Biochimica et Biophysica Acta 863 (1986) 139–155
Elsevier

BBA 72985

Visualization of domains in rigid ganglioside / phosphatidylcholine bilayers: Ca^{2+} effects

Ingrid E. Mehlhorn, Grace Parraga, Kathryn R. Barber and Chris W.M. Grant

Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1 (Canada)

(Received 30 July 1986)

Key words: Model membrane; Lectin binding; Ganglioside; Phospholipid bilayer; Ca^{2+} effect; Freeze-etching; Electron microscopy

We have considered the extent to which details of lectin binding directly visualized by freeze-etch electron microscopy are consistent with current concepts of ganglioside arrangement in phosphatidylcholine bilayer membranes. Native lectins in general seem appropriate labels for this type of study. Wheat germ agglutinin, *Ricinus communis* agglutinin, and peanut agglutinin are adequately resolved on membrane surfaces as spherical particles of diameters 6 nm, 10 nm, and 13 nm, respectively (uncorrected for platinum shadow thickness). The finite areas covered by these markers correspond to some 56, 157, and 265 lipid molecules, respectively, on the surfaces of the shadowed rigid phosphatidylcholine matrices employed here; and this constitutes a basic limitation to the precision with which one can localize a given glycolipid receptor. *Ricinus communis* agglutinin provides a marker whose size permits adequate quantitation of bound material while minimally obscuring detail. Using it we estimated the size limits of G_{M1}-enriched domains, since this is the ganglioside which has shown the greatest evidence of discontinuous distribution in our hands (Peters, M.W., Mehlhorn, I.E., Barber, K.R. and Grant, C.W.M. (1984) Biochim. Biophys. Acta 778, 419–428). Results of such analyses indicate the probable existence of phase separated domains selectively enriched in G_{M1} up to 60 nm in extent (5600 lipid molecules) for rigid dipalmitoylphosphatidylcholine membranes bearing up to 14 mol% G_{M1}. Similar observations were true of rigid bilayers of dimyristoylphosphatidylcholine; however, if domains enriched in G_{M1} exist in fluid dimyristoylphosphatidylcholine, they are on the order of 6 nm or less in diameter (or are dispersed by lectin binding). Employing the small lectin, wheat germ agglutinin, which binds to all gangliosides, we then examined the effect of exposure to Ca^{2+} ions (while in the fluid state) on the ganglioside 'domain structure' referred to above in rigid dipalmitoylphosphatidylcholine host matrices. G_{M1}, G_{D1a} and G_{T1b} were studied at 0, 2 and 10 mM Ca^{2+} concentrations. It was demonstrated by spin label measurements that the dipalmitoylphosphatidylcholine matrix retained its basic melting characteristics in the presence of added Ca^{2+} and ganglioside under these conditions. Within the technique's functional resolution limit of some 6 nm we were unable to identify any effect of Ca^{2+} in physiological concentration on

Abbreviations: RCA 60, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin; G_{M1}, Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc → Cer; G_{D1a}, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc → Cer; G_{T1b}, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glc → Cer; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Correspondence: Dr. C.W.M. Grant, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1.

ganglioside topography as reflected by bound lectin distribution. The rigid dipalmitoylphosphatidylcholine host matrix had been selected to minimize receptor redistribution (ganglioside aggregation or disaggregation) caused by lectin probe binding or sample preparation for electron microscopy. However the above Ca^{2+}-related observations were basically unaltered in a matrix of intermediate fluidity and zero cooperativity obtained by the addition of 30 mol% cholesterol. In none of our samples did we see bilayer disruption that might indicate significant patches of very high local glycosphingolipid concentration.

Introduction

Glycolipids and glycoproteins of the eucaryote plasma membrane have attracted considerable attention in recent years as specific binding sites for a wide range of macromolecules, infectious agents, and agents of the immune system. Their role as mediators of tissue recognition and attachment in morphogenesis and oncogenesis is of major research interest. Of these two families of carbohydrate-bearing molecules, glycolipids are physically restricted by their size to a region very close to (within some 2.5 nm of) the membrane surface [1,2]. Presumably as a result of this spatially restrictive location, glycolipid function as attachment or recognition sites for exogenous macrostructures depends critically not only upon their presence in the membrane, but also upon (as yet incompletely defined) local factors. An early example of this phenomenon was the observation that anti-globoside antibodies bind much more extensively to fetal erythrocytes than to those of later developmental stages although membrane antigen quantities are similar [3,4]. More recently Hakomori's group has demonstrated that high and low expressors of the tumor-associated antigen, gangliotriaosyl ceramide, identified amongst sublines of murine L5178Y lymphoma, do not bear a simple relation to analytical glycolipid content [5]. Certain glycolipids in transformed cells react more strongly with antisera directed against them than they do in the parent cell line [6]. In a similar vein, susceptibility of complex glycosphingolipid oligosaccharide headgroups to enzyme attack is often limited in the cell membrane, and can be affected by oncogenic transformation and cell cycle [7–10]. It is unlikely that any single factor would account for all such observations. However, one of the major possibilities being considered in a number of laboratories is that glycosphingolipid arrangement relative to other membrane structures significantly modulates their role as binding sites for macromolecules [11–16].

We have felt that interpretation of experiments designed to identify factors that control glycosphingolipid arrangement is often significantly limited by lack of detailed structural knowledge regarding the membrane systems employed [17]. Similar concerns have been raised by others (see, for example, Ref. 18). Freeze-etch electron microscopy offers a partial solution to this problem. It provides 100 times the resolution of light microscopy, and lends itself particularly well to the study of membrane surface features. Since oligosaccharide headgroups themselves are not detectable at the freeze-etch resolution limit of 2–4 nm, we have employed unmodified lectin molecules as topographic probes. In previous work we demonstrated the feasibility of the technique [19], and that lectin molecules marking the presence of the monosialo-ganglioside, G_{M1}, were less homogeneously distributed than those marking G_{D1a} in several simple bilayer systems [20]. In this article we consider the implications of interpreting such inhomogeneous distribution in terms of phase-separated regions enriched in ganglioside and address the extent of Ca^{2+} effects.

Ca^{2+} which is critical to membrane integrity in general has been suggested as a possible modulator of ganglioside arrangement and behaviour. Certainly the carboxyl-bearing *N*-acetylneuraminic acid residue characteristic of gangliosides might be expected to be significantly influenced by Ca^{2+} (reviewed in Refs. 21, 22). For instance Ca^{2+}-induced crosslinking of phosphatidylserine headgroup carboxyl groups provides a very significant driving force to phase separation and rigidification of this lipid in bilayer membranes [23–25]. A similar phenomenon has been claimed for phosphatidic acid [24,26] and phosphatidylglycerol [24,27]. We originally pointed out that the same effect could explain some of our experiments with

spin-labelled gangliosides (and neuraminic acid-rich glycoproteins), which we interpreted in terms of Ca^{2+}-induced increases in oligosaccharide packing density [28]. It has been demonstrated on the basis of monolayer experiments and theoretical modelling, that Ca^{2+} effects on gangliosides likely involve more than just crosslinking of carboxyl groups [21,22]. For one thing the glycerol side chain of *N*-acetylneuraminic acid is importantly involved in Ca^{2+} binding [29–31]. Nevertheless a single Ca^{2+} ion does seem capable of simultaneously binding to several ganglioside headgroups [29,30]. There now exist in the literature suggestions that Ca^{2+} may lead to important alterations in ganglioside arrangement relative to surrounding lipids [21,22,32,33a] (but see Ref. 33b), and to increased attractive forces between ganglioside molecules [22,28,32,34,35]. On the other hand, McDaniel and McLaughlin [36] have pointed out that some previous measurements of ganglioside affinity for Ca^{2+} may have been overestimated, with resultant overemphasis on its potential role in ganglioside behaviour.

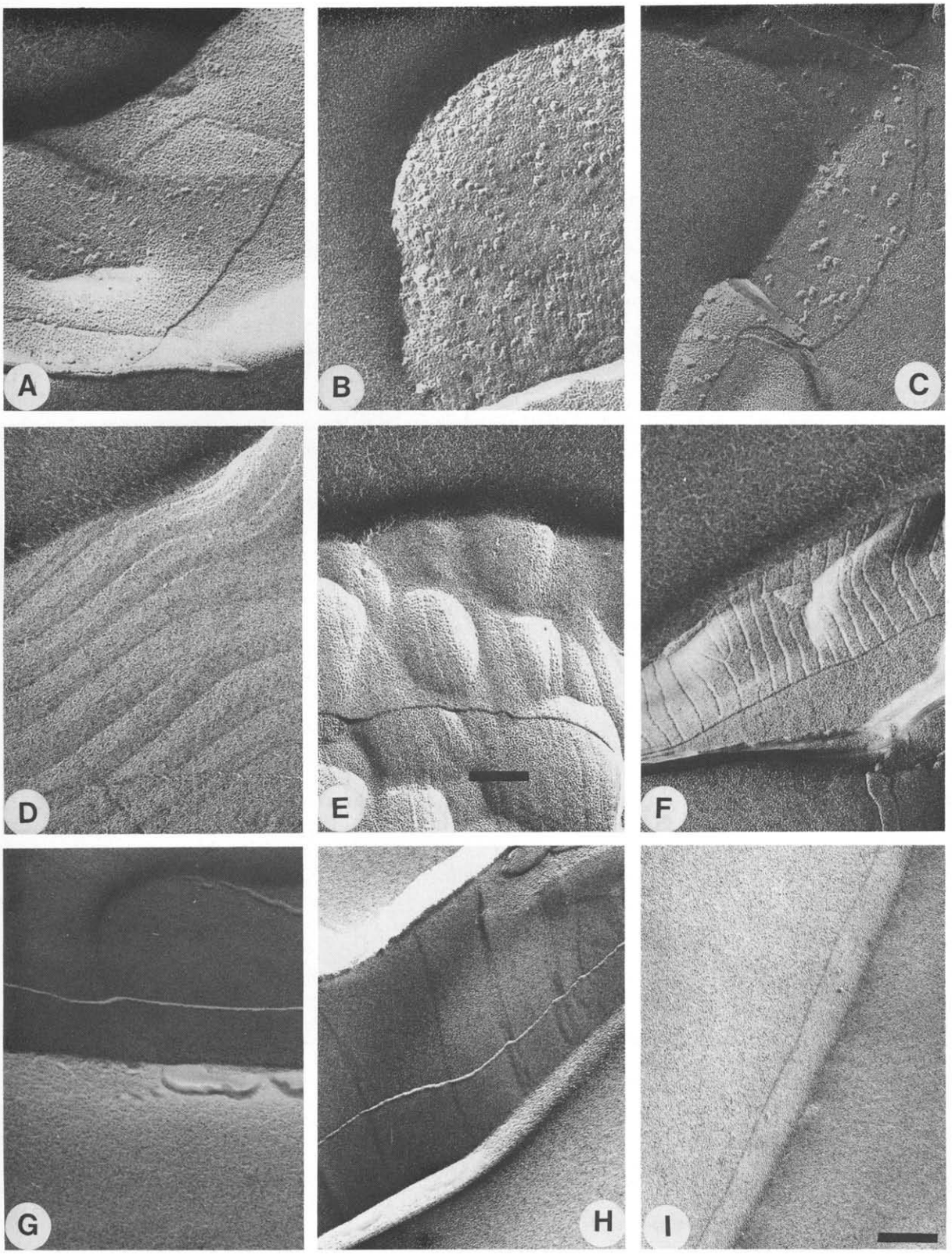
Given these concerns we have screened for any visible effect of Ca^{2+} on the putative domain structure of mono-, di- and trisialogangliosides, in bilayers of dipalmitoylphosphatidylcholine. Such bilayers may be incubated at temperatures above 41.5°C to permit diffusional equilibration, and then marked with lectin in the rigid state minimizing possible lectin-induced and quenching artefacts. Addition of cholesterol in excess of 20 mol% to such a (rigid) host matrix removes the cooperative phase transition of lipid acyl chains and imposes a relative freedom of lipid motion [37] more typical of eucaryote plasma membranes, without need for temperature alteration. Similar systems have been considered by a number of groups employing different techniques, so that it is possible to correlate our morphological findings with the results of other workers.

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-dimyristoylphosphatidylcholine (DMPC), wheat germ agglutinin (WGA), *Ricinus communis* agglutinin (RCA 60), peanut agglutinin, and dicetyl phosphate were obtained from Sigma (St. Louis, MO). Cholesterol was from Serdary Research (London, Canada). Gangliosides were obtained from Supelco (Mississauga, Canada). G_{T1b} was further purified of a G_{D1b} contaminant as described below. All lipids used were pure as judged by thin-layer chromatography on plates coated with silica gel GF_{254} (Stahl), eluted with 55 : 40 : 2 : 8 (v/v) chloroform/methanol/ammonia/water (gangliosides) or 65 : 25 : 4 (v/v) chloroform/methanol/water (phospholipids), and developed with sulfuric acid spray. G_{T1b} was further purified by an adaptation of the method of Hakomori and Siddiqui (38) on a 1 × 30 cm column of Anasil-S (Analabs, North Haven, CT). Briefly, 10 mg of commercial material was dissolved in 60 : 30 : 8 (v/v) chloroform/methanol/water and layered on a column prepared with the same solvent. Elution was with 60 ml of 60 : 40 : 10 (v/v) solvent followed by pure methanol. The spin label, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), was synthesized as described by Rozantsev [39].

Liposome preparation involved dissolving the desired ratio of lipid components in chloroform/methanol (1 : 1, v/v), and drying to thin films under a stream of nitrogen gas. Films were further dried in a vacuum desiccator for 2–3 h to ensure removal of residual solvent. Details of subsequent hydration and incubation are given in the figure and table captions. Ca^{2+} concentrations were tested by atomic absorption flame photometry. For freeze-etch electron microscopy liposomes were harvested by centrifugation at 1700 × *g* and quenched on gold alloy planchets in a slurry of freon cooled in liquid nitrogen. Freeze-etch replicas were prepared by platinum shadowing in a Balzers BAF 301 apparatus equipped with electron beam guns. Where possible, samples to be directly compared were handled simultaneously to avoid differences due to shadow variability. Etching was for 2 min at −103°C. Replicas were cleaned in $NaClO_4$, rinsed with distilled water, and exposed to ethanol/acetone (1 : 1, v/v) for 1 h to remove lipid traces. They were picked up on 400 mesh copper grids and viewed in a Philips EM300.

For EPR work lipid pellets were incubated with the TEMPO spin label [40] at a mol ratio of 125 : 1 lipid to spin label. Samples were placed in Corn-


A
B
C
D
E
F
G
H
I

ing 50 μl microsampling pipettes sealed at one end and held in the dewar insert of a Varian E12 (Tm_{110} cavity) using an insert described by workers in Harden McConnell's laboratory [40,41].

Results and Discussion

Sample fixation for freeze-etching is cryogenic: a small droplet of membrane suspension being frozen within a fraction of a second to liquid nitrogen temperatures, so that features present under the initial incubation conditions are preserved. A fracture induced through the frozen droplet tends to split bilayer membranes in the plane of their hydrophobic interiors. Membrane outer surfaces are then exposed by sublimation of overlying ice ('etching') in high vacuum. Subsequent shadowing with atomic platinum at a 45° angle highlights membrane features that protrude above the surface. The use of native lectin molecules as glycolipid location markers is illustrated in Fig. 1. Each micrograph shows at high magnification a portion of liposome hydrophobic interior and outer surface. In Figs. 1A–C, WGA, RCA 60, and peanut agglutinin, respectively, mark the presence of G_{M1} in rigid bilayers of DPPC. Their diameters reflect the gradation in size anticipated for macromolecules of increasing M_r (see below). Figs. 1D–I show typical controls that illustrate lack of the lectin-related features found in Figs. 1A–C when membranes bear the wrong receptor (D, E) or no receptor (F, G, H, I).

Fig. 1 also illustrates a very basic limitation to the use of specifically bound macromolecules as markers of receptor location: the size of the marker molecule itself. For instance, WGA is a plant protein of M_r 36000 with 4 equivalent binding sites for sialyl residues of glycolipids [42–44]. Assuming spherical shape and a partial specific volume of 0.69 ml/g [45], the diameter of this molecule would be 4.4 nm. This is only marginally greater than the 2–4 nm theoretical resolution limit imposed by the thickness of the platinum shadow, and approaches the smallest object one can hope to resolve distinctly by freeze-etching. Consistent with such considerations, the bilayer etch face in Fig. 1A shows particles 6 to 7.5 nm in diameter (uncorrected for shadow thickness). These are only well resolved as separate particles in particularly good replica regions. Given that a phospholipid molecule in rigid DPPC occupies an area of 0.5 nm^2 (i.e. has a size of about 0.7 nm in one dimension parallel to the bilayer surface) [46], a platinum-shadowed WGA molecule would cover a patch of 56 lipid molecules. At a lateral surface pressure of 33 dyn/cm as found in the human erythrocyte [47], G_{M1} in pure monolayers occupies an area of 0.7 nm^2 [21]. This area may be smaller when spaced with intervening phospholipids to decrease glycolipid headgroup steric interactions, hence the presence of G_{M1} will not greatly alter the above calculation. The oligosaccharide headgroup of G_{M1} is 2.2 nm in length, which might add yet more uncertainty to the receptor location marked by a given bound macromolecule in freeze-etch preparations if the headgroup is not simply extended straight up perpendicular to the plane of the membrane. However steric considerations from model building indicate that this factor probably does not significantly affect the estimate

Fig. 1. Freeze-etch electron micrographs of three native lectins and their use as markers for gangliosides in a rigid DPPC host matrix. (A, B, C) show liposomes of DPPC bearing 7 mol% G_{M1} and exposed to: native wheat germ agglutinin at 0.5 mg/ml (A), RCA 60 at 0.15 mg/ml (B), or peanut agglutinin at 2 mg/ml (C) in 10 mM phosphate buffer (pH 7.4). Native lectin molecules are visualized as spherical particles of diameter 0.6, 1.0 and 1.3 mm (corresponding to actual diameters of 6, 10 and 13 nm – uncorrected for shadow thickness) on membrane etch faces in A, B and C, respectively. (D, E) are controls for 1 B in which liposomes bore 7 mol% G_{D1a} which is not a receptor for RCA 60, while (F) shows a liposome of DPPC alone exposed to RCA 60. (G, H, I) are controls in which liposomes bore 7 mol% dicetyl phosphate instead of ganglioside, and were exposed to twice the concentration of WGA in the presence of 0, 2 and 10 mM $CaCl_2$, respectively. Samples were prepared by hydration of dry lipid films at 50°C (well above the 41.5°C host matrix phase transition temperature), vortexing, and allowing to cool in an 80 ml water bath to 22°C over 15 min. Exposure to lectin was for 10 min at 22°C, followed by harvesting of liposomes at 1700 × *g* for 10 min at the same temperature. Liposomes to be used in the WGA/Ca^{2+} experiments were suspended in large volumes (1 mg total lipid in 10 ml) of 5 mM Hepes buffer containing 20 mM NaCl and 0, 2 or 10 mM $CaCl_2$ (pH 7.4), incubated for 1 h at 50°C, harvested by centrifugation, and resuspended to a final lipid concentration of 0.5 mg/ml prior to lectin addition, to ensure equilibration with Ca^{2+} at the desired concentration. Magnification: ×100000. Shadow direction from bottom to top. Bar represents 100 nm.

of uncertainty already arrived at based on lectin size and shadow thickness.

RCA 60 is generally considered to be a monovalent plant protein of 60000 M_r [48]. Assuming sphericity and a partial specific volume of 0.75 ml/g [49], its diameter should be 5.2 nm. In Fig. 1B it is seen bound to G_{M1} receptors as spheres of diameter 10 nm (uncorrected for shadow thickness). This is in a size range quite readily resolved as single or multiple species, making it convenient for quantitation while keeping marker size to a minimum. Each platinum-shadowed RCA 60 monomer thus occupies a surface area equivalent to 157 phospholipid molecules in a rigid phosphatidylcholine matrix [46]. Theoretically in the case of RCA 60, a single bound marker could be indicating the location of between 1 and 112 G_{M1} molecules (assuming that they occupy a surface area of 0.7 nm^2 per molecule [21,47] in a phosphatidylcholine rigid lattice). However, phospholipid bilayer integrity is known to break down at G_{M1} concentrations in excess of 25 mol% in dipalmitoylphosphatidylcholine [31,50]. The electron micrographs which we have viewed of systems such as those described here show no evidence of bilayer disruption. Hence one may estimate at 34 the maximum number of G_{M1} molecules in the membrane region marked by a single platinum-shadowed RCA 60 molecule.

A larger lectin still is the 110000 M_r tetravalent peanut agglutinin [51] seen attached to G_{M1} in Fig. 1C. One may calculate its diameter to be 6.4 nm if spherical with a partial specific volume of 0.73 ml/g [51]. This species is quite easy to resolve as 13 nm particles (uncorrected for shadow thickness). A freeze-etch feature of this size would cover some 265 lipid molecules in the host matrix visualized in Fig. 1. Clearly there is potential advantage to employing the smallest probe molecule possible. In general we find that bound lectins are particularly well resolved on rigid bilayers of synthetic phospholipids, and less well resolved on fluid matrices (see later experiments with dimyristoylphosphatidylcholine). Perhaps highly uniform crystal packing of phospholipids permits little penetration amongst the headgroups.

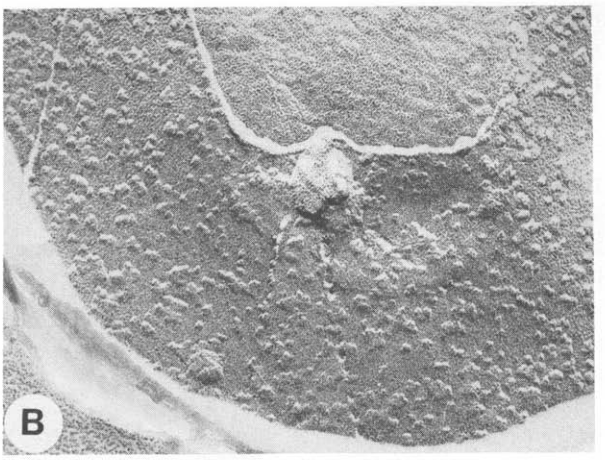
On the basis of size and steric considerations, it seems likely that two glycolipid molecules would have to be separated in the membrane by a distance equal to about one half to one lectin diameter in order to bind two different lectin molecules. If so, any membrane feature related to glycolipid arrangement would have to be larger than one half to one lectin diameters in extent to be identified by the criterion of binding two or more lectin molecules in recognizable proximity.

Points to be addressed in this manuscript are (a) to what extent are details of the lectin binding pattern significant, (b) what are the implications to glycolipid arrangement in bilayer membranes, and (c) how does Ca^{2+} affect these considerations? For instance the lectin molecules bound to G_{M1} in Figs. 1A–C often exist in small groups up to 60 nm in diameter. Is it reasonable to consider this a reflection of domains enriched in G_{M1}? Dealing with such questions requires use of the phase separation concept introduced by Chapman's [52] and McConnell's [40] groups (reviewed in Ref. 53). Any bilayer membrane has certain crystal characteristics. Theoretically a different type of lipid added to such a host matrix would diffuse uniformly throughout the bilayer only if it 'fit' in exactly the same way as the original host matrix molecules. In general this will not occur, and there will be a finite tendency for laterally separated regions of different composition to coexist in dynamic equilibrium. Probably one should be careful about using the term, 'clusters', in describing glycosphingolipids since it implies the existence of pure groups of a particular lipid molecule, whereas most often laterally separated domains only show selective enrichment. As already described, any significant region of membrane with greater than 25 mol% glycosphingolipid would be expected to show evidence of bilayer disruption [31,50]. In addition to lectin size, there are several factors which may limit the information content of lectin location as a reflection of such domain structure. Firstly there is the question of non-specific binding – which has already been addressed, and may be controlled for. Secondly there is the possibility of glycolipid/lectin redistribution subsequent to lectin binding, which in fact was originally suggested as a mechanism of action for cholera toxin (see, for example, Ref. 54). A tetravalent lectin should have no difficulty binding to at least two glycolipid molecules simultaneously in the model membranes described

here [55] so that aggregation is a possibility. Theoretically, however, further crosslinking should not be possible due to inability on steric grounds of two lectin molecules to bind to one G_{M1} headgroup. Certainly, although we have recorded electron microscopic evidence of glycoprotein rearrangement as a result of lectin crosslinking [19,56], we have not previously seen the same phenomenon with glycolipids [19]. However, even with a monovalent lectin, one cannot trivially discount the possibility that lectin binding may lead to rearrangement of receptors (aggregation or disaggregation). The selection of a rigid host matrix was intended to avoid this problem. Thirdly there is the possibility of lectin rearrangement during cryogenic fixation: when the sample is rapidly quenched to liquid nitrogen temperatures. For membranes that are already in a rigid (gel phase) lattice this should be minimal. Fluid membranes permit rapid lateral diffusion and it has been demonstrated that a fluid bilayer matrix allows significant lateral diffusional rearrangement of domain structure during the freeze-etch quenching process [57,58]. By the same token, glycolipids or glycolipid-lectin complexes might be laterally rearranged by being 'squeezed' out of an increasingly organized host lattice, as has been demonstrated for integral proteins (for review see Ref. 59). In fact this would be expected if there is a tendency to glycosphingolipid phase separation. This may be the phenomenon underlying the recent observation of Tillack et al. that a conventionally quenched sample of G_{M1} in a highly fluid host matrix showed patchiness of bound cholera toxin, while a similar sample 'slam frozen' in Dr. J. Heuser's laboratory showed uniform labelling [60].

In order to assess some of these factors we have attempted to analyse the distribution of bound lectin in samples whose nature might be expected to influence G_{M1} behaviour. The pattern of lectin binding as visualized in such experiments does seem to be related to significant membrane events. For instance the micrographs in Fig. 2 are all of rigid dipalmitoylphosphatidylcholine liposomes bearing 4.4 mol% G_{M1}. They were prepared at the same time and exposed simultaneously to the same lectin concentrations at 22°C. In each case the liposomes were produced by hydrating films dried from homogeneous organic solutions of lipid. However, those in Fig. 2A, were always maintained at 22°C (well below the 41.5°C main transition and 35°C pretransition temperatures of the host matrix [53]); while those in Fig. 2B, were warmed above 41.5°C and slowly cooled over a period of 15 min to 22°C prior to lectin addition. A qualitative difference is immediately apparent in that the samples which were not equilibrated (warmed above the phase transition temperature of the host matrix and slowly cooled) show relatively little evidence of lectin bound in groups. Although the samples were not prepared together, the same observation may be made in comparing Figs. 2A, B to Figs. 1A–C which were equilibrated by warming and slow cooling prior to lectin addition. Clearly lectin molecules do not bind in clusters simply by virtue of being aggregated in solution. Reliable quantitative assessment of lectin distribution over the membrane surface is a difficult problem, particularly in the face of the complications already described. The most straightforward quantitative method may be to note the number or fraction of lectin molecules in direct contact with other lectins in replica areas chosen for high technical quality, on the assumption that lectin molecules in direct contact may reflect the presence of underlying domains relatively enriched in G_{M1}. The results of such an approach to the data represented in Figs. 2A, B are shown in histogram form in Fig. 2C. From the histogram analysis, it is apparent that a measurable difference exists in the lectin binding pattern as a result of altering sample preparation. This may relate to glycosphingolipid organization within the bilayer as follows.

Given the structural differences between G_{M1} and dipalmitoylphosphatidylcholine, it would not be surprising if phase separated domains formed in mixed bilayers of the two species based on failure to be able to substitute exactly for one another in a crystal lattice. In fact formation of ganglioside-enriched domains in a dipalmitoyl phosphatidylcholine host matrix has been claimed previously [61–63] and a related phase diagram has been constructed [18]. Moreover the formation of such GM_1-enriched domains has been suggested by several groups to be most pronounced in rigid membranes [62,63]. On this basis a reasona-



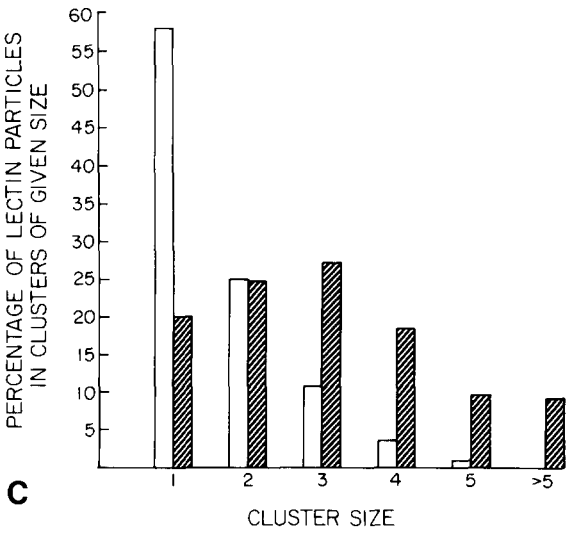


Fig. 2. Comparison of RCA 60 binding to liposomes of the same composition (4.4 mol% G_{M1} in DPPC) but different history. The sample in (A) was hydrated without warming above 22°C, while that in (B) was incubated at 50°C and allowed to cool over 15 min to 22°C (liposome phase transition temperature 41.5°C). Both samples were then simultaneously incubated with RCA 60 at 22°C and harvested as described in the caption to Fig. 1. Magnification: ×100000. Bar represents 100 nm. (C) is a histogram analysis of the surface distribution of receptor-bound RCA 60 on liposomes of the preparations illustrated in (A) and (B). Liposomes for histogram analyses were chosen solely on the basis of good replica quality (ability to distinguish surface features clearly). Lectin particles were counted manually at a magnification of ×100000 and grouped according to the number of others with which they made contact (defined as cluster size). Areas in excess of $120 \cdot 10^4$ μm^2 were counted on 6 or more liposomes from each sample using specimens prepared and freeze-etched together. Cross-hatched sections indicate data from the sample equilibrated at 50°C, while open sections refer to the sample which was not permitted to reach diffusional equilibrium. The samples which were not equilibrated show relatively little evidence of lectin bound in groups.

ble explanation would seem to be that the lipids dried down in organic solvent were fairly homogeneously dispersed. Hydration at 22°C produced bilayers which were too rigid to permit domain equilibration through lateral diffusion. As a result, the micrograph in Fig. 2A shows little evidence of G_{M1}-enriched domains larger than a lectin diameter (157 lipid molecules). When the same sample was allowed to equilibrate in the fluid state (above 41.5°C), and then cooled slowly prior to lectin addition, domains large enough to bind at least 6 lectin molecules in close proximity could form. This would seem to argue in favour of the meaningfulness of details of the lectin binding pattern. It also gives some idea of the size of domains involved: they are larger than 1 lectin diameter (157 lipid molecules) but probably smaller than some 10 lectin diameters (5600 lipid molecules) in a DPPC host matrix cooled slowly through its transition temperature.

The question of ganglioside behaviour in DPPC has been addressed by other workers using differential scanning calorimetry, a technique which extrapolates from heat uptake and melting behaviour to membrane domain composition. It permits estimation of 'cooperative unit size': the

number of lipid molecules which behave as an interdependent group, all melting or gelling simultaneously. Sillerud et al note that the addition of G_{M1} to dipalmitoyl phosphatidylcholine reduces the cooperative unit size from some 109 ± 9 to 39 ± 3 molecules [31]. Presumably a domain could not be smaller than one cooperative unit. The resolution of the freeze-etching lectin probe technique used here is from one to several cooperative units in the system under study. Hence there is the potential for visualization of features related to phase separation phenomena.

Another experiment relevant to the interpretation of lectin binding pattern in terms of underlying receptor arrangement is the effect of receptor concentration. An example is shown in Fig. 3 and the accompanying histogram. Liposomes of dipalmitoylphosphatidylcholine were prepared bearing either 1 or 14 mol% G_{M1}. They were warmed above the phase transition temperature and allowed to cool to 22°C over a period of 30 min. Each sample was exposed at the same time to identical concentrations (1 mg/ml) of RCA 60 in large excess. Note that there is visibly more lectin bound to the liposomes bearing 14 mol% receptor, and there appear to be more clusters. In fact though, the histogram distribution of particle cluster size is not as strikingly different as that in the previous experiment with equilibrated vs. unequilibrated liposomes. Also what difference there is may be partly due to the large difference in amount of lectin bound.

Host phospholipid matrix in the samples so far considered was selected to be well below its transition temperature to minimize glycolipid rearrangement related to lectin binding or sample quenching. Fig. 4 summarizes the results of an attempt at quantitation of lectin clusters as a reflection of G_{M1} domains in fluid vs. rigid dimyristoylphosphatidylcholine. Below 23°C this host matrix is rigid with an area per lipid molecule the same as that of dipalmitoyl phosphatidylcholine (0.50 nm^2 [46]). At 33°C it is extremely fluid and has an area per lipid molecule of 0.60 nm^2 [46,64]. Rigid dimyristoylphosphatidylcholine (Fig. 4A) shows the regular 'ripple' pattern characteristic of the $P_{\beta'}$ phase [65]. As judged by size of lectin clusters, G_{M1}-enriched domains in the rigid matrix seem to range up to some 1200 molecules. Lectin clusters are less obvious in the fluid phase dimyristoylphosphatidylcholine (and binding is less, consistent with the findings of Tillack et al. [66] for ferritin-conjugated RCA to asialo-G_{M1}). There is really no evidence of domain size greater than one lectin diameter (157 lipid molecules) in the fluid matrix (Fig. 4B). There is also the possibility of rearrangement of glycolipids over a distance of about 20 nm during the rapid freezing of fluid dimyristoylphosphatidylcholine prior to the fracture step, since irregular waves of this size are evident in the fluid membrane micrographs (Fig. 4B). The latter feature in fluid lipid has been noted to represent local rearrangement upon quenching [57,58].

Hence it appears that, within certain limits, there is justification for interpreting the visual details of native lectin bound to glycolipids in rigid bilayer matrices in terms of their arrangement within the membrane. We have applied the technique to three ganglioside families in order to add information from direct visualization to the controversial topic of Ca^{2+}-effects on gangliosides. We selected WGA as the lectin since it binds to all gangliosides, and is physically small yet distinguishable as a marker. Samples were handled in buffer of low overall ionic strength (which will tend to amplify any effect of the divalent cation). Similar results were obtained with isotonic saline, however, the presence of such high solute concentrations led to their precipitation during the etching step as a smooth layer of irregular depth that tends to obscure surface detail (and create new surface detail). As glycolipid lateral distribution may be sensitive to host matrix properties, membrane melting characteristics were documented by electron paramagnetic resonance spectroscopy. The small, amphiphilic spin label, TEMPO, is selectively excluded from rigid membranes; thus, by keeping track of the magnitude of the signal from TEMPO in the aqueous phase relative to that in the membrane, it is possible to follow the membrane phase state [40]. The results of this study are listed in Table I.

An examination of the data shows that the lipid transition temperature is not significantly altered relative to the study temperature of 22°C either by ganglioside incorporation or Ca^{2+} addition: the gross melting behaviour of the host matrix

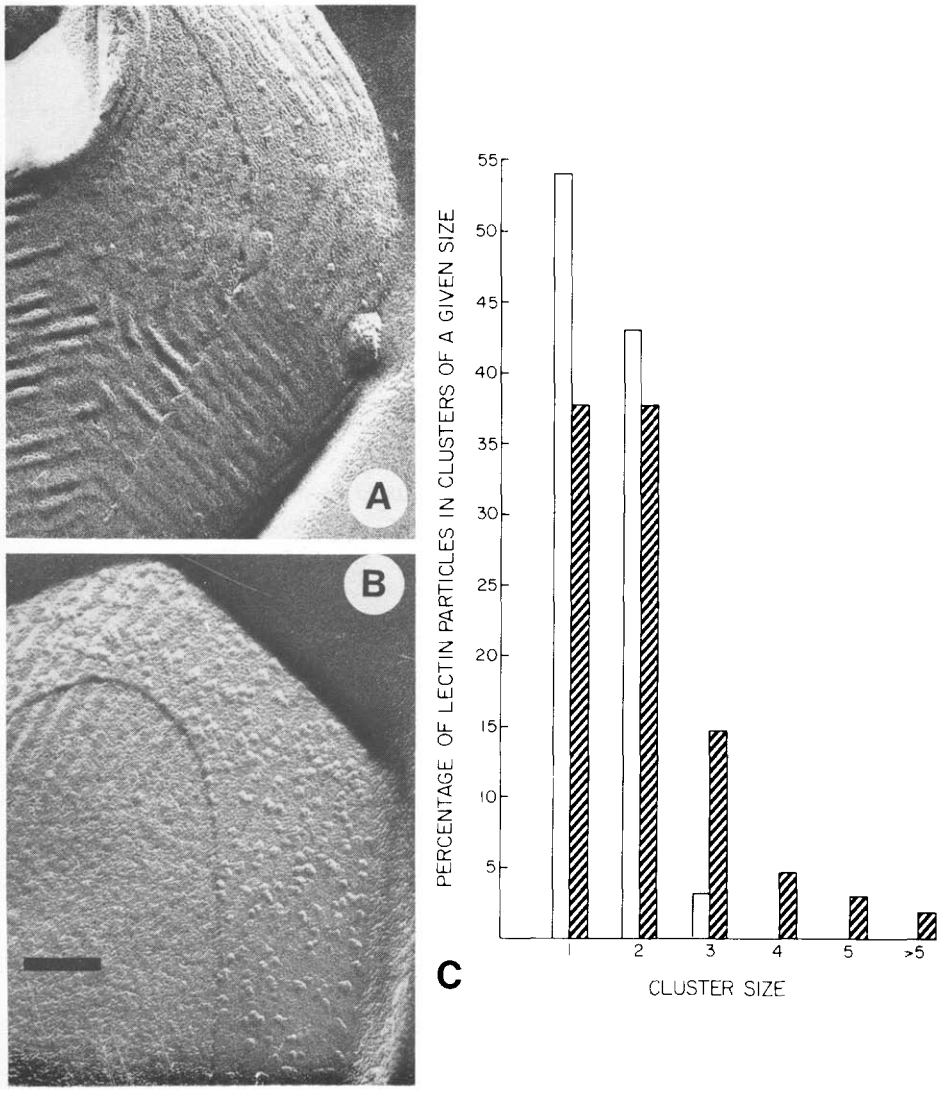


Fig. 3. Freeze-etch electron micrographs of RCA 60 binding to DPPC liposomes bearing different concentrations of G_{M1}. In each case liposomes were prepared and handled as described in the caption to Fig. 1. (A) shows a typical liposome with 1 mol% G_{M1} while that in (B) has 14 mol%. Magnification: ×100000. Bar represents 100 nm. (C) is a histogram analysis of receptor-bound RCA 60 on liposomes of the preparations illustrated in (A) and (B). Lectin particles in contact with one another were counted as 'clusters'. Cross-hatched sections represent data from the DPPC liposomes with 14 mol% G_{M1}, while open sections refer to the 1 mol% sample.

remains the same. More subtle details involve small changes in the transition temperature and pre-transition. Such changes in the melting behaviour of a DPPC multibilayer host matrix have been mentioned by other workers, including broadening of, and modest increases in, transition and pre-transition temperature [18,31,35]. We will not discuss these changes here since they are best dealt with in terms of a phase diagram as Barenholz et al. [67] and Maggio et al. [18] have done for glucosylceramide and gangliosides, respectively, in DPPC mixtures.

It was seen in Fig. 1 that liposomes bearing (negatively charged) dicetyl phosphate at 7 mol% instead of ganglioside show minimal evidence of WGA-related etch face particles when exposed to

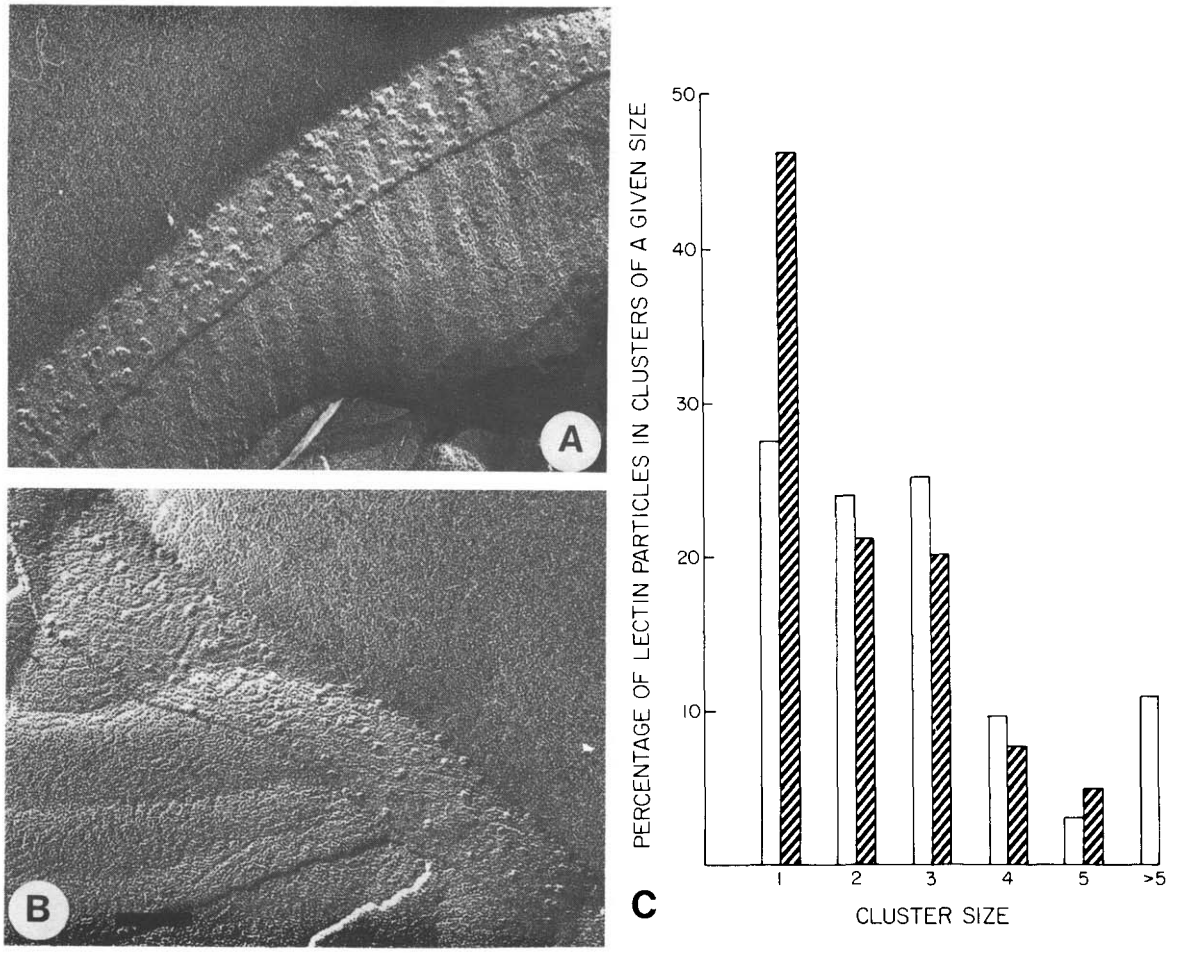


Fig. 4. Freeze-etch electron micrographs of liposomes of dimyristoylphosphatidylcholine bearing 7 mol% G_{M1}, and labelled with native RCA 60 above and below the host matrix phase transition temperature. In each case dry lipid films were hydrated at 35°C. The sample in 4A was subsequently allowed to cool in a 100 ml water bath to 4°C over a period of 15 min. Exposure to 0.15 mg/ml RCA 60 was for 10 min at 4°C (A) or 35°C (B) (2 mg total lipid in 2 ml 10 mM phosphate buffer (pH 7.4)), followed by harvesting of liposomes at 1700× *g* for 10 min at the same temperatures. Magnification is ×100000. Bar represents 100 nm. (C) represents a histogram analysis of receptor-bound RCA 60 on liposomes of the preparations illustrated in (A) and (B). Lectin particles in contact with one another were counted as 'clusters'. Cross-hatched sections represent data from the 35°C samples, while open sections refer to the 4°C samples.

lectin in the presence of 0, 2, or 10 mM $CaCl_2$ (Figs. 1G, H, I). Fig. 5 shows the appearance of liposomes bearing mono-, di-, or trisialo gangliosides after lectin labelling at the same Ca^{2+} concentrations. For the experiments illustrated, gangliosides were incorporated at 7 mol% into DPPC liposomes. The dried films of mixed lipids were hydrated in excess buffer above 41.5°C so that thermodynamic equilibrium was achieved in the presence of Ca^{2+} ions at the concentrations quoted. Samples were then allowed to cool over a period of 60 min to 22°C, at which point the host matrix should be rigid (Table I), and thus strongly inhibit glycolipid lateral rearrangement. Lectin was then added 15 min prior to quenching.

While the data shown are representative, there is an intrinsic variability amongst liposome surfaces. However, based on examination of large numbers of liposomes in repeated experiments, one may draw the following conclusions. Firstly the tendency for receptor glycolipid to be preferentially localized between $P_{\beta'}$ ripples (as was the

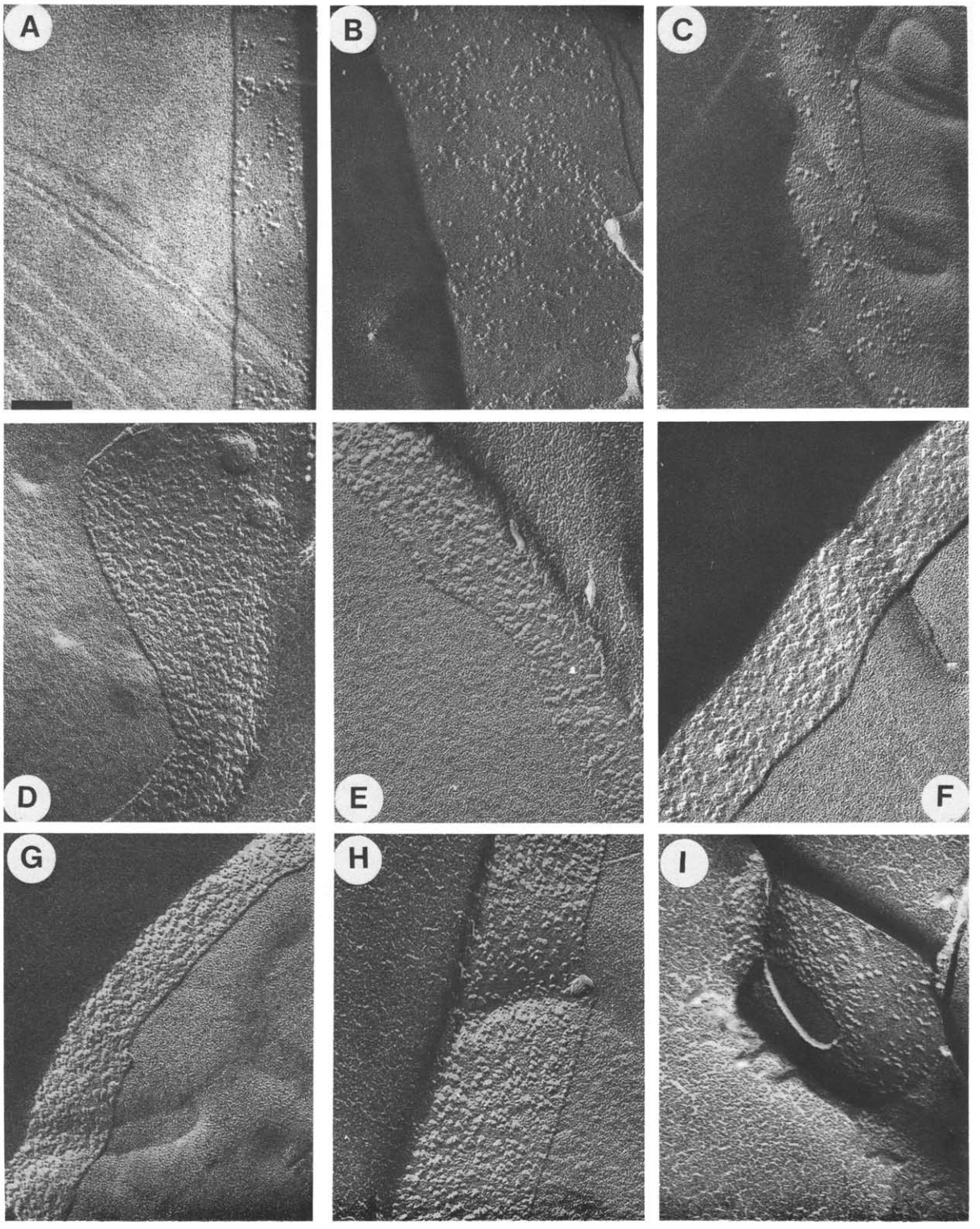


Fig. 5. Freeze-etch electron micrographs of DPPC liposomes bearing 7 mol% G_{M1} (A–C), G_{D1a} (D–F), or G_{T1b} (G–I), and exposed to WGA in the presence of various Ca^{2+} concentrations. 0 mM $CaCl_2$ (A, D, G). 2 mM $CaCl_2$ (B, E, H). 10 mM $CaCl_2$ (C, F, I). All samples in 20 mM NaCl buffered with 5 mM Hepes (pH 7.4). Sample preparation as in Fig. 1, except that after incubation with Ca^{2+} at 50°C for 1 h, samples were cooled over a period of 1 h to 22°C prior to lectin addition and quenching from 22°C. Magnification: ×100000. Bar represents 100 nm.

TABLE I

MELTING BEHAVIOUR

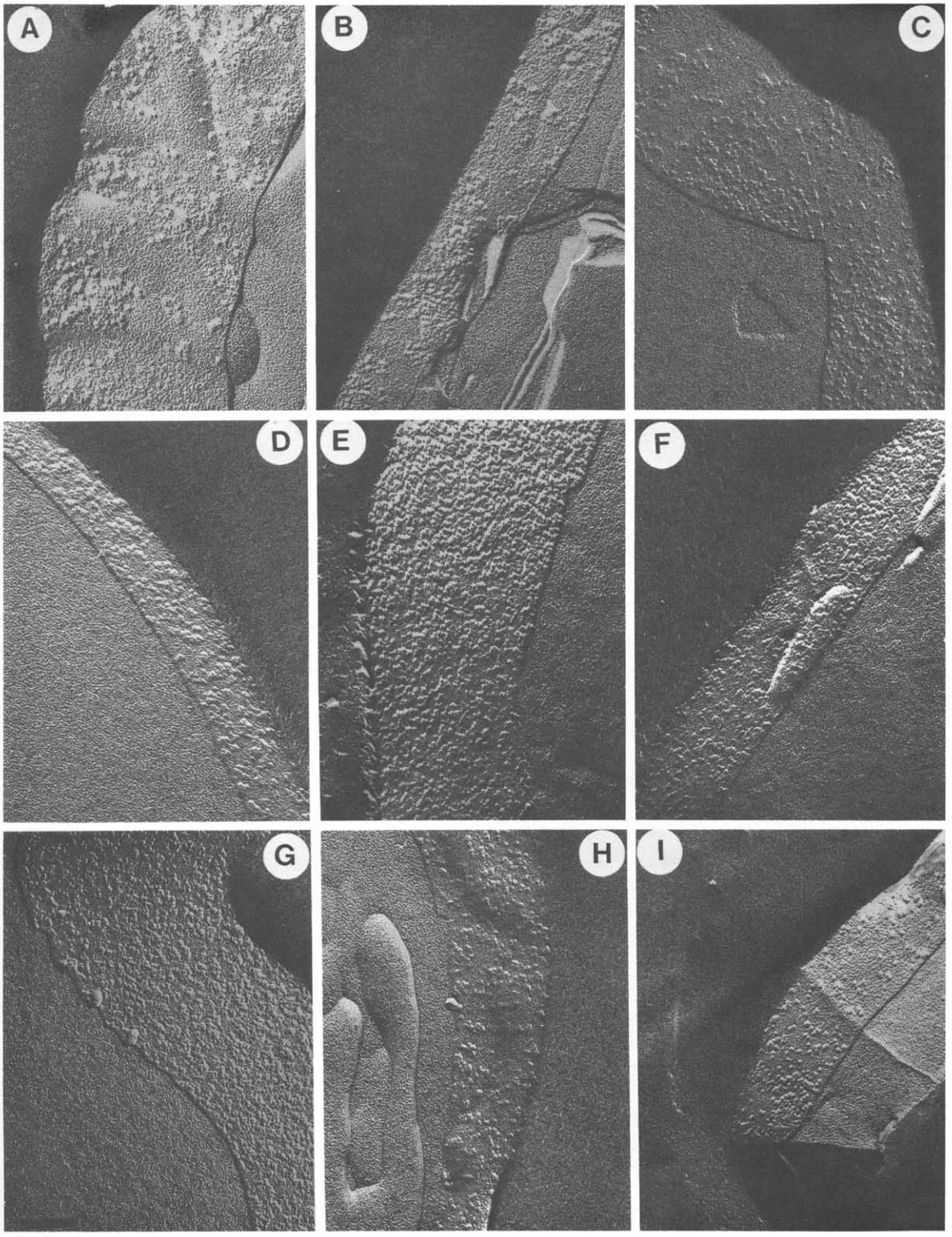
Melting behaviour of DPPC liposomes bearing 7 mol% G_{M1}, G_{D1a} or G_{T1b} in the presence and absence of Ca^{2+}. Values were determined from plots of EPR TEMPO data as outlined in Methods, and are averages of at least two independent experiments.

Lipid, DPPC +	Hydration and incubation buffer (pH 7.5), 5 mM Hepes, 20 mM NaCl +	Pre-transition temperature (°C ± 1.0 °C)	Transition temperature (°C ± 0.6 °C)
–	+ 0 mM Ca^{2+}	34.1	42.0
–	+ 2 mM Ca^{2+}	33.0	41.5
–	+ 10 mM Ca^{2+}	35.7	41.9
G_{M1}	+ 0 mM Ca^{2+}	33.6	40.6
G_{M1}	+ 2 mM Ca^{2+}	32.7	40.2
G_{M1}	+ 10 mM Ca^{2+}	34.0	44.0
G_{D1a}	+ 0 mM Ca^{2+}	34.9	41.6
G_{D1a}	+ 2 mM Ca^{2+}	37.7	41.8
G_{D1a}	+ 10 mM Ca^{2+}	broad	41.6
G_{T1b}	+ 0 mM Ca^{2+}	35.4	42.4
G_{T1b}	+ 2 mM Ca^{2+}	37.0	43.8
G_{T1b}	+ 10 mM Ca^{2+}	broad	44.3

case for G_{M1} in Fig. 2) was not seen for G_{D1a} or G_{T1b}. We have noted this previously with regard to G_{M1}/G_{D1a} in the absence of Ca^{2+} [20]. The result is unaltered by the presence of Ca^{2+} at 2 or 10 mM. Secondly, the existence of bound lectin in patches some 60 nm in diameter (5655 lipid molecules) that was noted in DPPC membranes bearing G_{M1}, is not nearly as striking for G_{D1a} or G_{T1b}. Rather both higher gangliosides show a more uniform pattern of labelling by lectin at 0, 2 and 10 mM Ca^{2+}. If one may take the small groups of lectin that are common to Figs. 5D–I as a valid reflection of domain structure, then domains selectively enriched in G_{D1a} or G_{T1b} must be in the range of 20 nm diameter or smaller. Domains of this size range would comprise some 630 or fewer lipid molecules. Once again since no evidence of bilayer disruption is visible, one must conclude that selective enrichment in such domains beyond 20–25 mol% ganglioside is unlikely.

It may be hoped that incubation with Ca^{2+} above the transition temperature, followed by slow cooling to 22°C, will permit ganglioside diffusional equilibrium which will then be preserved in a rigid matrix prior to lectin addition and prior to quenching in liquid freon. However, one might argue that the eucaryote plasma membrane exhibits greater fluidity and less cooperative melting behaviour than the systems described above. The incorporation of cholesterol above 20 mol% into a bilayer lattice of DPPC abolishes the 41.5°C phase transition, and induces a state of intermediate fluidity below this temperature (Refs. 68, 69 and references therein). Hence we applied the lectin labelling technique to bilayer membranes of 2 : 1 (mole ratio) DPPC/cholesterol bearing G_{M1}, G_{D1a} or G_{T1b} at 1 and 7 mol%. Typical 7 mol% samples are shown in Fig. 6. In spite of the very different characteristics of this membrane system, at physiological Ca^{2+} concentration the observations are qualitatively similar to those already described for bilayers of pure DPPC. G_{M1} domains marked by bound lectin are somewhat larger and more striking (Fig. 6A), up to 100 nm in diameter, than we recorded in DPPC alone. 'Domain size' (lectin cluster size) of G_{D1a} and G_{T1b} membranes with cholesterol appears the same as that in DPPC. Larger lectin clusters in the case of G_{M1} might be accounted for by lectin-induced G_{M1} patching in the more fluid matrix. However, if so, one would expect this to occur for all three ganglioside families, and lectin bound to the di- and trisialo species showed no such effect. Also, as mentioned earlier, we have not previously observed such redistribution where tested in matrices of intermediate or high fluidity [19,20]. Larger G_{M1} domains in the cholesterol-containing matrix might alternatively be accounted for on the basis of quenching artefact in the more fluid matrix, although this would presume a much greater effect upon the G_{M1}/lectin complex than upon the higher gangliosides. There was no visible effect of 2 mM Ca^{2+} vs. 0 mM Ca^{2+}. Increasing Ca^{2+} concentration beyond the physiological range to 10 mM did lead to the presence of some very large lectin patches (see, for example, Fig. 6F), but these were atypical. As in all cases, binding was reversed by the inhibitory sugar, *N*-acetyl-D-glucosamine at a concentration of 30 mM.

Although cholesterol-phospholipid mixtures are non-cooperative above 20 mol%, they nevertheless are liquid crystal in nature and have been demonstrated as a result to exclude integral proteins from phase-separated domains [70]. As already


A
B
C
D
E
F
G
H
I

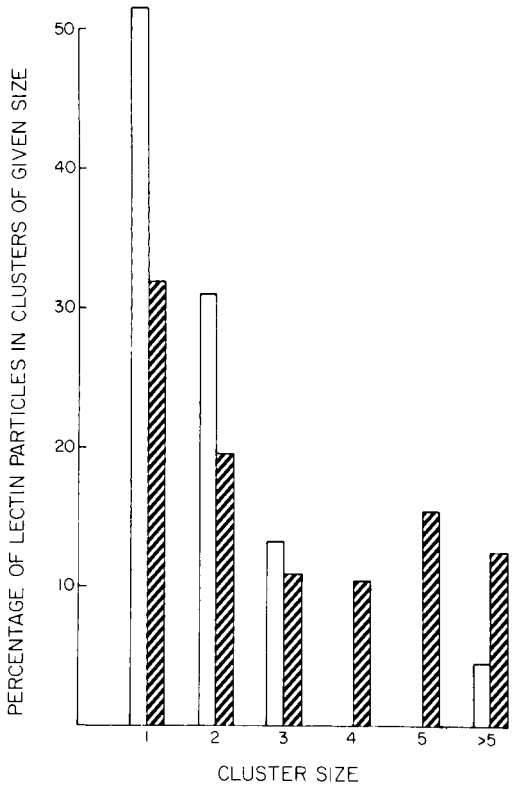


Fig. 7. Histogram analysis of the arrangement of RCA 60 bound to G_{M1} (7 mol%) in 2:1 (mole ratio) DPPC/cholesterol as seen in the same sample labelled and quenched at two different states of fluidity. Lectin particles in direct contact with one another were counted as 'clusters'. 22°C (less fluid): cross-hatched bars; 35°C (more fluid): open bars. Sample preparation as in caption to Fig. 1 (both samples cooled from 50°C prior to lectin labelling). Liposomes were exposed to RCA 60 in 10 mM phosphate buffer (pH 7.4) for 10 min at 22°C or 35°C. Magnification: ×100000. Bar represents 100 nm.

described, domain formation and quenching artefact may derive from the same phenomenon. The pattern seen for lectin bound to G_{M1} is in fact very similar to that of the integral protein, $(Ca^{2+} + Mg^{2+})$-ATPase of rabbit sarcoplasmic reticulum, in similar bilayers reported by Kleemann and McConnell [70] and related by these authors to the existence of lipid domains. We studied the effect of incubation temperature on liposomes bearing 7 mol% G_{M1} in a host matrix of 2:1 (mole ratio) dipalmitoylphosphatidylcholine/cholesterol by quantitating bound RCA 60. Liposomes were initially hydrated and then incubated at 50°C for 10 min prior to cooling to the temperature at which they were labelled with lectin. Samples were marked with RCA 60 at 35°C and quenched from the same temperature, or cooled slowly to 21°C prior to lectin addition and quenching (from 21°C). DPPC/cholesterol bilayers are markedly more fluid at 35°C than 21°C, yet the specimens quenched from these temperatures were not obviously different. Overall there was a more heterogeneous lectin distribution in the 21°C sample, which is reflected in the histogram (Fig. 7). Such a result is in line with the concept already discussed of the existence of G_{M1}-enriched domains; however, given the limitations involved in fluid matrices, it is not a compelling argument. Certainly though it is inconsistent with lectin-induced clustering of receptors or with quenching artefact, both of which should be more marked in the 35°C samples.

Acknowledgements

This research was supported by grants from the Medical Research Council of Canada and the Academic Research Foundation.

References

1 Spiegel, S., Skutelsky, E., Bayer, E.A. and Wilchek, M. (1982) Biochim. Biophys. Acta 687, 27–34
2 Grant, C.W.M. (1984) Can. J. Biochem. Cell Biol. 62, 1151–1157
3 Koscielak, J., Hakomori, S. and Heanoz, R.W. (1968) Immunochemistry 5, 441–455
4 Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733–764
5 Kannagi, R., Stroup, R., Cochran, N.A., Urdal, D.L., Young, Jr., W.W. and Hakomori, S. (1983) Cancer Res. 43, 4997–5005
6 Hakomori, S., Teather, C. and Andrews, H. (1968) Biochem. Biophys. Res. Commun. 33, 563–568
7 Weinstein, D.B., Marsh, J.B., Glick, M.C. and Warren, L. (1970) J. Biol. Chem. 245, 3928–3937

Fig. 6. Freeze-etch electron micrographs of liposomes of 2:1 (mole ratio) DPPC/cholesterol bearing 7 mol% G_{M1} (A–C), G_{D1a} (D–F), or G_{T1b} (G–I) and exposed to WGA in the presence of various Ca^{2+} concentrations. 0 mM $CaCl_2$ (A, D, G). 2 mM $CaCl_2$ (B, E, H). 10 mM $CaCl_2$: (C, F, I). All samples in 20 mM NaCl buffered with 5 mM Hepes (pH 7.4). Sample preparation as in Fig. 5. Magnification: ×100000. Bar represents 100 nm.

8 Gahmberg, C.G. and Hakomori, S. (1973) Proc. Natl. Acad. Sci. USA 70, 3329–3333
9 Gahmberg, C.G. and Hakomori, S. (1975) J. Biol. Chem. 250, 2438–2446
10 Moss, J., Manganiello, V.C. and Fishman, P.H. (1977) Biochemistry 16, 1876–1881
11 Urdal, D.L. and Hakomori, S. (1983) J. Biol. Chem. 258, 6869–6874
12 Alving, C.R., Urban, K.A. and Richards, R.L. (1980) Biochim. Biophys. Acta 600, 117–125
13 Cestaro, B., Barenholz, Y. and Gatt, S. (1980) Biochemistry 19, 615–619
14 Thomas, P.D. and Podder, S.K. (1982) Biochim. Biophys. Acta 688, 453–459
15 Peters, M.W., Singleton, C., Barber, K.R. and Grant, C.W.M. (1983) Biochim. Biophys. Acta 731, 475–482
16 Utsumi, H., Suzuki, T., Inoue, K. and Nojima, S. (1984) J. Biochem. 96, 97–105
17 Thompson, T.E. and Tillack, T.W. (1985) Annu. Rev. Biophys. Biophys. Chem. 14, 361–386
18 Maggio, B., Ariga, T., Sturtevant, J.M. and Yu, R.K. (1985) Biochim. Biophys. Acta 818, 1–12
19 Peters, M.W. and Grant, C.W.M. (1984) Biochim. Biophys. Acta 775, 273–282
20 Peters, M.W., Mehlhorn, I.E., Barber, K.R. and Grant, C.W.M.. (1984) Biochim. Biophys. Acta 778, 419–428
21 Maggio, B., Cumar, F.A. and Caputto, R. (1981) Biochim. Biophys. Acta 650, 69–87
22 Probst, W., Nobius, D. and Rahmann, H. (1984) Cell. Mol. Neurobiol. 4, 157–176
23 Sklar, L.A., Miljanich, G.P. and Dratz, E.A. (1979) J. Biol. Chem. 254, 9592–9597
24 Van Dijck, P.W.M., De Kruijff, B., Verkleij, A.J., Van Deenen, L.L.M. and De Gier, J. (1978) Biochim. Biophys. Acta 512, 84–96
25 Tilcock, C.P.S. and Cullis, P.R. (1981) Biochim. Biophys. Acta 641, 189–201
26 Galla, H.-J. and Sackmann, E. (1975) Biochim. Biophys. Acta 401, 509–529
27 Findlay, E.J. and Barton, P.G. (1978) Biochemistry 17, 2400–2405
28 Sharom, F.J. and Grant, C.W.M. (1978) Biochim. Biophys. Acta 507, 280–293
29 Behr, J.-P. and Lehn, J.-M (1973) FEBS Lett. 31, 297–299
30 Probst, W., Rosner, H., Wiegundt, H. and Rahmann, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 979–986
31 Sillerud, L.O., Schafer, D.E., Yu, R.K. and Konisberg, W.H. (1979) J. Biol. Chem. 254, 10876–10880
32 Brewer, G.J. and Thomas, P.D. (1984) Biochim. Biophys. Acta 776, 279–287
33 a. Goins, B. and Freire, E. (1985) Biochemistry 24, 1791–1797
33 b. Goins, B., Masserini, M., Barisas, B.G. and Freire, E. (1986) Biophys. J. 49, 849–856
34 Myers, M., Wortman, C. and Freire, E. (1984) Biochemistry 23, 1442–1448
35 Bertoli, E., Masserini, M., Sonnino, S., Ghidoni, R., Cestaro, B. and Tettamanti, G. (1981) Biochim. Biophys. Acta 467, 196–202
36 McDaniel, R.V., and McLaughlin, S. (1985) Biophys. J. 47, 424a
37 Rubenstein, J.L.R., Smith, B.A. and McConnell, H.M. (1979) Proc. Natl. Acad. Sci. USA 76, 15–18
38 Hakomori, S. and Siddiqui, B. (1974) Methods Enzymol. 32(b), 354–355
39 Rozantsev, E.G. (1970) Free Nitroxyl Radicals, Plenum Press, New York
40 Shimshick, E.J. and McConnell, H.M. (1973) Biochemistry 12, 2351–2360
41 Gaffney, B.J. and McNamee, C.M. (1974) Methods Enzymol. 32, 161–198
42 Bhavanandan, V.P. and Katlic, A.W. (1979) J. Biol. Chem. 254, 4000–4008
43 Kronis, K.A. and Carver, J.P. (1982) Biochemistry 21, 3050–3057
44 Boldt, D.H., Speckart, S.F., Richards, R.L. and Alving, C.R. (1977) Biochem. Biophys. Res. Commun. 74, 208–214
45 Nagata, Y. and Burger, M. (1974) J. Biol. Chem. 249, 3116–3122
46 Janiak, M.J., Small, D.M. and Shipley, G.G. (1979) J. Biol. Chem. 254, 6068–6078
47 Zwaal, R.F.A., Demel, R.A., Roelofsen, B. and Van Deenen, L.L.M. (1976) Trends Biochem. Sci. 112–114
48 Brown, J.C. and Hunt, R.C. (1978) Int. Rev. Sytol. 52, 277–349
49 Kabat, E.A., Heidelberger, M. and Bezer, A.E. (1947) J. Biol. Chem. 168, 629–639
50 Bunow, M.R. and Bunow, B. (1979) Biophys. J. 27, 325–327
51 Lotan, R., Skutelsky, E., Danon, D. and Sharon, M. (1975) J. Biol. Chem. 250, 8518–8523
52 Phillips, M.C., Graham, G.E. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35–44
53 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237–344
54 Fishman, P.H. and Atikkan, E.E. (1980) J. Mem. Biol. 54, 51–60
55 Grant, C.W.M. and Peters, M.W. (1984) Biochim. Biophys. Acta 779, 403–422
56 Ketis, N.V. and Grant, C.W.M. (1983) Biochim. Biophys. Acta 730, 359–368
57 Ververgaert, P.H.J.T., Verkleij, A.J., Verhoeven, J.J. and Elbers, P.F. (1973) Biochim. Biophys. Acta 311, 651–654
58 Grant, C.W.M., Wu, S.H. and McConnell, H.M. (1974) Biochim. Biophys. Acta 363, 151–158
59 Grant, C.W.M. (1983) in Membrane Fluidity in Biology (Aloia, R.C., ed.), Vol. 2, pp. 131–150, Academic Press, New York
60 Thompson, T.E., Allietta, M., Brown, R.E., Johnson, M.L. and Tillack, T.W. (1985) Biochim. Biophys. Acta 817, 229–237
61 Lee, P.M., Ketis, N.V., Barber, K.R. and Grant, C.W.M. (1980) Biochim. Biophys. Acta 601, 302–314
62 Delmelle, M., Dufrane, S.P., Brasseur, R. and Ruysschaert, J.M. (1980) FEBS Lett. 121, 11–14
63 Bach, D., Miller, I.R. and Sela, B. (1982) Biochim. Biophys. Acta 686, 233–239
64 Cornell, B.A. and Spearovic, F. (1983) Biochim. Biophys. Acta 733, 189–193

65 Tardieu, A., Luzzati, C. and Reman, F.C. (1973) J. Mol. Biol. 75, 711–733
66 Tillack, T.W., Wong, M., Allietta, M. and Thompson, T.E. (1982) Biochim. Biophys. Acta 691, 261–273
67 Barenholz, Y., Freire, E., Thompson, T.E., Correa-Freire, M.C., Bach, D. and Miller, I.R. (1983) Biochemistry 22, 3497–3501
68 Demel, R.A. and De Kruÿff, B. (1976) Biochim. Biophys. Acta 457, 109–131
69 Pink, D.A., Green, T.J. and Chapman, D. (1981) Biochemistry 20, 6692–6698
70 Kleemann, W. and McConnell, H.M. (1976) Biochim. Biophys. Acta 419, 206–222
71 Tillack, T.W., Allietta, M., Moran, R.E. and Young, Jr., W.W. (1983) Biochim. Biophys. Acta 733, 15–24
72 Bach, D., Miller, I.R. and Sela, B. (1982) Biochim. Biophys. Acta 686, 233–239