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LECTIN-MEDIATED AGGLUTINATION OF LIPOSOMES CONTAINING GLYCOPHORIN

EFFECTS OF ACYL CHAIN LENGTH

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Glycophorin from human erythrocytes has been incorporated into liposomes of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC). The thermal properties of unsonicated liposomes with glycophorin/lipid molar ratios up to $4 \cdot 10^{-3}$ have been studied by differential scanning calorimetry and the numbers of lipids withdrawn from participation in the gel-to-lamellar phase transition were found to be 42±22 (DMPC), 197±28 (DPPC) and 240±64 (DSPC). The initial rates of agglutination of sonicated liposomes with glycophorin/lipid molar ratios up to $4 \cdot 10^{-3}$ by wheat germ agglutinin in the concentration range 0–7 μM have been measured over a range of temperature. Below the gel-to-lamellar phase transition (T_c) the rates of agglutination increase with acyl chain length in the sequence DMPC<DPPC<DSPC. Agglutination is found to be second order in liposome concentration and is completely reversed on saturation of the wheat germ agglutinin-binding sites by *N*-acetylglucosamine. Agglutination rates decrease with increasing temperature below T_c and are largely independent of temperature above T_c. The results are discussed in relation to the clustering of glycophorin in the phospholipid bilayers and its effect on binding and subsequent interliposomal bridge formation by wheat germ agglutinin.

Introduction

The determination of the structure and transmembrane disposition of the glycophorins [1,2], the major sialoglycoproteins of the human erythrocyte membrane, has led to studies on the effects of glycophorin incorporation on the properties of liposomes [3,–10]. Liposomes incorporating glycophorin serve as useful model systems to study the effects of local lipid environment on the behaviour of glycophorin in the bilayer as well as the changes in overall liposomal properties resulting from the presence of a glycoprotein at the bilayer/aqueous interface. One significant change in liposomal behaviour is the susceptibility to agglutination (or aggregation) by lectins conferred by glycophorin. Wheat germ agglutinin, a lectin of molecular weight 36000, has multiple binding sites for the *N*-acetylneuraminic acid [11–14] present in the sialoglycoproteins and will agglutinate glycophorin-containing liposomes by a bridging mechanism. Lectin agglutination of liposomes incorporating glycolipids has been reported [15–20] but there has been little work on lectin agglutination of glycoprotein-containing liposomes [21,22].

In this paper we report a study of the agglutination by wheat germ agglutinin of glycophorin-containing liposomes made from a homologous series

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of synthetic diacylphosphatidylcholines covering a range of glycophorin/lipid molar ratios. The effects of wheat germ agglutinin concentration and temperature on the agglutination process have also been investigated.

Materials and Methods

Materials. Glycophorin was isolated from human erythrocyte membranes either by the method of Marchesi and Andrews [1] or by the modified procedure described by Segrest et al. [23]. The purity of the material was assessed from the periodic acid-Schiff-stained and Coomassie-stained polyacrylamide gel patterns.

The gel pattern showed only the bands characteristic of the glycophorins [24,25]. No attempt was made to separate the different glycophorins [26,27]. Synthetic diacylphosphatidylcholines were purchased from Sigma Chemical Co. (London). Their purity was checked by thin-layer chromatography and was in excess of 98% and they were hence used as supplied. Wheat germ agglutinin from *Tritcum vulgaris* was obtained from Sigma and used as supplied. All other reagents were of analytical grades.

Liposome preparation. Liposome dispersions were prepared at a lipid concentration of 0.02% (w/v) in buffer (10 mM Tris-HCl plus 0.1 M NaCl, pH 7.4) and glycophorin/lipid molar ratios up to $4 \cdot 10^{-3}$. Desired amounts of lipid (5 mg $\cdot$ cm^{-3} in chloroform) and glycophorin (1 mg $\cdot$ cm^{-3} in water) were added along with 50 cm^3 of chloroform/methanol solution (4:1, v/v) to a 1 litre round-bottom flask. The solvent was removed by rotary evaporation at a temperature at least 10°C above the chain-melting temperature (T_c) of the lipid. The resulting glycophorin-lipid film was flushed with nitrogen to eliminate trace solvent and the required quantity of nitrogen-saturated buffer was added and the film was dispersed at $(T_c + 10)$°C. For thermal analysis the liposomes were pelleted by centrifugation at $80000 \times g$ for 1 h and portions of the pellet were analysed (see below). For the agglutination experiments the liposome dispersions were saturated with nitrogen, sealed in a boiling tube and sonicated above T_c for $2\frac{1}{4}$ h in a bath sonicator.

Differential scanning calorimetry. This was carried out by use of a Perkin-Elmer DSC 1-B instrument calibrated with lauric acid and indium. Liposome pellets (approx. 15 μl) were thermally analysed at a scan rate of 4°C $\cdot$ min^{-1}. After thermal analysis the contents of the pans were dispersed in 10% (w/v) sodium *n*-dodecyl sulphate solution and analysed for glycophorin using the protein assay of Lowry et al. [28] and for phospholipid [29].

Auto-correlation spectroscopy. The *z*-average diffusion coefficients ($\bar{D}_z$) of sonicated DPPC liposomes incorporating different amounts of glycophorin were measured by auto-correlation spectroscopy [30–33] and the corresponding hydrodynamic diameter $(\langle d_z^{-1} \rangle)^{-1}$ [34] calculated from the Stokes-Einstein equation:

$$\left(\langle d_z^{-1} \rangle\right)^{-1} = \frac{kT}{3\pi\eta\langle D_z \rangle} \tag{1}$$

at 25°C ($T = 298$ K) with a solvent viscosity η ($= 8.874 \cdot 10^{-4}$ N $\cdot$ s $\cdot$ m^{-2}). Liposome suspensions were filtered through a 0.22 μm Millipore filter to remove dust particles and diluted within the concentration range covered by dilute-solution theory. A 1 W argon ion laser (Spectra Physics 164, $\lambda_0 =$ 514.5 nm) source was used and light scattered at 90° to the incident beam was collected and analysed using a 48-channel auto-correlator (Malvern K7023) operating in scaling mode. The data were analysed by the method of cumulants [33].

Agglutination assay. Agglutination was assessed by spectroscopically measuring the time course of the increase in turbidity on addition of wheat germ agglutinin using a Cary 219 spectrophotometer. Sonicated liposome dispersions (0.6 cm^3, lipid concentration 0.02% (w/v)) were added to each of a pair of 1 cm^3 quartz cuvettes. After temperature equilibration, agglutination was initiated by the addition of the required amount of wheat germ agglutinin (0–200 μl, concentration 1 mg $\cdot$ cm^{-3} in buffer) to the sample cuvette and an identical volume of buffer to the reference cuvette. The absorbance (A) was recorded at 340 nm up to 500 s from mixing. Initial rates (dA/dt) were determined from the absorbance vs. time plots by fitting the curves to orthogonal polynomials.

Results

Effect of acyl chain length on the enthalpy of chain-melting

The enthalpies of the gel-to-lamellar phase transition in liposomes decreases linearly as a function of the glycophorin/lipid molar ratio (GP/L) and can be expressed by the equation [7]:

$$\Delta H = \Delta H_0[1 - N(\mathrm{GP/L})] \qquad (2)$$

where ΔH_0 is the enthalpy of chain melting in the absence of glycophorin and N the number of lipid molecules per glycophorin molecule withdrawn from participation in the transition. Table I gives the values of ΔH_0 and N determined from plots of ΔH vs. (GP/L) in the range $0–4 \cdot 10^{-3}$. The plot for (DPPC) liposomes has been previously reported [10]. The values of ΔH_0 are approx. 30% lower than those reported by Hinz and Sturtevant [35] but a more recent report by Chen et al. [36] gives 28.5 ± 2 kJ · mol^{-1} for DPPC liposomes and Petri et al. [37] found 33 ± 4 kJ · mol^{-1}. Our value is in good accord with the more recent reports.

The number (N) of withdrawn lipids increases with acyl chain length; we find a lower value for DMPC than Van Zoelen et al. [7] who obtained 86 ± 10. For DPPC liposomes incorporating vesicular stomatitis virus glycoprotein $N = 270 \pm 150$ [37]. There is good evidence that below the chain-melting temperature glycophorin is clustered into a glycoprotein-rich phase [3,9,38] in bilayers and the values of N reflect the increasing concentration of lipid in the glycophorin clusters with increasing acyl chain length.

Auto-correlation spectroscopy

Table II shows the z-average diffusion coefficients and corresponding hydrodynamic diameters of liposomes incorporating glycophorin. There is a very significant increase in size of the liposomes on incorporation of glycophorin relative to pure DPPC liposomes.

TABLE II

DIFFUSION COEFFICIENTS AND DIAMETERS OF LIPOSOMES INCORPORATING GLYCOPHORIN

Glycophorin/DPPC mole ratio ($\times 10^3$)	$\langle D_z \rangle$ ($m^2 \cdot s^{-1}$) ($\times 10^{12}$)	$(\langle d_z^{-1} \rangle)^{-1}$ (nm)
0.0	13.39±0.18	36.7±0.5
1.5	4.10±0.22	120.0±6.8
2.5	3.59±0.08	137.0±3.0
3.5	3.52±0.06	139.7±2.5
4.5	3.72±0.04	132.3±1.3

Dependence of agglutination rate on glycophorin/lipid molar ratio and wheat germ agglutinin concentration

Fig. 1a shows typical absorbance vs. time curves for DPPC liposomes (GP/L ratio $2 \cdot 10^{-3}$) on addition of increasing concentrations of wheat germ agglutinin. The measurements were made using a reference of liposomes suspensions at the same concentration. Similar data were obtained for DMPC and DSPC liposomes covering GP/L molar ratios from 1 to $4 \cdot 10^{-3}$ and at different temperatures. The data were processed by computer fitting of the plots to determine the initial slopes. We found that addition of wheat germ agglutinin to the liposomes caused an instantaneous increase in absorbance (Fig. 1b), of which only a small fraction could be attributed to the absorbance of the lectin at 340 nm. Systematic

TABLE I

THERMOCHEMICAL PROPERTIES OF LIPOSOMES INCORPORATING GLYCOPHORIN

The number of independent experiments is given in parentheses.

Phospholipid	T_c(°C)	ΔH_0(kJ · mol^{-1})	N	E_{act}(kJ · mol^{-1})
DMPC	24.5	17.2±0.5	42±22 (2)	−34±14
DPPC	42.5	27.4±1.3	197±28 (5)	−64±10
DSPC	55.0	33.0±2.0	240±64 (3)	−75± 8

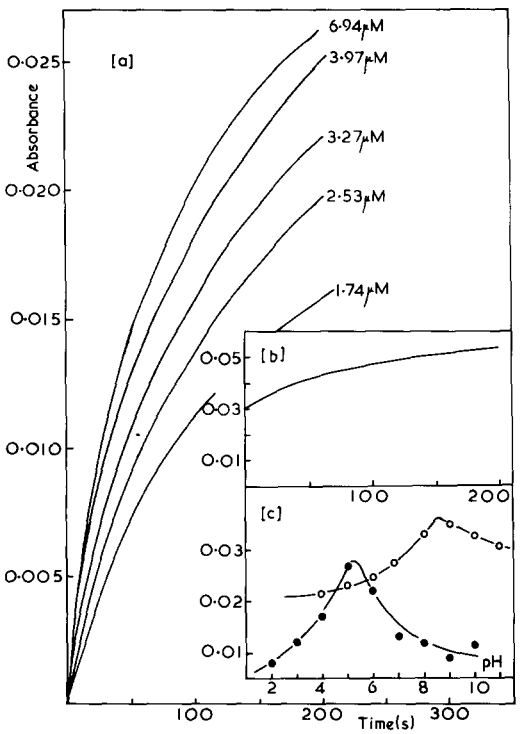

Fig. 1. Agglutination of DPPC liposomes incorporating glycophorin by wheat germ agglutinin at 37°C. (a) Absorbance (340 nm) as a function of time on addition of wheat germ agglutinin (concentration range 1.74–6.94 μM). Glycophorin/DPPC molar ratio $2 \cdot 10^{-3}$. Liposome concentration range 0.019–0.015% (w/v). (b) Typical absorbance vs. time curve showing the initial change in absorbance on addition of 3.27 μM wheat germ agglutinin to DPPC liposomes as in a. (c) Initial absorbance change as a function of pH on addition of 2.53 μM wheat germ agglutinin (○) and 1.36 μM bovine serum albumin (●) to DPPC liposomes (0.0176% (w/v)) in the absence of glycophorin.

investigation of this initial change in absorbance showed that it was pH dependent, almost independent of temperature in the range 10–50°C, was not specific to wheat germ agglutinin and occurred for liposomes without glycophorin. As shown in Fig. 1c, a similar instantaneous absorbance change was found on addition of bovine serum albumin. These absorbance changes go through maxima at the isoelectric points of the proteins (pH 5.4 for bovine serum albumin [39]; pH 8.7 for wheat germ agglutinin [12]) and possibly reflect changes in the absorbance of the liposomes due to non-specific adsorption of the proteins to the liposomal surface.

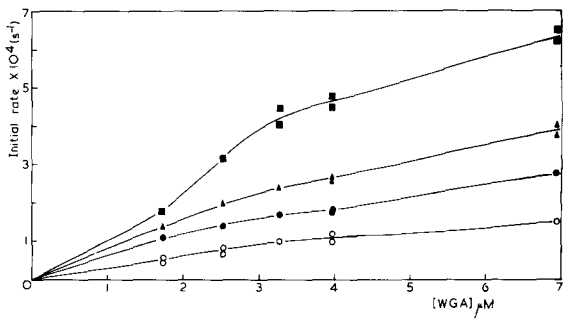

Fig. 2. Initial rates of agglutination of DMPC liposomes incorporating glycophorin as a function of wheat germ agglutinin (WGA) concentration at 37°C. Glycophorin/DMPC molar ratio (○) $1 \cdot 10^{-3}$, (●) $2 \cdot 10^{-3}$, (▲) $3 \cdot 10^{-3}$, (■) $4 \cdot 10^{-3}$.

In general, protein adsorption is often maximal at the isoelectric point [40]. As these initial absorbance changes were not related to the rate of glycophorin-mediated agglutination, they were

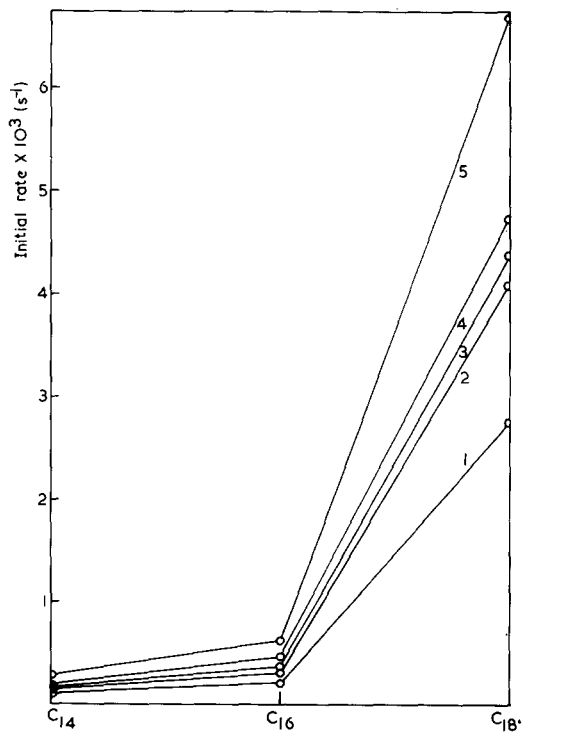

Fig. 3. Chain-length dependence of the initial rates of agglutination of phosphatidylcholine liposomes incorporating glycophorin at 37°C. The wheat germ agglutinin concentrations range from 1.74 to 6.94 μM, the glycophorin/phospholipid molar ratio is constant at $2 \cdot 10^{-3}$. Curve 1, 1.74 μM; 2, 2.53 μM; 3, 3.27 μM; 4, 3.97 μM; 5, 6.94 μM.

subtracted from the measured absorbance at zero time before processing the absorbance vs. time curves (Fig. 1a).

Fig. 2 shows the initial rates of agglutination (dA/dt) as a function of wheat germ agglutinin concentration (0–7 μM) for DMPC liposomes at different GP/L molar ratios. At these GP/L molar ratios and liposome concentrations the concentration of glycophorin in the system is in the range 0.3–1.2 μM so that the rate of agglutination does not reach a limiting value until the wheat germ agglutinin is in excess. Similar data were obtained for DPPC and DSPC liposomes. The initial rate of agglutination at 37°C was found to increase in the sequence DMPC < DPPC < DSPC, as illustrated in Fig. 3 where initial rates are plotted against acyl chain length at constant GP/L molar ratio. At 37°C DSPC is below and DMPC is above the corresponding chain-melting temperature.

Dependence of rate on liposome concentration

The initial rate of agglutination was measured as a function of lipid concentration (liposome concentration) for DSPC liposomes where the rate was sufficiently fast to measure over a range of lipid concentration. Fig. 4 shows that the initial rate increases linearly with the square of the lipid concentration in the range 0–0.01% (w/v). The initial rates at the higher lipid concentration of 0.02% (w/v) also fall on these lines (not shown). Collision between pairs of liposomes would be expected to lead to a rate equation which is second order in liposome (and hence lipid) concentration.

Light-scattering theory leads to the following relationship between absorbance (A) and the numbers of scattering particles (N) of volume v [41].

$$A = 0.434Bd \qquad (3)$$

where $B = \text{constant} \times Nv^2$ and d is the optical path length provided the particles are small compared with the wavelength of light. From Eqn. 3 it follows that for dimerization A should double, since the scattering volume doubles but the number of particles halves. The liposome suspensions had absorbances relative to solvent in the range 0.04–0.15. The maximum changes in absorbance observed at the highest GP/L ratio and wheat germ agglutinin concentrations after 200 s were 0.024 (DMPC), 0.10 (DPPC) and 0.24 (DSPC). Although some interference effects will occur, the changes in absorbance over which the initial rates were computed correspond predominantly to the dimerization of the liposomes.

Reversibility of agglutination

The reversibility of agglutination was assessed by the addition of *N*-acetylglucosamine to an ag-

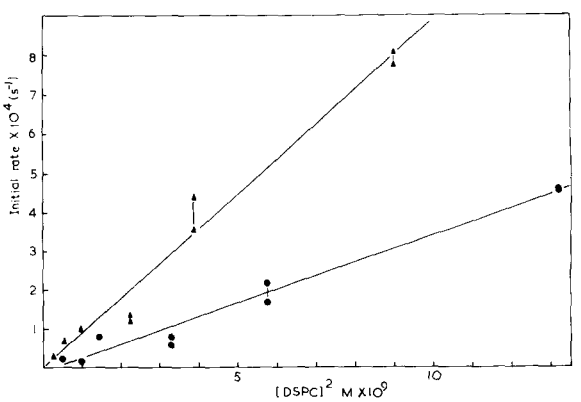

Fig. 4. Initial rate of agglutination of DSPC liposomes incorporating glycophorin as a function of liposome concentration at 37°C. Wheat germ agglutinin concentration 2.53 μM (●) and 6.94 μM (▲).

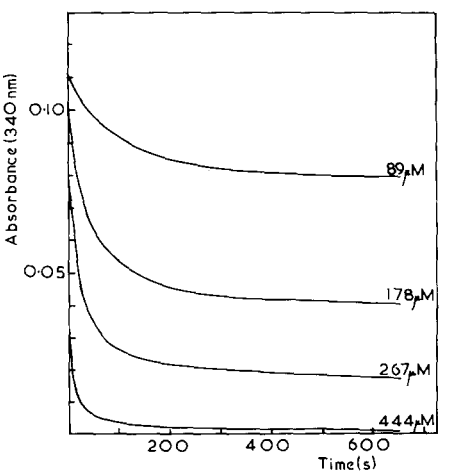

Fig. 5. The effect of *N*-acetylglycosamine on the dissociation of agglutinated DPPC liposomes at 37°C. DPPC liposomes (glycophorin/DPPC molar ratio $2 \cdot 10^{-3}$, lipid concentration 0.017% (w/v)) were agglutinated by 3.97 μM wheat germ agglutinin; *N*-acetylglucosamine (0.1 M) was added to the concentrations indicated on the curves.

glutinated suspension of DPPC liposomes (GP/L molar ratio $2 \cdot 10^{-3}$), as shown in Fig. 5. In these experiments a liposome suspension (0.017% (w/v) lipid) was agglutinated by wheat germ agglutinin (3.97 μM) and after approx. 1 h the disaggregation process was followed after addition of increasing amounts of *N*-acetylglucosamine (0.1 M). The absorbance was measured relative to a blank containing no wheat germ agglutinin. After addition of 0.44 mM *N*-acetylglucosamine agglutination was almost completely reversed, indicating that there was no significant fusion on agglutination.

Effect of temperature on agglutination

Fig. 6 shows the initial rate of agglutination as a function of temperature for DMPC, DPPC and DSPC liposomes (0.018% (w/v), GP/L molar ratio $2 \cdot 10^{-3}$) agglutinated with 2.53 μM wheat germ agglutinin. The rate of agglutination decreases with temperature and shows a transition in the region of the onset of chain melting as detected by DSC. The constancy of concentrations in these experiments means that the initial rates will be directly proportional to the rate constants for agglutination and hence Arrhenius plots of the data below T_c were used to determine activation energies. Decreasing rates with increase in temperature give negative activation energies as shown in Table I, which imply that there is no potential energy barrier to agglutination. Decreasing rate constants with increasing temperature have been observed for lectin agglutination in other liposomal systems [16,19].

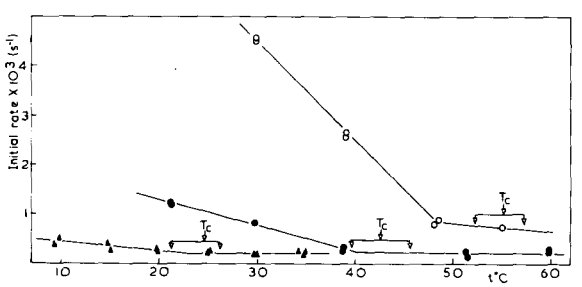

Fig. 6. Temperature dependence of the initial rate of agglutination of phosphatidylcholine liposomes incorporating glycophorin. Glycophorin/phospholipid molar ratio $2 \cdot 10^{-3}$. Wheat germ agglutinin concentration 2.53 μM. (○) DSPC, (●) DPPC, (▲) DMPC. The arrows denote the temperature range over which chain melting is observed by DSC. T_c denotes the chain-melting temperature (DSC peak temperature).

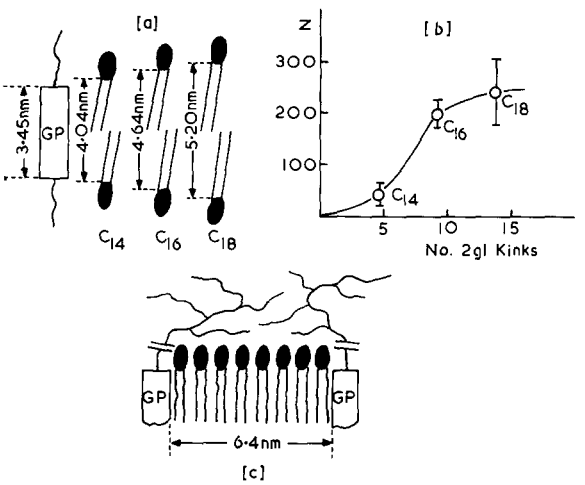

Fig. 7. (a) Relative dimensions of the transmembrane section of glycophorin and phospholipid bilayers (to scale). (b) The number of disordered lipid molecules (N) determined by DSC as a function of the number of 2gl kinks required to bring the acyl chains into register with the dimensions of the transmembrane section of glycophorin. (c) Relative spacing of glycophorin molecules in clusters in DMPC bilayers. (The N-terminal oligosaccharide-bearing segment of glycophorin is not to scale.)

Discussion

The data show that the initial rate of agglutination of phosphatidylcholine liposomes incorporating glycophorin depends markedly on the phospholipid acyl chain length and temperature. The fastest rates are found for DSPC liposomes in which glycophorin withdraws the largest number of lipid molecules from participation in the gel-to-lamellar phase transitions. The clustering of glycophorin molecules in the liposomal bilayer below T_c [3,9,37,38,42] may well play a significant role in the agglutination process. The rates of agglutination of the liposomes are comparable above the chain-melting temperatures which implies that when glycophorin clusters are largely broken up and the glycoprotein is free to diffuse laterally in the mobile lamellar phase, discrimination between the different phospholipids is almost lost. It might be envisaged from steric considerations that the binding of wheat germ agglutinin to dispersed glycophorin molecules will be more favourable than binding to glycophorin in clusters, so that fewer free binding sites which are necessary for the

bilayer through interaction with the phospholipid head groups. Such an interaction might clearly involve much larger numbers of lipid molecules. However, NMR studies on the dynamics of the oligosaccharide-bearing segment [49,50] indicate that its motion is largely unrestricted and there is no strong evidence for interaction with phospholipid head groups. Should such an interaction predominate in liposomes, then the number of lipids withdrawn would perhaps be expected to be much less dependent on acyl chain length than we observe here.

It is not entirely clear why the rates of agglutination of DPPC liposomes are closer to those of DMPC than to those of DSPC liposomes. It should be noted that the above simple steric considerations relate primarily to the situation close to T_c (the temperature at which the number of withdrawn lipids was determined) and that we have assumed that glycophorin clustering in unsonicated and sonicated liposomes changes similarly with acyl chain length, i.e., glycophorin is inserted uniformly in the liposomes formed from the different phospholipids. The consequence of limited association of the transbilayer segments of glycophorin has also been ignored. The decrease in agglutination rates with increasing temperature below T_c implies that there are gradual changes in the system with temperature as well as a cooperative effect at the phase transition. These temperature effects probably relate to the independent behaviour of the sialoglycopeptide [25,49,50], so that although the bilayer conditions may dictate the relative proximity of the glycophorin molecules they do not entirely control the complex series of events associated with the agglutination.

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