (10 μl), and the solvent was removed in vacuo. 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 hexokinase/ATP-catalyzed phosphorylation of the mannose, to the enzyme system of the pyruvate kinase/phosphoenolpyruvate and lactate dehydrogenase/NADH. Liberated galactose was assayed with NAD^+ and galactose dehydrogenase.

The permeability of the liposomes was checked by ^{14}C-sucrose release experiments [13]. In the absence of either cholesterol or one of its sugar derivatives, the rate of sucrose efflux was measured to be 0.7%/h. In the presence of 20 mole % cholesterol or 14 mole % AHMC, the rate of efflux was 0.2% and 0.3%/h, respectively [3]. In addition, spin-labeling studies using spin-labeled egg phosphatidyl choline (PC) (7.6) showed the following order parameters:

PC liposome alone: S = 0.514 ± 0.0044 (25°)

PC liposome + 10% AHGC: S = 0.575 ± 0.0035 (25°)

The S = +0.061 for the 10% AHGC liposome [3]. The condensing effect is about the same as for liposomes prepared with 20% cholesterol [14]. Finally, 10% AHGC broadens but does not eliminate the phase transition of DPPC liposomes. The effect is similar to that observed when 10% cholesterol is added to DPPC-containing liposomes (R. R. Rando, unpublished results).

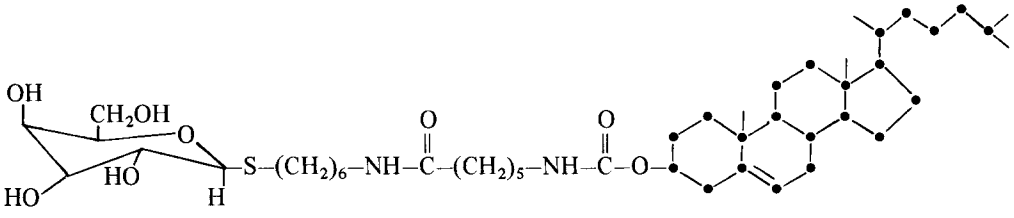
Kinetics of the Lectin-Mediated Aggregation of the Liposomes

The glycolipid-containing liposomes in pH = 7.4 tris buffer (50 mM + 140 mM NaCl) were placed in standard 1-ml quartz cuvettes in a temperature-thermostated Gilford model 240 spectrophotometer. The temperature of the liposomal solutions was accurately maintained with a Lauda model RC-20S circulatory bath. The concentrated lectin solutions were added at the desired temperature and the absorbance at 360 nm was recorded with time. Generally, the concentration of phospholipid used in the experiment was 0.06 μmole/ml. Any deviations from this value are appropriately recorded.

RESULTS

Characterization of the Liposomal Populations

The β-D-thiogalactosyl cholesterol carbamate (II) (AHGC):



AHGC (II)

was prepared as indicated in Materials and Methods. The ^{35}S analog of this compound was incorporated into lecithin-based small unilamellar liposomes according to the procedure of Barenholz et al [11]. In Figure 1 a plot of the percentage of AHGC incorporated as a func-

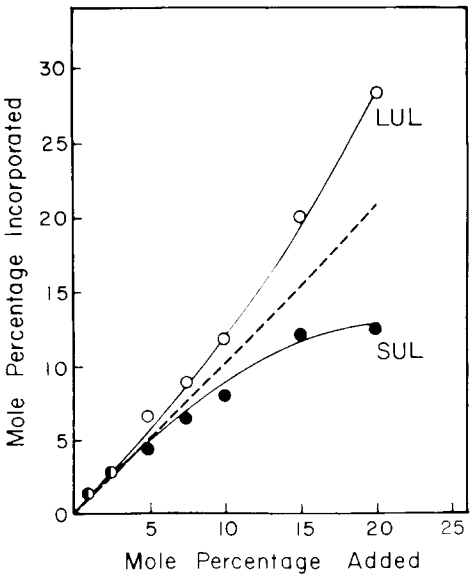


Fig. 1. Quantitative relationship between added and incorporated AHGC. Liposomes were prepared with varying initial concentrations of ^{35}S-AHGC. The liposomes were separated on a Sepharose 2B column and counted in Aquasol (New England Nuclear, Inc.) after phosphate determination. SUL, small unilamellar liposomes used in the studies reported here; LUL, large unilamellar liposomes.

tion of the amount added is shown. It is apparent that the radius of curvature of small liposomes imposes a limit on the level of incorporation of glycolipid. The same effect was observed with the α-mannosyl-containing analog AHMC [3]. Approximately 13% of the glycolipid can be maximally incorporated into these liposomes. The larger liposomes incorporate AHGC at much the same level as they do cholesterol itself, namely 50%. Chromatography of the small unilamellar liposomes prepared here on a Sepharose 2B show a symmetrical peak centered at about 400 Å diameter (Fig. 2). This column was sized with Dow latex beads of diameter 380 and 800 Å.

An important point to be clarified is what percentage of the sugar is on the outside of the liposome and whether this percentage is invariant with the extent of glycolipid incorporation. To these ends, 10% ^{35}S-AHGC-labeled liposomes were prepared and treated with galactose oxidase (mol wt = 42,000) for varying periods of time. This enzyme oxidizes the 6-methylene hydroxy group of galactose to an aldehyde [15]. To quantitate the amount of aldehyde produced under these conditions, α-^{3}H-acethydrazide ($^{3}CH_3-\overset{\overset{O}{\|}}{C}-NH-NH_2$) was added as a titrant. This compound, like other hydrazides, forms a stable hydrazone with aldehyde moieties [10]. The resulting ^{3}H/^{35}S ratios then gave the actual amount of galactose on the outside (accessible) of the liposome. When these experiments were performed, the results shown in Figure 3 were obtained. The curve levels off when 57% of the galactose is titrated. Calculations show that with liposomes of 200-Å radius, the relative surface area is 64% on the outside to 36% on the inside. Thus, the cholesterol carbamates described here are distributed more or less randomly in the bilayer, much the same as cholesterol itself [16]. Flip-flop of the cholesterol carbamates does not seem to occur on the time scale of these experiments. It would be predicted that it should be a very slow process here anyway, owing to the polarity of the sugar moiety. In the liposomes discussed here it can be calculated

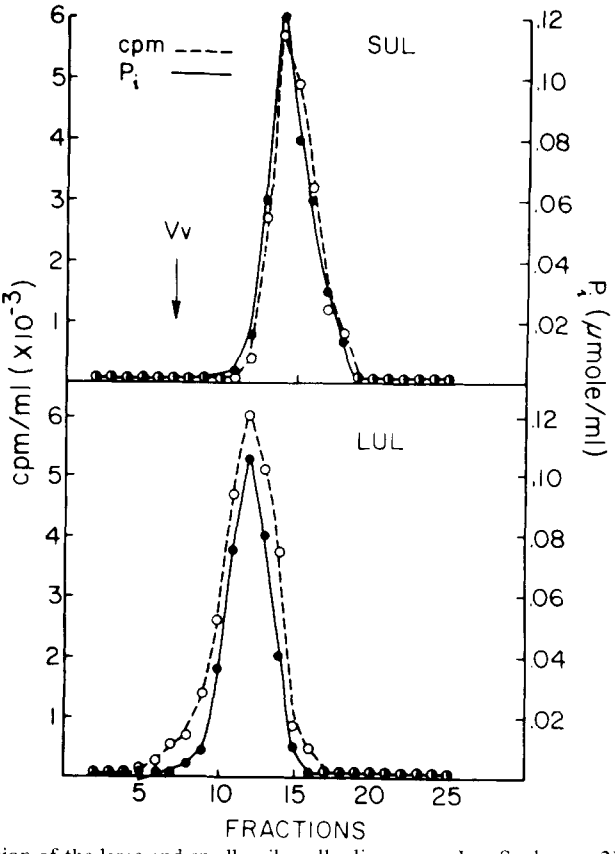


Fig. 2. Size distribution of the large and small unilamellar liposomes. In a Sepharose 2B column 10% ^{3}H-AHGC-containing large and small lecithin liposomes were chromatographed and eluted with 0.05 M tris buffer, pH 7.4, containing 0.14 M NaCl. This buffer was used in the preparation of the liposomes. The various fractions were analyzed both for inorganic phosphate after ashing and for radioactivity. The column was sized as before with 380-Å and 800-Å latex microsphases (Dow Chemical Co.) [3]. The peak fractions for the small liposomes occur at approximately 400 Å.

that there are 6.3×10^3 lipid molecules per liposome; hence in 10% AHGC-containing liposomes there are approximately 403 outside glycolipid molecules per liposome, and the density of these receptors is 1.25×10^3 Å^2/sugar. As shown in Figure 3, the limiting ^{3}H/^{35}S ratio is 3.497 with 10% vesicles. This ratio was determined at 2.5, 5.0, 7.5, and 10% glycolipid, and proved to be invariant (Table I). Therefore, insofar as galactose oxidase is a probe for accessible sugar residues, the relative amount of outside sugars does not change with different concentrations of glycolipid in the liposomes.

Rates of Aggregation of the AHGC-Containing Small Unilamellar Liposomes

Aggregation of the AHGC-containing liposomes was accomplished with the castor bean lectin ricin [17]. This lectin, of mol wt 120,000, is a tetramer containing two β-D galactoside-binding sites per oligomer [18]. Since this lectin binds galactosides more tightly at lower temperatures, it was decided to first study the temperature dependence of the aggregation of AHGC-containing liposomes [18]. The V_{max} occurs at approximately 10°C. Experiments showing the temperature profile are shown later in Figure 10. The dependence of the aggregation rates of the 10% AHGC-containing liposomes on the ricin concentration

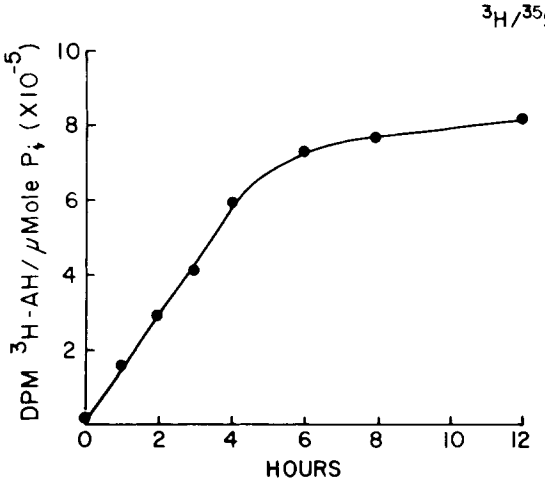


Fig. 3. Galactose oxidase-α-^{3}H-acetylhydrazide labeling of AHGC-containing liposomes. Small unilamellar liposomes (0.5 ml, 0.065 μmole/ml), containing 10% ^{35}S-AHGC (specific activity = 0.22 mCi/mmole) and dissolved in 0.05 M Tris buffer (pH 7.4) + 0.14 M NaCl per tube, were treated with 25 units galactose-oxidase and 10 units of catalase. The galactose oxidase was left out of control liposomes. At the indicated times, diethyldithiocarbamic acid was added at a final concentration of 0.1 mM to triplicate tubes to block the galactose-oxidase [24]. Then 5.5 ml of 10 mM α-^{3}H-acetylhydrazide (specific activity 1.35 mCi/mmole) was added to the tubes and the tubes were incubated for an additional 12 h and then dialyzed against a 1,000-fold excess of buffer with five changes; 0.2 ml of the dialysate was counted and the $^3H/^{35}S$ ratios were determined. An additional 0.2 ml of the dialysate was used for a phosphate determination. There was no incorporation of ^{3}H above background in tubes lacking the galactose-oxidase.

TABLE I. Amount of Galactose vs Percentage Glycolipid in Liposomes

% AHGC in liposome	$^3H/^{35}S$
2.5	3.56
5.0	3.51
7.5	3.55
10.0	3.47

Experiments were performed identically to that shown in Figure 3, starting with different percentages of AHGC incorporated in the liposomes.

at 10°C is given in Figure 4. Pseudo-first order rates occur at ricin concentrations above approximately 125 μg/ml. The aggregation rates observed by the turbidometric assay were checked against a radiochemical assay. The 10% AHGC-containing liposomes were aggregated to different end points with varying concentrations of ricin. The aggregated liposomes were centrifuged and the amount of radioactivity in the pellet was determined. A plot of the extent of aggregation as determined by this method as well as by light scattering was determined to be a straight line, as shown in Figure 5. It is clear that the light-scattering assay can be taken as a good measure of the extent of liposomal aggregation. It should be mentioned that the ricin-mediated aggregation of these liposomes is reversible in the presence of β-galactosides. Ricin-aggregated liposomes were treated with α-lactose at different concentrations and an immediate decrease in light scattering was observed (Fig. 6). Even after remaining aggregated over night, the liposomes could still be easily disaggregated in the presence of α-lactose. Thus, substantial fusion of the liposome does not occur under the conditions of these experiments.

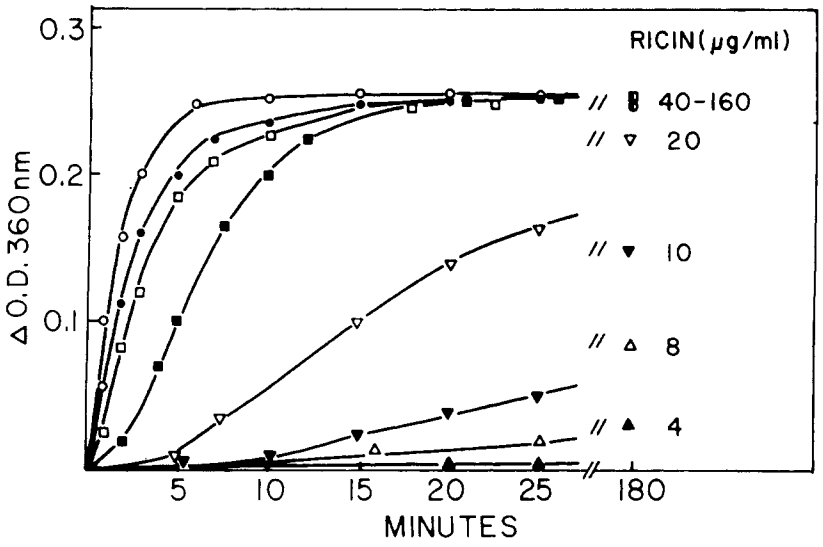


Fig. 4. Aggregation of 10% AHGC liposomes at varying ricin concentrations. Liposomes containing 10% AHGC were made up at a concentration of 0.06 μmole/ml and their rates of aggregation were followed at 10°C in the presence of 0, 2, 4, 8, 10, 20, 40, 80, 120, and 160 μg/ml ricin. The above plot shows that the rates and amplitudes of the aggregation reaction are dependent on the concentrations of ricin.

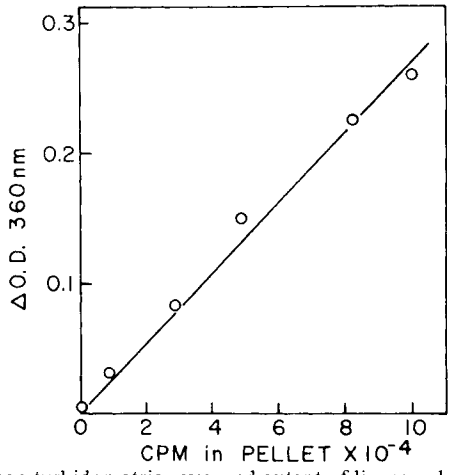


Fig. 5. Relationship between turbidometric assay and extent of liposomal aggregation. As in Figure 4, 10% ^{3}H-AHGC-containing liposomes were aggregated to completion in the presence of 4, 8, 10, 20, 40, 80, 120, and 160 μg/ml ricin. The samples were spun down at 100,000g for 2 h and the amount of radioactivity remaining in the pellet was determined. These values are plotted against the amplitude of the OD determined by light scattering. From 40 μg/ml on up, 87% of the total radioactivity is found in the pellet after centrifugation.

Nonlinear Effects of Glycolipid Concentration on Rates of Ricin-Mediated Aggregation of Derivatized Liposomes

AHGC-containing liposomes were prepared containing varying concentrations of the glycolipid. These liposomes were then treated with a saturating concentration of ricin, and the extents and rates of their aggregation were measured at 10°C (Fig. 7). Under the

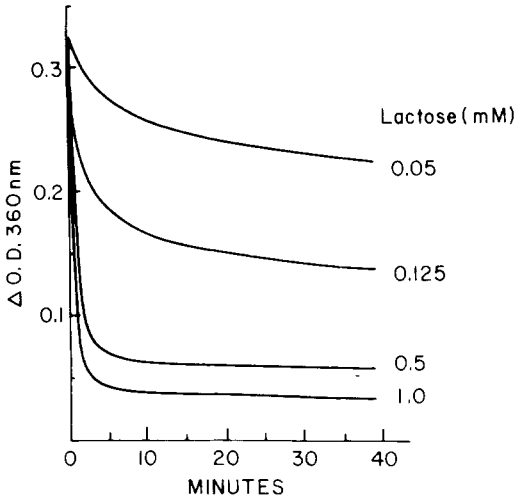


Fig. 6. Reversibility of ricin-mediated aggregation of AHGC-containing liposomes in presence of α-lactose. Liposomes (0.06 μmole/ml) containing 7.5% AHGC were aggregated to completion by 160 μg/ml ricin at 15°C in a total volume of 1 ml. After the amplitude of the aggreagtion had become maximal, 10 μl of α-lactose solution was added to final concentrations of 0.05 mM, 0.125 mM, 0.5 mM, and 1 mM. The graph shows the disappearance of the turbidity at 360 mM as a function of time in the presence of α-lactose.

conditions of these experiments the kinetics are clearly biphasic. Neither the lag period nor the pseudo-first-order rates of aggregation are linearly dependent on the sugar concentrations introduced into the liposomes. Figure 8 shows a plot of the lag periods and the first-order rate constants measured at the different glycolipid concentrations. A clear break occurs at approximately 5% glycolipid. Below this concentration the lag period markedly increases and pseudo-first-order rate constants markedly decrease. It should be noted that at higher phospholipid concentrations (approximately 0.2 μmole/ml) the lag period disappears and pseudo-first-order kinetics are observed (unpublished experiments). However, strongly nonlinear effects of the glycolipid concentrations on the rates of aggregation are again observed. Since the rates of aggregation are too rapid to measure conveniently at the higher glycolipid concentrations, kinetics were routinely studied at the lower phospholipid concentrations (0.06 μmole/ml).

Aggregation of Liposomes Formed From Mixed Glycolipids

Since the observed nonlinear aggregation behavior of the glycolipid-containing liposomes was not caused by nonlinear glycolipid incorporation, we sought to determine whether functional differences in the glycolipids introduced at the different concentrations could be measured. The experiments with galactose oxidase suggested, of course, that these putative differences do not in fact exist. However, more direct experiments were required in order to completely eliminate this possibility, since it could be argued that at low glycolipid incorporation the sugars are introduced in such a way that they are inaccessible to the lectin or in some way bound so that they cannot support aggregation, and only at higher glycolipid concentrations can fruitful incorporation occur. A model of this type would predict that glycolipids containing different sugars would exert a synergistic effect on each other when incorporated into liposomes in terms of the sensitivity of these liposomes to

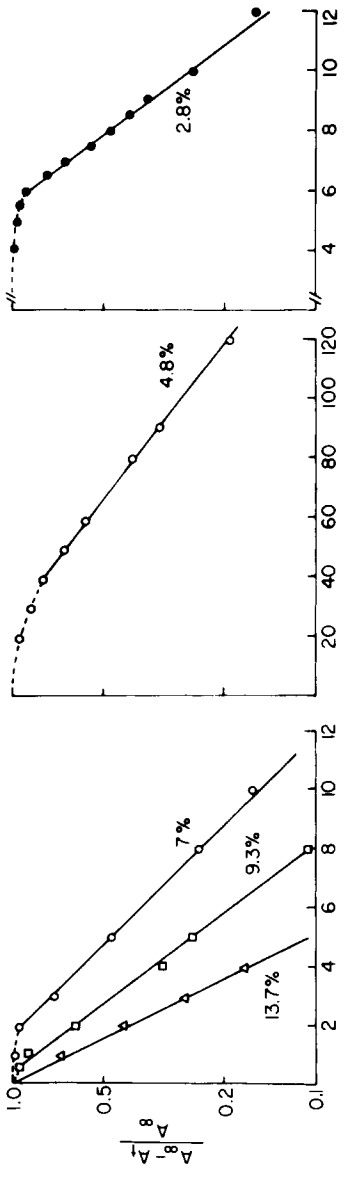


Fig. 7. Ricin-mediated aggregation of AHGC-containing liposomes at varying AHGC concentrations. Liposomes containing 2.8%, 4.8%, 7.0%, 9.3%, and 13.7% ^{3}H-AHGC were prepared. Pseudo-first-order rate plots for the rates of aggregation of these liposomes (0.06 mole/ml) by 160 μg/ml ricin at 10°C are shown. Accurate end points (A_∞) were obtained by allowing the ricin-mediated aggregation to proceed for several hours until no further increase in absorbance occurred. In all cases the same extents of aggregation were obtained.

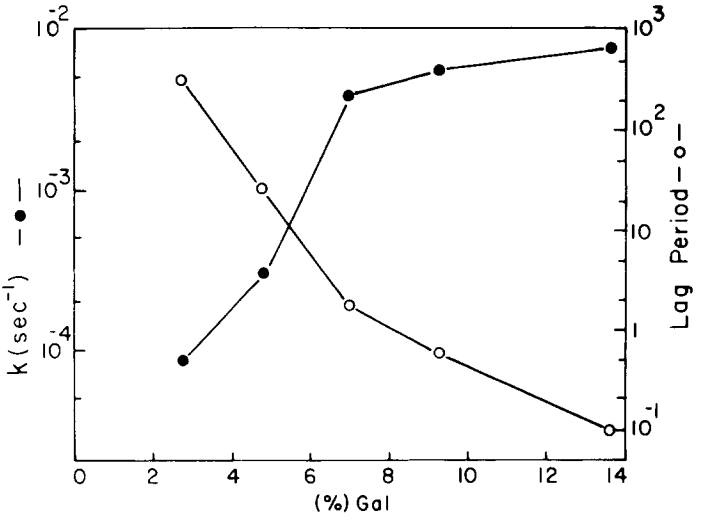


Fig. 8. Lag times and pseudo-first-order rate of aggregation as a function of AHGC concentrations. The lag time and calculated pseudo-first-order rate constants from Figure 7 are plotted as a function of the AHGC concentration.

aggregation by the appropriate lectins. Alternatively, if the glycolipids are introduced into the bilayer in a unimodal way, it would be predicted that the mixed glycolipids would behave independently of each other. Liposomes were prepared from 3.5% AHMC + 8% AHGC, and 5% AHMC + 8% AHGC. These mixed liposomes were treated with either ricin or Con A (Fig. 9). The rates of aggregation of these liposomes show that the glycolipids are behaving independently of each other. That is, the rates of aggregation of the liposomes by a particular lectin are unaffected by the second glycolipid. Both lectins could not be added to the same liposomal population because ricin precipitates Con A [19]. In addition to demonstrating the unimodal incorporation of glycolipids into these liposomes these experiments control for the effects of increasing the concentration of the cholesterol anchor on the rate of the lectin-mediated aggregation of these liposomes. There is no measurable effect.

Effect of Membrane Fluidity on the Rate of Aggregation of the Liposome

A question that arises in these experiments is "What is the effect of glycolipid mobility on the lectin-mediated aggregation rates of the liposomes?" To approach this question 7.5% and 10% AHGC- and AHMC-containing liposomes were prepared from dipalmitoyl phosphatidyl choline (DPPC). Liposomes prepared from the latter material alone show a phase transition at approximately 40°C [20]. Below this temperature lipid is in solid phase, and mobility within this membrane is sharply restricted; hence if mobility of the glycolipid is important for aggregation, these rates should be sharply decreased below the transition temperature [21]. The rates of aggregation of the DPPC-based liposomes were determined at temperatures between 4° and 35°C. As a control, the rates were also determined for the corresponding lecithin-based liposomes. As already mentioned, the rate of the ricin-mediated aggregation of AHGC-containing licithin liposomes achieves a maximum at ~10°. Corresponding experiments with Con A and AHMC-containing liposomes do not show the same profile. In these instances the rates increase with temperature in the range

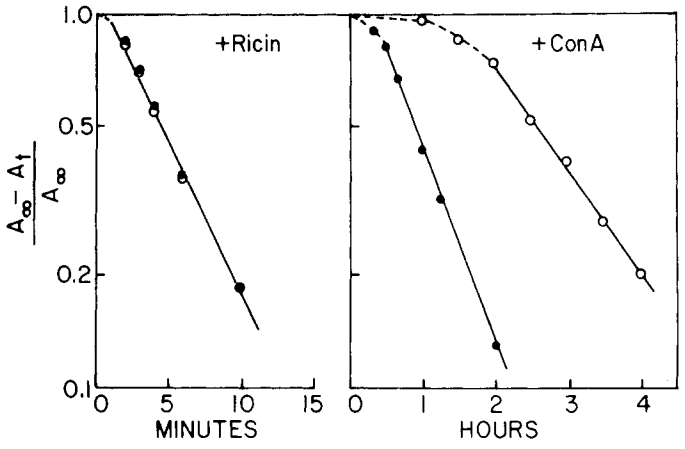


Fig. 9. Aggregation of liposomes prepared from AHGC and AHMC. Liposomes were prepared containing (3.5% AHMC + 8% AHGC) (○) and (5% AHMC + 8% AHGC) (●). Ricin (160 μg/ml) and Con A (100 μg/ml) were added, both at 10°C and rates of aggregation of these liposomes are followed at 360 nm.

studied in DPPC and more or less level off in lecithin. First-order rate plots for the 7.5 and 10% AHGC and AHMC liposomes treated with ricin and Con A, respectively, were made. In Figure 10 plots of the pseudo-first-order rate constants for the aggregation for the DPPC- and lecithin-based liposomes are given as a function of the temperature. As can be seen here, the rates of aggregation for the DPPC liposomes never show a sharp drop with temperature as would be expected if glycolipid mobility were required for aggregation to occur. The temperature profiles for the aggregation of the DPPC liposomes and lecithin liposomes appear similar. Clearly no phase transition can occur in the temperature ranges studied in the lecithin-based liposomes. Interestingly, the pseudo-first-order rate constants for the ricin-mediated aggregation of the AHGC-containing liposomes are actually larger in DPPC. As mentioned earlier, the phase transition for DPPC is still evident in the presence of 10% AHGC.

DISCUSSION

The incorporation of synthetic, cholesterol-containing glycolipid into small unilamellar liposomes renders these liposomes agglutinable by the appropriate lectin. These glycolipids are incorporated into the membrane bilayer in much the same way as cholesterol itself. For example, the cholesterol carbamates are distributed evenly on both sides of the bilayer, they show a condensing effect above the phase transition of the membrane, and they render the liposomes less permeable to small molecules. The only major difference noted so far between cholesterol and the cholesterol analogs is the decreased incorporation of the latter into egg yolk lecithin-based small unilamellar liposomes. This feature is shared by other cholesterol analogs having a blocked oxygen group [22]. Interestingly, the relationship between the concentration of glycolipid incorporated and the rates of the lectin-mediated aggregation of these liposomes was not linear. Below approximately 5% glycolipid, the lag period was markedly increased and the pseudo-first-order rates of aggregation were markedly decreased. This behavior seems typical in a variety of glycolipid-liposome systems which have been studied. For example, the ricin-mediated aggregation lactosyl

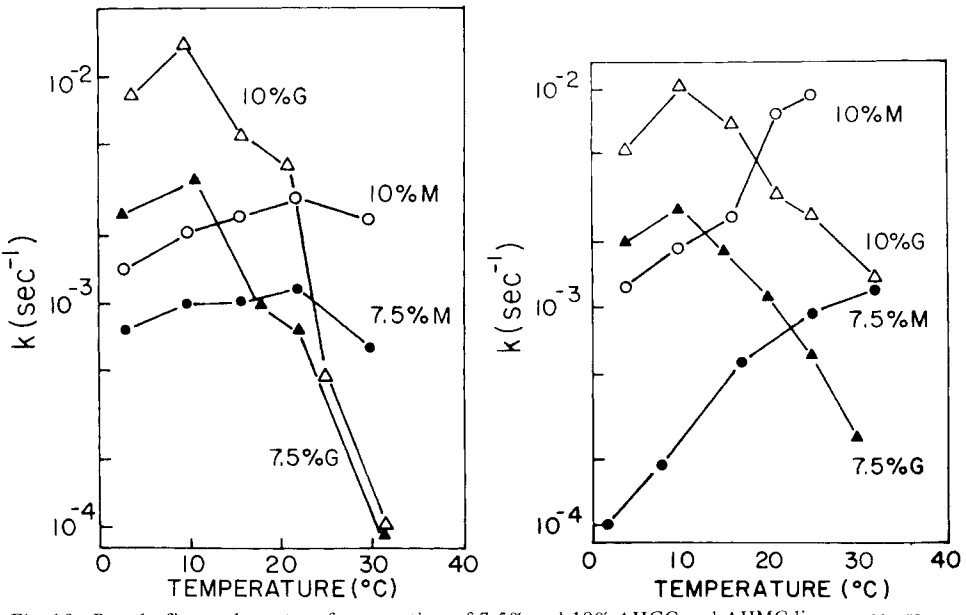


Fig. 10. Pseudo-first-order rates of aggregation of 7.5% and 10% AHGC and AHMC liposomes prepared from lecithin and DPPC at varying temperatures. Small unilamellar liposomes containing 7.5% and 10% AHGC as well as 7.5% and 10% AHMC were prepared from both lecithin and DPPC. The AHGC-containing liposomes (at 0.06 μM/ml) were treated with 110 μg/ml ricin and the AHMC-containing liposomes with 125 μg/ml Con A in the usual buffer at varying temperatures. At the left the pseudo-first-order data constants for the aggregation of the lecithin-based liposomes are shown and at the right the same data are shown for the DPPC liposomes.

ceramide-containing liposomes show a threshold at approximately 5% glycolipid as do liposomes containing monosialoganglioside GM_1 when treated with wheat germ agglutinin [1, 4]. Since the nature of these threshold effects are of some interest as models for cellular membrane-membrane interaction and recognition, we sought to study the underlying mechanisms.

The simplest mechanism possible for the observed nonlinear effects would result from the differential incorporation of glycolipids into the membrane at different concentrations. The experiments reported here with galactose oxidase (which show that the same inside-outside ratio is obtained at the different glycolipid concentrations), as well as our studies with liposomes prepared from mixtures of AHGC and AHMC (which show that the glycolipids behave independently), rule out this possibility. This suggests that the relationship between the aggregation response and the liposomal sugar levels is inherently cooperative. The kinetics that we have observed are reminiscent of those observed during the gelation of sickle cell hemoglobin [23]. In this case, the highly cooperative kinetics were interpreted to mean that a series of thermodynamically unfavorable nucleation steps occurred prior to the formation of a large enough aggregate whose further polymerization is highly favored [23]. The same phenomena could obtain here.

At the very least our data suggest that a minimal critical level of glycolipid is required in the membrane before aggregation will proceed at a measurable rate. Perhaps the simplest mechanistic hypothesis starts with the assumption that multiple bonds are required between liposomes to hold them together and that the radius of curvature of the liposomes imposes a limit to the number of possible bonds formed. The local density of

glycolipids would then become very important because the closer they are together the easier it would be for the lectin to bridge the gap between the liposomes. As the local density of the glycolipids decreased, the curvature of the liposome would move the sugar residues far enough apart on adjacent liposomes so that only a strained, high-energy form of the lectin could bridge the gap. However, once nucleation had occurred and small aggregates were formed, further aggregation should proceed rapidly to the same extent at various glycolipid concentrations. This is what is found. A mechanism of this type assumes that the mobility of the cholesterol analog in the membrane is not going to be important, since even at the lowest glycolipid concentrations studied (2.5%) there are more than enough external galactosyl moieties to cover the surface of the liposomes with ricin. That mobility is indeed not important is suggested by the temperature studies on the aggregation of the AHGC- and AHMC-containing DPPC and lecithin-based liposomes. The fact that mobility is not important here probably reflects the fact that the dissociation rate constant for the sugar-lectin complex far exceeds the rate of glycolipid mobility in the membrane.

It is of interest to note that threshold effects of the type described here have also been found in the Con A-mediated agglutination of erythrocytes which have had α-mannose residues randomly incorporated into the cell surface at varying levels [10]. If effects of this type can be demonstrated in naturally adhering cells, it would mean that large qualitative changes in recognition patterns could be based on small quantitive changes in cell surface sugar content. This would be a very simple and attractive way of arranging for great selectivity in cell-cell recognition without requiring there to be extended sequence information at the surface of cells. Threshold phenomena allow for the generation of enormous numbers of unique arrays with monomeric codons. This would be of particular interest in a developing system where adhesive patterns are time-dependent.

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