Lectin-Induced Agglutination of Phospholipid/Glycolipid Vesicles†

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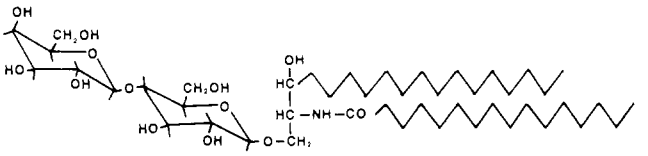
ABSTRACT: A model membrane system is described which exhibits characteristics similar to those observed for ligand binding to cell surfaces, in the absence of cellular energetic machinery or a cytoskeleton. *Ricinus communis* agglutinin (RCA), a plant lectin, reversibly agglutinates model membrane vesicles composed of egg yolk lecithin (EYL) and the glycolipid lactosylceramide (LC). The initial velocity of this agglutination depends upon pH, temperature, and the EYL/LC mole ratio. Vesicles containing less than 5 mol % LC are not agglutinated by RCA. At higher LC contents, agglutination increases with increased LC content. The temperature profile of RCA-induced agglutination exhibits a maximum at 25 °C. Decreased agglutination at higher temperatures may be due to a temperature-dependent reversible transition of the lectin. Decreased agglutination at low temperatures is correlated with differential scanning calorimetry studies of the EYL/LC vesicles, which suggest that a glycolipid lateral phase separation occurs at low temperatures. RCA-agglutinated EYL/LC vesicles can be deagglutinated by addition of sugars which compete with the vesicles for the lectin. Electron microscopy of deagglutinated vesicles indicates that extensive vesicle fusion has not taken place. Vesicles composed of EYL and bovine spinal cord cerebroside (galactocerebroside), a glycolipid which possesses the requisite terminal galactose for RCA binding, are not agglutinated by RCA, indicating that the glycosyl receptor must extend out from the bilayer surface to support lectin-induced agglutination.

The ability of mammalian cell-surface receptors to move in the membrane plane is affected by transformation (Rosenblith et al., 1973; Nicolson, 1973) and differentiation (Moscona, 1971). Various lines of evidence have suggested that receptor topography is controlled both by cytoskeletal components (Edelman et al, 1973; Ji and Nicolson, 1974; Poste et al., 1975; Bales et al., 1977; Aubin et al., 1975) and by membrane fluidity (Horwitz et al., 1974; Ben-Bassat et al., 1977; Maccecchini and Burger, 1977; Hatten et al., 1978). The determination of the extent to which each of these factors influences receptor mobility will depend upon the development of model systems in which these effects can be separated.

The role of glycolipids as mobile cell-surface receptors has been suggested by their ability to interact with lectins and hormones in liposomes prepared from total lipid extracts (Rendi et al., 1976) or from pure glycolipids and lecithin (Surolia et al., 1975; Redwood and Polefka, 1976; Aloj et al., 1977; Boldt et al., 1977; Curatolo et al., 1977). In this paper, we characterize the interactions of a lectin from castor beans (*Ricinus communis*) with sonicated vesicles composed of egg yolk lecithin (EYL)[1] and lactosylceramide[2] (LC), a neutral glycolipid whose structure is shown as structure I.



I

Materials and Methods

Materials. Ricinus communis agglutinin (RCA) was purified according to Nicolson and Blaustein (1972), as modified by Podder et al. (1974). RCA was stored in 0.01 M Tris-HCl–0.2 M NaCl (pH 7.2). EYL was extracted and purified according to Litman (1973). Lactosylceramide (*N*-palmitoyldihydrolactocerebroside, lot no. 3) was purchased from Miles Laboratories, Elkhart, Ind., and was repurified by silicic acid chromatography. After repurification, LC exhibited one spot on TLC in $CHCl_3/CH_3OH/H_2O$ (65:25:4). Bovine spinal cord cerebroside (galactocerebroside) was from Supelco, Inc., Bellefonte, Pa. Galactose (Gal) and lactose (Lac) were from Sigma Chemical Co., St. Louis, Mo. All other reagents were reagent grade. Water was doubly distilled. [*N-methyl*-^{3}H]-Choline-labeled EYL ([^{3}H]EYL) was synthesized using egg phosphatidic acid and [*N-methyl*-^{3}H]choline (New England Nuclear, Boston, Mass.) as previously described (Sears et al., 1976).

EYL/LC Vesicles. Solutions of EYL (in 2:1 chloroform/methanol, v/v) and LC (in chloroform) were mixed in appropriate proportions in a round-bottomed flask, and the solvent was removed in a rotary evaporator. After vacuum desiccation overnight, 8 mL of a buffer composed of 0.01 M Tris-HCl (pH 7.2) and 0.2 M NaCl was added to the flask, and the lipid was suspended by shaking for ~15 min at room temperature. This dispersion was sonicated for 20 min at 4 °C under a N_2 atmosphere, followed by centrifugation at 12 000*g* for 20 min at 4 °C to remove titanium fragments and multilamellar liposomes.

Lectin-Induced Agglutination. Ricinus communis agglutinin, in 0.01 M Tris-HCl (pH 7.2)–0.2 M NaCl, was added rapidly to an EYL/LC vesicle suspension and vortexed. The

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[1] Abbreviations used: EYL, egg yolk lecithin; LC, lactosylceramide; DSC, differential scanning calorimetry; RCA, *Ricinus communis* agglutinin; LDL, low-density lipoprotein; Gal, galactose; Lac, lactose; [^{3}H]EYL, [*N-methyl*-^{3}H]choline-labeled EYL; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

[2] Note that the glycolipid used in this study is not, strictly speaking, lactosylceramide but rather dihydrosphingosyllactosylceramide, in which the sphingosine moiety is completely saturated.

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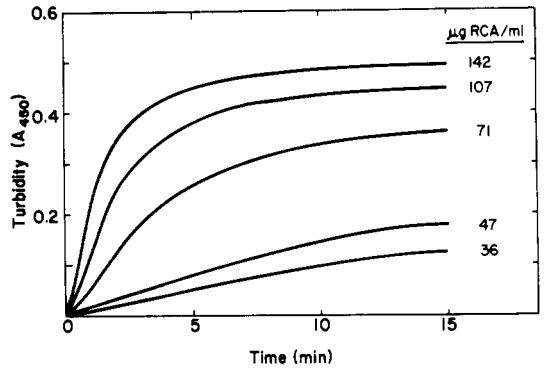

FIGURE 1: Turbidity increase observed upon the addition of RCA to a suspension of sonicated EYL vesicles containing 10 mol % LC. Incubation mixtures contained vesicles at an EYL concentration of 0.24 μmol/mL and various amounts of RCA, in a total volume of 2.1 mL.

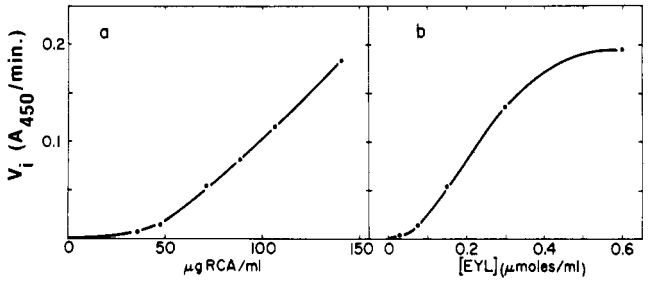

FIGURE 2: (a) Initial velocity of agglutination of EYL vesicles containing 10 mol % LC as a function of RCA concentration. Incubation conditions are identical to those in Figure 1. (b) Initial velocity of agglutination as a function of vesicle concentration. 100 μL of RCA (at 1.5 mg/mL) was added to various amounts of EYL/LC vesicles (10 mol % LC), in a total volume of 2.1 mL.

mixture was immediately transferred to a spectrophotometer, and agglutination was followed by the turbidity (A_{450}) increase with time. Lectin-induced agglutination was studied systematically as a function of lectin concentration, vesicle concentration, phospholipid/glycolipid ratio, pH, and temperature. Throughout this study, the initial velocity of agglutination is reported because prolonged incubation (>30 min) of EYL/LC vesicles with RCA results in the formation of very large aggregates (~0.5-mm diameter) which sediment to the bottom of the tube.

Electron Microscopy. Sonicated vesicles were negatively stained with 2% sodium phosphotungstate (pH 7.4) on Formvar-coated copper grids. Electron micrographs were obtained with an AEI-GB electron microscope, calibrated with a catalase standard.

Differential Scanning Calorimetry (DSC). EYL/LC vesicles for DSC were prepared as above, concentrated in an A-75 minicon dialyzing apparatus (Amicon Corp., Lexington, Mass.), and hermetically sealed in stainless-steel pans (50 μL capacity). Scanning calorimetry traces were obtained on a Perkin-Elmer DSC-2 differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, Conn.) at a heating/cooling rate of 5 °C/min at 0.2 mcal/s sensitivity. Peak areas were determined by planimetry, and enthalpies were calculated on the basis of phosphorus assays of the contents of the DSC pans (Gomori, 1942).

Results

Addition of *Ricinus communis* agglutinin to a clear sonicated suspension of EYL containing 10 mol % LC results in an increase in the turbidity of the suspension which can be monitored by absorbance at 450 nm. The time course of the

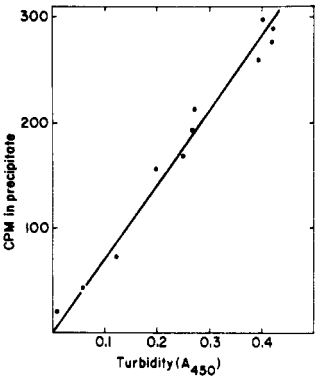

FIGURE 3: Relationship between the turbidity (A_{450}) of RCA-agglutinated EYL/LC vesicle suspensions after a 1 h incubation and the mass of agglutinated material which can be sedimented at 23 000*g* (cpm). Incubation mixtures contained EYL/LC vesicles (10 mol % LC) at an EYL concentration of 0.24 μmol/mL and various amounts of RCA, in a total volume of 2.1 mL.

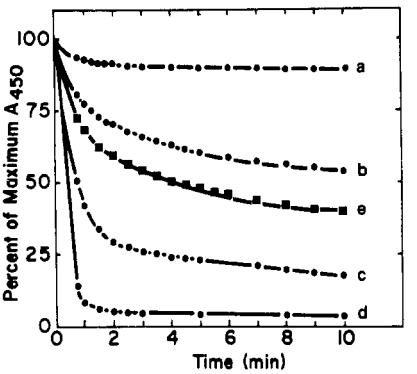

FIGURE 4: Deagglutination of RCA-agglutinated EYL/LC vesicles (10 mol % LC) by lactose (● - - ●) and galactose (■ - - ■). Sugar concentrations are: (a) 4.6, (b) 46, (c) 150, and (d) 460 μM lactose and (e) 460 μM galactose.

lectin-induced agglutination at various RCA concentrations (at 24 °C) is shown in Figure 1. Both the rate and the extent of the agglutination depend upon RCA concentration. The initial velocity of agglutination (A_{450}/min) increases linearly with RCA concentration over the range 50–150 μg of RCA/mL, while very little agglutination is observed below 50 μg/mL (Figure 2a). The dependence of the initial velocity of agglutination upon vesicle concentration shows a similar lag at low vesicle concentrations, as shown in Figure 2b. To validate the use of turbidity as a quantitative measure of agglutination, [^{3}H]EYL vesicles containing 10 mol % LC were agglutinated with various amounts of RCA, and the turbidity (A_{450}) was measured following a 1-h incubation at 24 °C and vigorous vortexing to break up large aggregates. The suspensions were centrifuged at 23 000*g* for 20 min (24 °C), and the radioactivity in the clear supernatants was measured. Figure 3 is a plot of counts per minutes in the precipitate (obtained by subtraction) as a function of turbidity after a 1-h incubation. The plot is linear (r = 0.987) and extrapolates to the origin, indicating that turbidity at 450 nm is directly proportional to the mass of material agglutinated.

The agglutination of EYL/LC vesicles can be reversed by the addition of sugars which compete with the glycolipid in the vesicles for sugar-binding sites on the lectin. In Figure 4 is shown the effect of the addition of lactose and galactose on the turbidity of lectin-agglutinated vesicle suspensions. The rate and extent of deagglutination are dependent upon sugar concentration, as shown for lactose (the more effective of the two

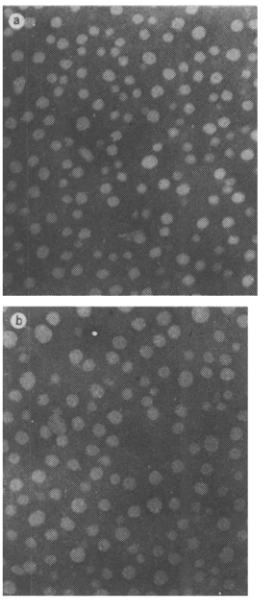

FIGURE 5: Electron micrographs (negative stain) of (a) sonicated EYL/LC vesicles (10 mol % LC), and (b) same after agglutination with RCA and deagglutination with lactose (×60 000).

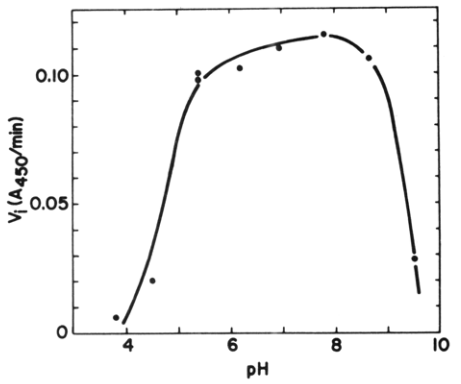

FIGURE 6: Effect of pH on the initial velocity of agglutination of EYL/LC vesicles (10 mol % LC).

sugars). Electron microscopy of lactose-deagglutinated vesicle suspensions shows only small particles, verifying that lectin-induced agglutination does not result in extensive vesicle fusion (Figure 5).

The pH dependence of the initial velocity of agglutination of EYL vesicles containing 10 mol % LC is presented in Figure 6. The agglutination exhibits a broad pH maximum, ranging from pH 5.4 to 8.7. The decrease in agglutination at pHs below and above this range is presumably due to conformational changes in the protein, since neither EYL nor LC exhibit a pK_a in this range. The initial velocity of agglutination undergoes a significant variation with the phospholipid/glycolipid ratio, as illustrated in Figure 7. No agglutination is observed at LC contents less than 5 mol %, with minimal agglutination observed at 5 mol %. At higher LC contents, the initial velocity of agglutination increases with increasing LC content.

The temperature dependence of the initial velocity of agglutination of EYL vesicles containing 10 mol % LC was determined and is shown in Figure 8. Agglutination velocity increases with increasing temperature, reaching a maximum at 25 °C, and decreases at higher temperatures. When a sample containing EYL and RCA is incubated at 37 °C (a temperature at which minimal agglutination is observed) and then decreased in temperature to 25 °C, the turbidity increases.

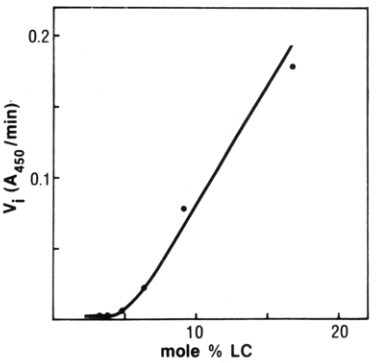

FIGURE 7: Effect of LC content on the initial velocity of RCA-induced agglutination of EYL/LC vesicles.

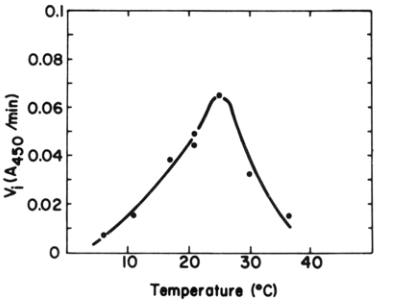

FIGURE 8: Temperature dependence of the initial velocity of RCA-induced agglutination of EYL/LC vesicles (10 mol % LC).

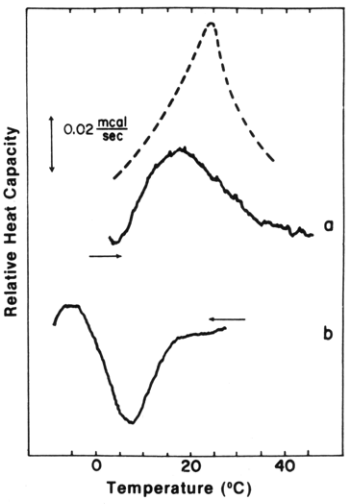

FIGURE 9: Differential scanning calorimetry traces of sonicated EYL/LC vesicles (10 mol % LC), obtained at a heating/cooling rate of 5 °C/min: (a) heating run; (b) cooling run. The temperature dependence of agglutination (from Figure 9) is reproduced for comparison (dashed curve).

If this sample is returned to 37 °C, a decrease in turbidity is observed, indicating deagglutination. However, if vesicles are agglutinated at 25 °C, they do not deagglutinate when the temperature is lowered to 4 °C and remain as a stable aggregate at this temperature for several days. In order to determine whether the temperature dependence of agglutination is related to some property of the EYL/LC vesicles, the vesicles were studied by differential scanning calorimetry. DSC thermograms of sonicated EYL vesicles containing 10 mol % LC are presented in Figure 9. (The temperature dependence of agglutination is superimposed on the calorimetric heating run.) A broad transition is observed, which spans the temperature range 0–32 °C, with a single peak at 17 °C on heating. The calorimetric transition coincides with the ascending portion

(5–25 °C) of the temperature profile of agglutination.

RCA was unable to agglutinate EYL vesicles containing 20 mol % bovine spinal cord cerebroside (galactocerebroside) at 25 °C, even though this glycolipid possesses the requisite terminal galactose for RCA binding. It appears that the terminal galactose must be located at least two sugar residues out from the ceramide backbone to support vesicle agglutination. EYL vesicles which lack glycolipid are not agglutinated or fused by RCA.

Discussion

The development of model systems for the study of cell-surface phenomena is a necessary prerequisite for separation of the various effects involved in ligand binding to cell surfaces and in cell–cell interaction. The phospholipid/glycolipid vesicle system described in this paper allows the study of the interactions of a lectin with a simple model membrane in the absence of the cellular energetic machinery and cytoskeleton.

When *Ricinus communis* agglutinin is added to a suspension of sonicated phospholipid/glycolipid vesicles, agglutination of the vesicles into large aggregates occurs, indicated by an increase in the turbidity of the suspension. The possibility that this turbidity increase represents irreversible fusion of vesicles to form larger particles is eliminated by the observation that the process can be completely reversed by the addition of saturating amounts of lactose. This deagglutination is evidenced by a decrease in the turbidity to the level observed before addition of lectin and is corroborated by the observation that no large fused vesicles are seen by electron microscopy. The agglutination presumably occurs via RCA cross-bridges between glycolipid molecules on different vesicles. Since RCA is divalent (Olsnes et al., 1974; Podder et al., 1974), one RCA molecule may be able to bridge two vesicles, or, alternatively, self-aggregated multimers of RCA may form the cross-bridges.

The agglutination of EYL/LC vesicles by RCA exhibits specificity: RCA can agglutinate EYL/LC vesicles but not EYL/cerebroside vesicles or vesicles composed of EYL alone. Space-filling models of galactocerebroside and lecithin indicate that when in a bilayer the monoglycosyl moiety of galactocerebroside is positioned at the level of the lecithin choline head groups and so may be inaccessible to RCA (Figure 10). The terminal galactose residue of lactosylceramide, on the other hand, projects out above the phospholipid choline head groups and so is more accessible to binding by RCA.

The surface concentration and distribution of lactosylceramide in the EYL/LC vesicles would be expected to be important determinants of the extent of RCA-induced agglutination. No agglutination of vesicles containing less than 5 mol % LC occurs, as was also observed by Redwood and Polefka (1976) for wheat germ agglutinin-induced fusion of EYL/ganglioside vesicles. A similar requirement for a threshold concentration of surface receptors has also been reported for antibody-induced agglutination of red blood cells (Hoyer and Trabold, 1970; Tsai et al., 1978). Above 5 mol % LC, the velocity of agglutination of EYL/LC vesicles increases with increasing LC content. This suggests that the formation of stable lectin bridges between vesicles is a function of the surface density of glycolipid receptors. This dependence can be explained by the hypothesis that a stable vesicle aggregate requires more than one attachment point between two vesicles, as has been suggested for lectin-induced agglutination of transformed cells (Nicolson, 1971).

The temperature dependence of agglutination described in Figure 8 indicates that factors other than gross receptor surface concentration are involved in lecitin-induced agglutination.

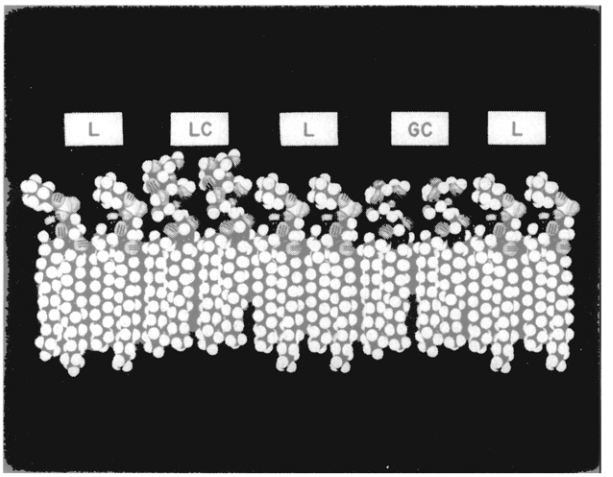

FIGURE 10: CPK model of one leaflet of a lecithin (L) bilayer illustrating the difference in the protrusion beyond the bilayer surface of lactosylceramide (LC) compared to galactosylceramide (GC). The terminal galactose of lactosylceramide clearly extends beyond the phosphorylcholine head groups of the lecithin, whereas the galactose of galactosylceramide lies in the plane of the lecithin polar head groups. Extension of the glycosyl receptor moiety beyond the plane of the lecithin head groups appears to be necessary for agglutination by *Ricinus communis* agglutinin.

Sonicated EYL vesicles containing 10 mol % LC undergo a broad thermal transition which spans the temperature range 0–32 °C. EYL alone exhibits a transition at ca. −5 °C. If all the lipid in the vesicles (EYL + LC) is undergoing an acyl chain order–disorder transition, the calculated enthalpy (ΔH) for this transition is 1.02 ± 0.12 cal/g. This is an unusually small ΔH for an acyl chain order–disorder transition (Ladbrooke and Chapman, 1969). If, on the other hand, the transition involves only the lactosylceramide, a ΔH of 11.9 ± 1.27 cal/g is calculated, which is a more reasonable enthalpy for an acyl chain order–disorder transition. We infer that, over the temperature range 0–32 °C, the LC in these EYL/LC sonicated vesicles undergoes an acyl chain transition. By analogy with studies of mixtures of phospholipids (Shimshick and McConnell, 1973; Grant et al., 1974), this transition may be accompanied by a lateral phase separation into LC-rich regions at low temperatures (below the transition). Above the transition, a relatively homogeneous distribution of LC in the EYL bilayer would exist. Since DSC cannot provide *direct* evidence for lateral phase segregation, we are currently studying phospholipid/glycolipid phase behavior by ^{2}H NMR, using deuterated lipids.

The maximum initial velocity of agglutination is observed at a temperature (25 °C) at which the calorimetric transition is almost complete (on heating), while at lower temperatures less agglutination takes place. The failure of RCA to agglutinate EYL/LC vesicles at low temperatures may be due to either (1) the inability of LC to diffuse laterally at temperatures below its acyl chain order–disorder transition or (2) the inability of RCA to bind to the lactosyl head group of LC when the glycolipid is in a laterally segregated surface. In such a state lateral interactions between the glycosyl head groups could serve to prevent binding.

The decrease in agglutination with increasing temperature above 25 °C may not be related to the thermal behavior of the EYL/LC vesicles. RCA is also able to agglutinate human serum low-density lipoprotein (LDL) (Yau A., Curatolo, W., Small, D. M., and Sears, B., manuscript in preparation) and the temperature dependence of LDL agglutination shows a similar decrease at temperatures greater than 25 °C (and a plateau below 25 °C). This decreased agglutination observed

in both systems may be due to a reversible temperature sensitivity of the lectin. Preliminary quasielastic laser light-scattering studies of RCA indicate that the lectin may undergo a small conformational transformation at about 25 °C (Young, C. Y., personal communication), while UV difference spectroscopy reveals no large temperature-dependent changes (Curatolo, W., unpublished observation).

In summary, we have described a model membrane system which exhibits characteristics similar to those observed for lectin-induced cell–cell agglutination: specificity and crypticity. We have shown that such characteristics do not necessarily rely on cellular energetic machinery or the presence of a cytoskeleton, although we would certainly not understate the importance of these components in cell-surface phenomena such as capping. A recent fluorescence photobleaching recovery study of bilayer lipid membranes has reached similar conclusions (Wolf et al., 1977). Furthermore, we have shown that the physical state of the glycolipid receptor in the EYL/LC model system plays an important role in the lectin-induced agglutination. The complexity of factors involved in agglutination in this relatively simple model system suggests that the molecular basis of agglutination in natural systems is quite complicated. Therefore, a systematic study of model systems should aid in assigning the relative roles of various membrane components and membrane-associated components in cell–ligand and cell–cell interactions.

Acknowledgments

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