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Complex carbohydrate–lectin interaction at the interface: a model for cellular adhesion. I. Effect of vesicle size on the kinetics of aggregation between a fatty acid conjugate of lectin and a liposomal asialoganglioside

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Two types of phospholipid vesicles capable of mutual recognition have been tailor-made to serve as a model system for the study of carbohydrate-mediated cellular adhesion. One of the vesicles contained a fatty acid conjugate of a galactose specific lectin (lectin vesicle) and the other an asialoganglioside with a reactive terminal galactose residue (galactose vesicle). The kinetics of aggregation of these two types of vesicles was followed by monitoring time-dependent change in turbidity. A 10–100-fold enhancement in the forward rate constant (k_f ranging from $7.1 \cdot 10^5$ to $4.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at 27°C) was observed when compared with that for the lectin-galactose system in solution (k_f being $4.5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$), reported in the literature. A study of the influence of vesicle size on the rate of aggregation showed that enhancement depended on the curvature of the galactose vesicle rather than the density of asialoganglioside suggesting a possible diffusion in the plane of the membrane. The ratio, k_f/k_d is found to be approx. 10^{10} M^{-1} indicating that the formation of multiple bonds plays a role for stable adhesion.

Introduction

The presence of glycoconjugates at the membrane surface of cells is considered to be important in a variety of membrane-mediated phenomena. At the level of the plasma membrane their role as recognition sites for hormones, toxins and viruses is well established [1–3]. Lately glycoconjugates, e.g., gangliosides have been found to play an important role in cell-cell adhesion [4,5]. Various theoretical models have been proposed to elucidate the cellular adhesion at the molecular level [6]. The theory of specific interactions [7] in the antigen-antibody or lectin-receptor systems have been invoked in order to explain selectivity in cell-cell adhesion. Experimental verification of the above model is difficult since they involve simulation of well-defined membrane-bound receptor–ligand interactions. The ideal choice to study such interactions should have been 'cell adhesion molecules' (CAM). But the limited availability of such molecules needed for reconstitution have impeded quantitative studies. The functional reconstitution of multivalent proteins brings about additional complexities. However, antigen-antibody reactions offer excellent model system to study mutual recognition because of their high affinity and diffusion controlled rates when studied in form of two apposing particles bearing complimentary molecules [8,9].

In our laboratory, we have taken lectin-ganglioside as complimentary molecules because of their availability in large quantities and possibly they play a major role in the carbohydrate-mediated cellular adhesion [10]. At the same time, a liposomal model system perhaps is the best surface for bringing out the details in the cellular adhesion phenomena. It has already been shown that lectins can recognise the complex carbohydrate sequence of gangliosides embedded in liposomes. As a result polyvalent lectins give rise to precipitin reactions resembling cell-cell agglutination which can be monitored by following the change in turbidity. The rate of this aggregation is strongly dependent on the density of the ganglioside component present on the liposomal surface [11].

The main objective of using the liposomal system for modelling the carbohydrate-mediated cell-cell adhesion

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in the present study is to demonstrate the formation of bonds between specific components on the juxtaposed membranes and thus estimate the relative stability of bonds formed. Here we report the reactivity of a ganglioside embedded in a liposome (galactose vesicle) as a function of the density and vesicle size, towards a fatty acid conjugate of the complimentary lectin molecule incorporated in the apposing liposome (lectin vesicle). Results presented here show that the kinetic control of aggregation is very sensitive to the vesicle size and increases by one order of magnitude for a change in diameter from 200 Å to 600 Å. It may be emphasised that the size-dependent kinetics of the ligand–receptor interaction reported there for the first time, has an important bearing in understanding the molecular mechanism of membrane-mediated phenomena in general.

Materials and Methods

Dimirystoylphosphatidylcholine (DMPC), cholesterol, galactose, sodium cholate, octyl glucoside, Tris and lauryl sulphate (SDS) were obtained from Sigma Chem. Co. Rhodamine 6G was purchased from Eastman Kodak Co. The other chemicals used were all of analytical grade and commercially available. A mixture of gangliosides was isolated from sheep's brain [12] and purified by passing through a DEAE-Sephadex column (acetate form). AsialoGM$_1$ was prepared by hydrolysing the ganglioside mixture with 1 M formic acid and collected after passing through a DEAE-Sephadex column.

The *Ricinus communis* agglutinin (RCA$_1$) was isolated from castor seeds using DEAE-Sephadex followed by a G-100 Sephadex column chromatographies [13]. The protein concentration was determined spectrophotometrically at pH 7.4 in a buffer containing 10 mM Tris-HCl, 100 mM NaCl (Tris buffer) using an absorbance value $A^{1\%}_{280\ nm} = 11.8$ [14].

Preparation of octadecylrhodamine 6G (R_{18}). Stearoyl chloride was reacted with Rhodamine 6G in dry benzene in the presence of pyridine with constant stirring at room temperature for 2 h. The mixture was kept overnight in a dessicator. The product was purified by thin-layer chromatography on Silica gel G coted plate in a solvent mixture of chloroform/ethyl acetate (2 : 1, v/v). The product (R_{18}) was stored with silica gel as dry powder at −20 °C.

Preparation of conjugated lectin. Stearoyl chloride, obtained by reacting stearic acid (crystallized from dry acetone) and thionyl chloride according to the method described by Ralston and Selby [15], was added (50–80 μl) to a buffer (0.1 M phosphate, 0.15 M NaCl, pH 8.) containing 2 percent sodium cholate (cholate buffer), dispersed by vortexing and to that mixture an equal volume of lectin solution (10–20 μM) in phosphate-buffered saline (0.05 M phosphate, 0.15 M NaCl, pH 7.4) containing 0.1 M galactose was added. The resulting mixture was incubated for 20 min at 4 °C [16,17]. The excess cholate was then dialysed against Tris buffer.

Preparation of lectin vesicles. For the preparation of vesicles containing fatty acid conjugate of the lectin, conjugated RCA$_1$ in cholate buffer was added to the sonicated lipid vesicles (10–20 μmol of phospholipid) and the excess cholate (2.5-times more than that of the phospholipid) was then dialysed against Tris buffer to get the vesicle containing the fatty acid conjugate of RCA$_1$.

Preparation of galactose vesicles. A DMPC/asialoGM$_1$ lipid film, made in a Brosil glass tube by passing nitrogen through the lipid solution in chloroform/methanol (1 : 1, v/v), was dessicated under vacuum for 24 h. The dry film was then hydrated with Tris buffer, dispersed by vortexing and the dispersion was sonicated in a bath-type Elama Trans Sonic (T410) sonicator to clarity. The vesicles were centrifuged at 22 000 × g for 20 minutes to remove large multilamellar vesicles.

The self quenching probe (R_{18}) was incorporated in the liposome while making the lipid film and the same was followed for cholesterol.

Molecular weights were determined form the data given by Zumbuehl and Weder [18] by constructing a calibration line plotting molecular weights vs diameters of vesicles. For vesicles containing cholesterol, molecular weights were taken from Johnson [19]. A batch of vesicles was prepared by the dialysis of octyl glucoside (lipid/detergent 1 : 15, mol/mol) following the method of Mimms et al. [20].

Estimations of sugar and lipid phosphate were done according to methods described earlier [21,22].

Turbidity measurements. The change in turbidity was measured by a Shimadzu UV-190 double beam spectrophotometer equipped with U-135 recorder at a wavelength of 340 nm.

Fluorescence measurements. Fluorescence spectra were taken in a Hitachi 650-60 spectrofluorimeter with asialoGM$_1$ bearing DMPC liposomes containing R_{18}. The excitation wavelength was 520 nm with a bandwidth of 5 nm operating in ratio mode. The emission maximum was found at 550 nm. The vesicles were disrupted with 5–10 mM SDS to get the fluorescence intensities at infinite dilution for determining the self quenching of the probe [23].

Calculation of rate constants.

$$\text{Lectin vesicle} + \text{galactose vesicle} \underset{k_d}{\overset{k_f}{\rightleftharpoons}} \text{aggregate}$$

The semilogarithmic plots of change in absorbance at 340 nm with time are linear indicating a binary association of the vesicles. The slope of the semilog plot of the

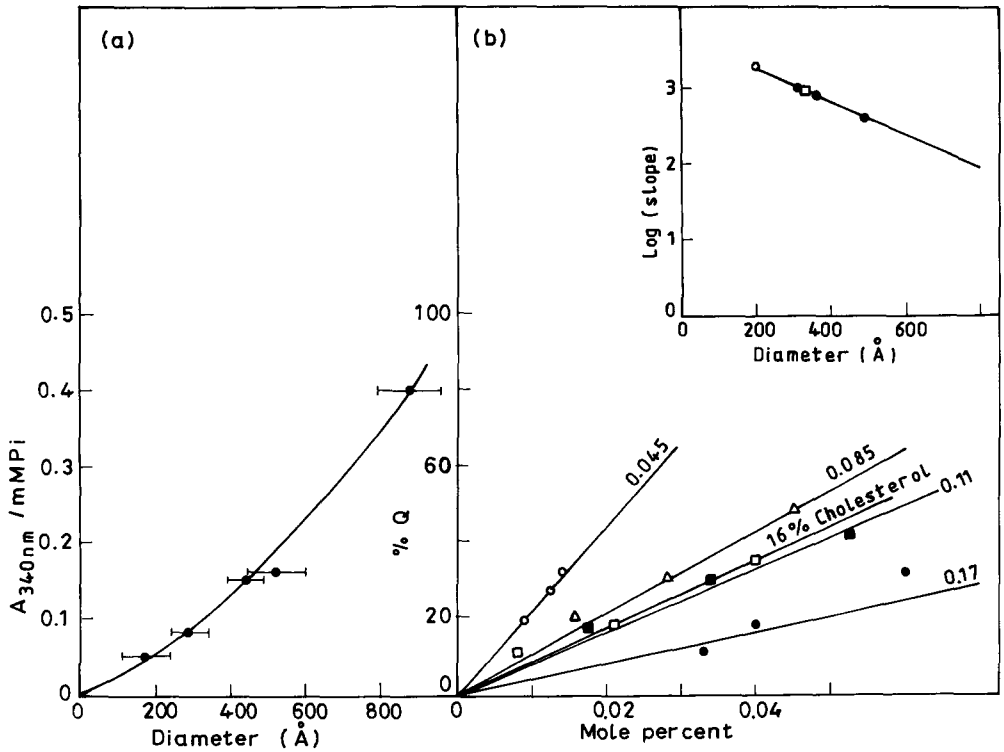

Fig. 1. Determination of size of the galactose vesicle by electron microscopy and by the self-quenching of R_{18} incorporated in the liposome. (a) Liposomes with different A_{340nm}/mM P_i values are viewed under the electron microscope after negative staining. Measured diameters are plotted against their respective A_{340nm}/mM P_i values. (b) The percentages of self-quenching of R_{18} incorporated in vesicles with different A_{340nm}/mM P_i values (as indicated by the numbers) are plotted against the mole percent of R_{18} with respect to DMPC. The inset shows a plot of the logarithm of slopes of the straight lines versus diameter as determined from Fig. 1a.

rate curve gives the pseudo-first-order rate constant (k'_d s^{-1}). The second-order rate constant is given by

$$k'_f = \frac{k'_d}{[\text{asialoGM}_1]} M^{-1} \cdot s^{-1} \quad (1)$$

where [asialoGM$_1$] is the molar concentration of the excess component. The normalised second-order rate constant becomes

$$k_f = k'_f \cdot \frac{\text{molecular weight of gal-vesicle}}{\text{molecular weight of asialoGM}_1} M^{-1}(\text{vesicle}) \cdot s^{-1} \quad (2)$$

When RCA_I is free in solution, the bimolecular rate constant (k_{app}) is calculated according to Haynes and Westine [24] as

$$k_{app} = \frac{N_{\text{asialoGM}_1}}{2 \cdot t_{0.5} \cdot [\text{asialoGM}_1]} M^{-1}(\text{receptor}) \cdot s^{-1} \quad (3)$$

where $N_{\text{asialoGM1}}$ is the number of asialoGM$_1$ molecules per vesicle and $t_{0.5}$ being the time corresponding to 50 percent formation (as reflected in the turbidity measurements).

The dissociation rate constant (k''_d) was calculated from the slope of the semilog plot of galactose-induced dissociation profile. The normalised k_d value is obtained as

$$k_d = \frac{k''_d}{[\text{galactose}] \cdot K_{eq}} s^{-1} \quad (4)$$

where K_{eq} is known to be $1.2 \cdot 10^3$ for RCA_1-galactose system [25].

Results

In order to provide a convenient assay of vesicle diameter, light scattering from absorbance and concentration measurements was calibrated by electron microscopy. Vesicles of different batches with different A_{340nm})/mM P_i values were examined under an electron microscope (Philips 301) after negative staining with 2 percent of phosphotungstic acid at pH 7.0. From the average diameter of the vesicles a calibration curve was obtained by plotting A_{340nm}/mM P_i against the diameter as shown in Fig. 1a. In addition, the self quenching of the fluorescent probe (R_{18}) was used as a further measure of the size. By plotting the self quenching of R_{18} fluorescence against the mole percent, different lines were obtained as shown in Fig. 1b for different populations of vesicles having different values of A_{340nm}/mM P_i. The slope of the stright lines gave a measure of the size of liposomes containing asialoGM$_1$ as shown in the inset. Fig. 2 shows a representative electron micrograph of the galactose vesicles.

Fig. 3 shows the time-dependent changes in absorbance due to the aggregation of vesicles. Subsequently a disaggregation upon addition of galactose is

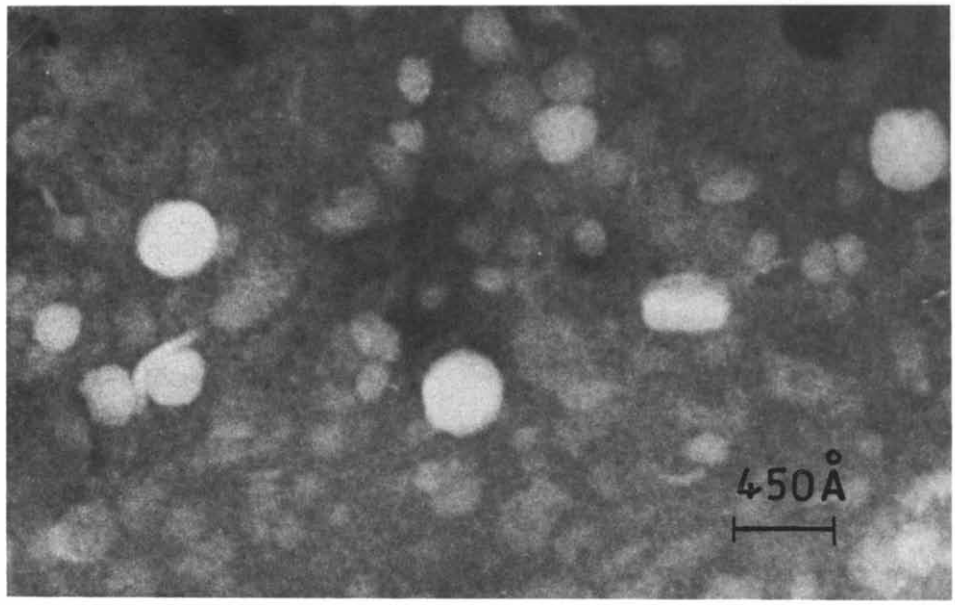

Fig. 2. An electron micrograph of galactose vesicles containing 6% of asialoGM$_1$ prepared by sonication. The A_{340nm}/mM P_i value was 0.15.

also evident. In the case of vesicle-bound RCA_I or the free conjugated RCA_I the concentration of galactose needed for complete disaggregation was more compared to that required in the case of the native lectin. This could result from the non-uniform distribution of fatty acid conjugates leading to the formation of multiple bonds. Hence we have defined a term CD_{50} (concentration required to bring about 50 percent disaggregation) to serve as a parameter for affinity. The semilog plot of the rate curves for the aggregation of lectin vesicles (or the conjugated RCA_I) and galactose vesicles are linear indicating a pseudo-first-order reaction, characterized by a single time constant. The kinetic data obtained at the initial stage is considered as representing the binary association. Fig. 4 shows a linear plot of k'_d versus the concentration of the excess component i.e., asialoGM$_1$ in galactose vesicle. From the slope and the intercept k'_f and k''_d values are determined. It is evident from Fig. 5 that normalised k_f M(ves)$^{-1} \cdot s^{-1}$ values, where M^{-1} (ves) refers to the molar concentration of galactose vesicles, depend strongly on the diameter of the vesicle. k_f increases from $7.1 \cdot 10^5$ to $2.75 \cdot 10^7$ for a change in the vesicle diameter from 250 Å to 800 Å. Table I lists a few forward and backward rate constants showing a

TABLE I

Normalised forward and backward rate constants of the aggregation reaction between lectin vesicle and galactose vesicle

Values in parenthesis are determined from the intercept of Fig. 4. All numbers are averages of at least two determinations at 27°C. Molecular weight and diameter of lectin vesicles are $6.4 \cdot 10^7$ and 1000 Å, respectively [16].

Aggregating species	Galactose vesicle		Density of asialoGM$_1$	k_f (M^{-1}(vesicle)$\cdot s^{-1}$)	k_d (s^{-1})
	diameter (Å)	molecular weight			
Lectin vesicle	190 [a]	$1.5 \cdot 10^6$	5	$3.6 \cdot 10^5$	–
DMPC (1.2–1.5 mM)	250 [a]	$5.5 \cdot 10^6$	2	$7.1 \cdot 10^5$	$(5.1 \cdot 10^{-3})$
RCA_I (1.2–1.5 μM)	520 [a]	$2.7 \cdot 10^7$	5	$1.73 \cdot 10^7$	$(1.5 \cdot 10^{-3})$
Galactose vesicle aGM$_1$ (12.0–25.0 μM)	790 [a]	$4.9 \cdot 10^7$	8	$2.75 \cdot 10^7$	$4.7 \cdot 10^{-3}$
Conjugated RCA_I (0.5–1.0 μM)	330 [b]	$7.95 \cdot 10^6$	3	$5.35 \cdot 10^6$	–
	250 [c]	$5.5 \cdot 10^6$	5	$8.1 \cdot 10^5$	$9.14 \cdot 10^{-3}$
Galactose vesicle aGM$_1$ (12.0–25.0 μM)	600 [d]	$3.71 \cdot 10^7$	8	$2.37 \cdot 10^7$	$1.26 \cdot 10^{-3}$
	2200 [e]	$2.84 \cdot 10^8$	7	$4.54 \cdot 10^7$	$4.9\ 10^{-3}$

[a] Sonicated liposome.
[b] Sonicated liposome containing 16 percent cholesterol.
[c] Sonicated liposome whose trapped volume was measured to be 0.323 μl/μmol of lipid phosphate.
[d] Vesicles obtained by the dilution of a 1:1 DMPC/cholate mixed micelles.
[e] Vesicles obtained by the dialysis of octyl glucoside.

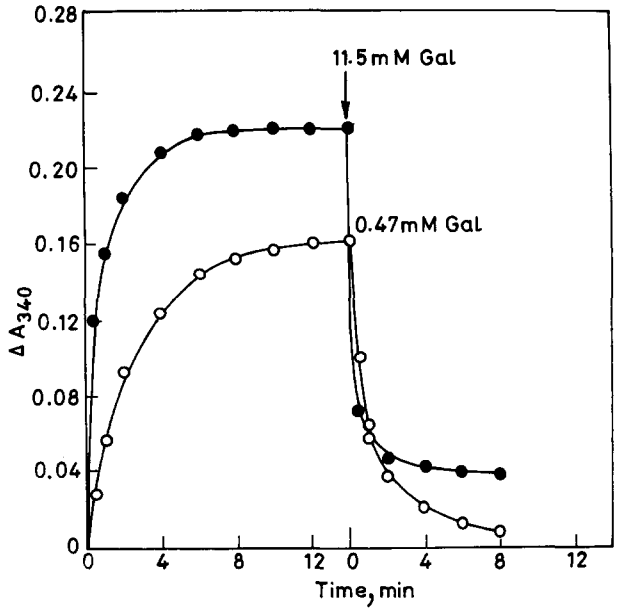

Fig. 3. Kinetics of the formation and galactose-induced dissociation of precipitin complex between lectin vesicle and free RCA_I with galactose vesicle. ●, Vesicle-bound RCA_I ($2.14 \cdot 10^{-6}$ M); and ○, free RCA_I ($1.8 \cdot 10^{-6}$ M) with asialoGM_1 ($12.4 \cdot 10^{-6}$ M). The density of asialoGM_1 was 5.0 percent.

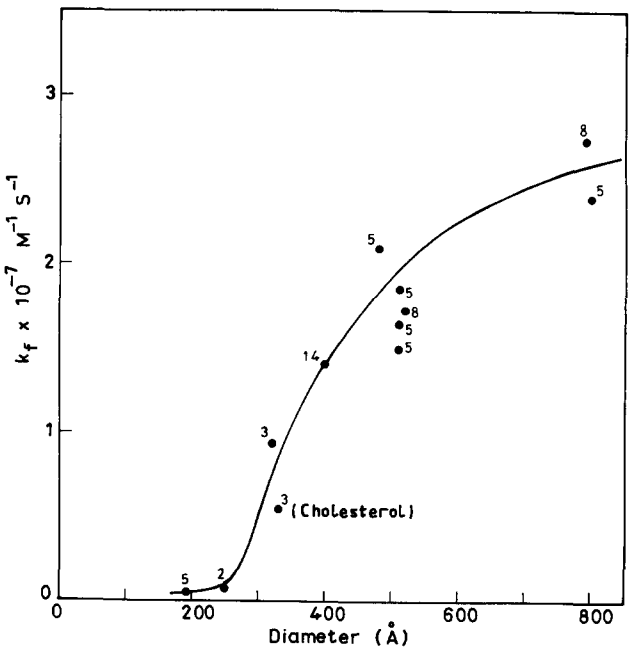

Fig. 5. Dependence of forward rate constant (k_f) with vesicle diameter. The numbers represent density of asialoGM_1.

strong size dependence which seemingly reaches a limiting value at 800 Å or higher.

In order to detect the dependence of the k_f' on the size of the galactose vesicles, we have followed the method of Almog et al. [26] to prepare different populations of galactose vesicles with a fixed density of asialoGM_1. A mixed film containing DMPC/cholate/asialoGM_1 (22:20:2, μmol/μmol) was dried under nitrogen atmosphere, hydrated with Tris buffer and equilibrated for 12 h at 27°C. Aliquots were taken, diluted suitably and incubated for 2–3 h until the change in turbidity became negligible. Those different populations of galactose vesicles, thus obtained, were triggered with the fatty acid conjugate of RCA_I. Fig. 6 shows that the sizes of the populations are different as reflected from the values of A_{340nm}/mM P_i. k_f' values also show a similar pattern when calculated from the slope of the semilog plot shown in the inset. Table II summarises the data showing that the rate of aggregation of lectin vesicles and galactose vesicles is independent of the density of asialoGM_1 (mole percent of asialoGM_1 with respect to the bulk phospholipid). But in the case of the same induced by the free lectin, the

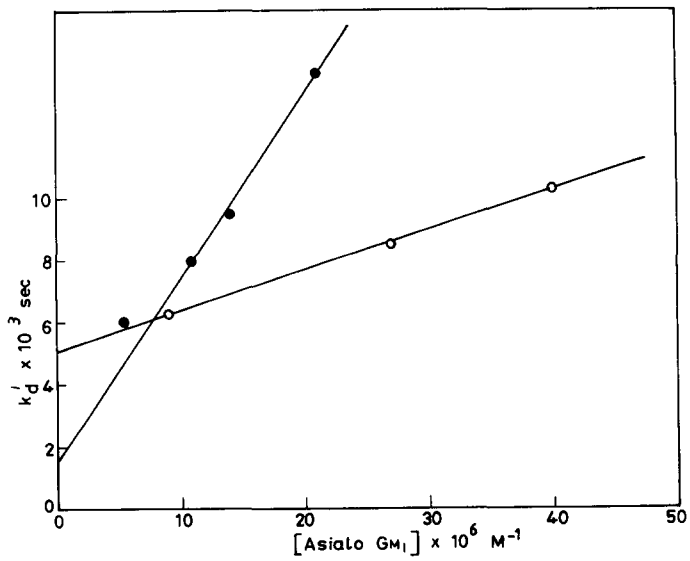

Fig. 4. Plot of k_d' against the excess component (asialoGM_1). The galactose vesicles had a diameter of 450 Å (●) and 250 Å (○), respectively, with a density of 5 percent.

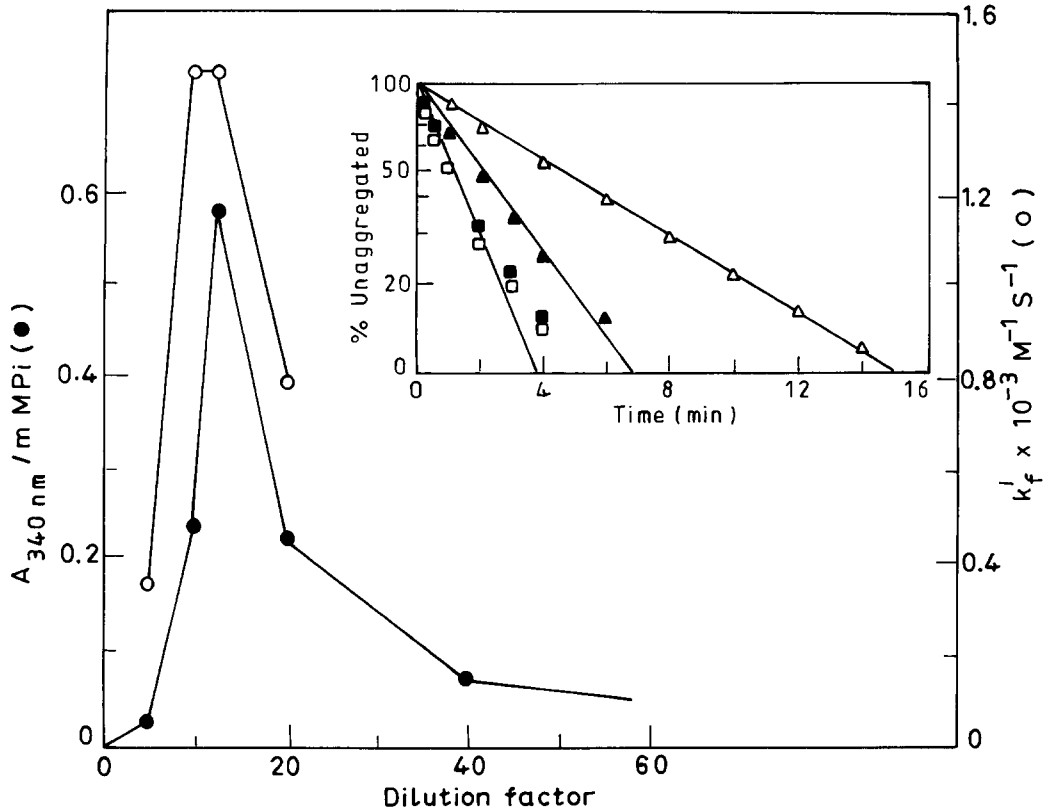

Fig. 6. Dilution of a mixed phospholipid/cholate/asialoGM$_1$ micelle to get different size population of mixed micelles/vesicles. Plot of the dilution factors against A_{340nm}/mM P_i (●) and k_f' $M^{-1} \cdot s^{-1}$ (○) as determined from the slope of the semilog plots shown in the inset. The precipitin reaction is carried out using conjugated RCA$_I$ ($0.35 \cdot 10^{-6}$ M) and asialoGM$_1$ ($7.14 \cdot 10^{-6}$ M). Dilution factors are: △, 5; □, 10; ■, 12.5; and ▲, 20.

k_{app} values depend remarkably on the density. Finally the ratio, k_f/k_d and CD_{50} remain invariant with the vesicle diameter as shown in Fig. 7.

Discussion

It is clear from the data presented there that the apposition of two vesicles, one containing the fatty acid conjugate of RCA$_I$ and the other bearing asialoGM$_1$ results in the true 'heterophilic' adhesion mediated by carbohydrate and protein. Similarly, a 'homophilic' adhesion between two spherical model membranes containing mixed gangliosides have been reported by Brewer and Thomas [27]. The measured association rate constant of RCA$_I$ with galactose is $4.5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ [28]. In the present study the normalised forward rate con-

TABLE II

Effect of density on the forward rate constant of the aggregation of lectin vesicle with galactose vesicle and free lectin

Aggregating species	Diameter of galactose vesicle (Å)	Density (%)	No. of aGM$_1$ per vesicle	$k_{f\,cor}$ (M^{-1} (receptor)$\cdot s^{-1}$)	k_{app} (M^{-1} (receptor)$\cdot s^{-1}$)
Lectin vesicle	190 [a]	5	111	$3.25 \cdot 10^3$	–
DMPC (1.2–1.5 mM)	250 [a]	2	162	$4.38 \cdot 10^3$	–
RCA$_I$ (1.2–1.5 μM)	520 [a]	5	1991	$8.69 \cdot 10^3$	–
Galactose vesicle aGM$_1$ (12.0–25.0 μM)	790 [a]	8	5781	$4.76 \cdot 10^3$	–
Conjugated RCA$_I$	250 [c]	5	406	$2.00 \cdot 10^3$	–
(0.5–1.0 μM)	600 [d]	8	4380	$5.41 \cdot 10^3$	–
	2200 [e]	7	29300	$1.55 \cdot 10^3$	–
RCA$_I$ (1.5 μM)	220 [a]	7	340	–	$6.9 \cdot 10^4$
Galactose vesicle aGM$_1$ (11.4 μM)	350 [a]	19	3363	–	$3.49 \cdot 10^6$

For footnotes, a, c, d and e, see Table I. $k_{f\,cor}$ values are calculated according to Kitano et al. [8].

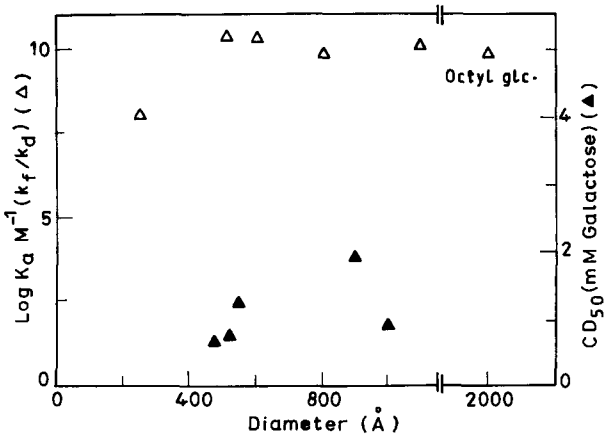

Fig. 7. Plot of logarithm of k_f/k_d (△) and C_{50} (▲) against the diameter of galactose vesicle.

stant is enhanced by a factor of 10–100 upon changing the vesicle diameter from 250 Å to 800 Å. However, it reaches a limiting value above 800 Å. Kitano et al. have reported a similar enhancement in the antigen-antibody reaction when the interacting molecules were coupled to latex beads [8]. The rate constant fo the dimeric association process of polymer latex particles carrying antigens or antibodies was estimated to be $1.3 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ which is larger than the rate constant of association reaction between antigen and antibody (k_f being $1.9 \cdot 10^6$ $M^{-1} \cdot s^{-1}$). The enhanced rate in the present case is possibly due to the increase in the local density of the receptors in going from the solution to the surface and also due to the surface diffusion of the receptors. It has been shown by Astumian and Schelly [29] that if the possibility of surface diffusion is excluded, then the reduction of dimensionality leads to a decrease of the specific rates. Hence, in this case, the observed rate enhancement may be attributed to the diffusion of the receptors on the vesicle surface.

When RCA_I is free in the solution, a density dependence for smaller vesicles is reflected in the k_{app} values summarised in Table II. In the case of the liposomal RCA_I-asialoGM_1 system this dependence is only marginal, as k_f M^{-1}(receptors) $\cdot$ s^{-1} values remain more or less constant as a function of the number of receptors (Table II). The density dependent rate enhancement is only experienced when each receptor is stereochemically approachable as in the case of free RCA_I. However, unlike free protein receptors, membrane-associated receptors are clustered on a large particle and hence very little density effect is observed in the case of liposomal RCA_I-asialoGM_1 system.

Now it is worthwhile to compare these rate data to that of phospholipid vesicle aggregation and the Ca^{2+}-induced vesicle aggregation [30,31]. The latter is fast and approaches the diffusion controlled rate ($k_f \approx 10^8$ $M^{-1} \cdot s^{-1}$) whereas the phospholipid vesicle aggregation is slow due to the repulsive forces acting between them. In the case of oppositely charged latex particles the rate of association is also very fast with a rate constant of $1.9 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ [32]. The rate of lectin-induced aggregation falls in between these two values indicating a weak interaction between the phospholipid bilayers as the carbohydrate receptors are extended from the surface.

The size effect which increases with the increase in the curvature resulting in optimal surface dilution of the receptors seems to be a consequence of facile stereochemical orientation of the conjugated/vesicle-bound RCA_I. This size-dependence of the rate of aggregation, however, is opposite to that observed in the case of blood clotting factor Va-Lc binding to phospholipid vesicles. In the latter case it has been shown that the k_{app} varies inversely with size [33]. Since association and dissociation processes are altered to the same extent with size, equilibrium binding constants of collisionally limited reactions should remain unaltered. Fig. 7 shows that the value of k_f/k_d remains more or less constant and can therefore be used as the stability constant of vesicle-vesicle aggregation.

The information about the relative stability of the bonds formed is obtained from the measurements of k_f, k_d and CD_{50}. The ratio CD_{50}/k_d is found to be sensitive to the time of incubation of precipitin complexes, i.e., more galactose is needed for the disaggregation to the same extent at a later point of time. This clearly suggests that the surface diffusion and redistribution of components subsequent to the formation of stable adhesion changes with time. The same is evident from the resonance energy transfer experiments done with galactose-specific peanut agglutinin (unpublished data from our lab). The ratio k_f/k_d is found to be approx. 10^{10} M^{-1} (Fig. 7) which clearly indicate the formation of multiple bonds by the multivalent lectin. The free energy of activation (ΔG^*) of binary association of lectin vesicle and galactose vesicle is determined as 7.3 kcal $\cdot$ mol^{-1} (k_f being $2.7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at 25 °C). This is comparable to that of latex-bound antigen-antibody reaction ($\Delta G^* = 7.7$ kcal $\cdot$ mol^{-1}), but greater than that of the association of oppositely charged latex particles ($\Delta G^* = 4.8$ kcal $\cdot$ mol^{-1}). The large activation energy in the present study may be due to the conformational changes of the proteins during the aggregation process [8].

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