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GLYCOLIPIDS IN MODEL MEMBRANES
SPIN LABEL AND FREEZE-ETCH STUDIES

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SUMMARY

1. Several types of glycolipid are examined in lipid bilayer model membranes as part of a program to clarify their fuction in living cells.

2. Data obtained with three spin labelled derivatives of galactosyl ceramide is reported showing a fatty acid fluidity gradient similar to that obtained with phospholipid spin labels. Some possible structural implications of the observed differences are considered.

3. Results obtained using Freeze-Etch electron microscopy and hemagglutination inhibition are given showing beef brain gangliosides in lipid vesicles to be effective receptors for influenza virus.

INTRODUCTION

Lipid bilayer structures obtained by hydrating dry phospholipids have been a popular model membrane system for some years now. Their usefulness stems from the fact that bilamellar regions are thought to be a common structural feature of many biological membranes [1]. In recent years these hydrated lipid bilayers have been seen as a (partial) solution to one of the problems facing researchers who would study isolated membrane components, viz. their loss of function when removed from the membrane context. In some cases it has been possible to support or reconstitute the activity of sensitive membrane constituents using bilayer structures derived from purified lipids (e.g. refs. 2–6).

We have been interested in lipid bilayers as a support matrix for carbohydrate-bearing components (neutral glycolipids, gangliosides and glycoproteins). In general the meaningful incorporation of isolated membrane components into the walls of lipid vesicles under non-denaturing conditions is a challenging problem. But in the case of lipids it is only necessary to dissolve the dried components in an organic solvent prior to drying and subsequent hydration. One is left (presumably) with a symmetric bilayer, which will be an important shortcoming for certain studies

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in view of the growing interest in membrane assymmetry [7–9]. Nevertheless such systems are appealing in that they offer the possibility of investigating fuctional carbohydrate-bearing recognition sites in a "biological membrane" devoid of any other complex constituents. One can control the lipid composition and fluidity of such systems very precisely and even modify their domain structure to some extent. [10–14].

MATERIALS AND METHODS

Materials. Dipalmitoyl phosphatidylcholine was obtained from Calbiochem. Egg phosphatidylcholine, cholesterol, dioleoyl phosphatidylcholine and palmitoyl lyso-phosphatidylcholine were obtained from Serdary Research Laboratories. All lipids were pure by the criterion of thin layer chromatography on silica gel G (Stahl). Gangliosides were prepared from fresh beef brain grey matter by the method of Kanfer [15] and analysed as a mixture of mono-, di- and trisialo species (with a few percent higher gangliosides).

Influenza virus, strain A/PR8/34 (HONI), purified by erythrocyte adsorption and elution followed by preparative ultracentrifugation, was a gift of Dr. Alan P. Kendal, currently of the Respiratory Virology Section, Center for Disease Control, Atlanta, G. The Port Chalmers strain was grown with the help of Dr. Margaret Creighton of the Department of Bacteriology and Immunology at the University of Western Ontario and was isolated by differential centrifugation. Viruses were inactivated by heating to 56 °C for 30 min, or by ultraviolet irradiation.

Spin labels. The stearic acid derivative with nitroxide-containing ring in the C-12 position (the (5, 10) fatty acid) was prepared by a combination of the methods of Waggoner [16] et al and Hubell and McConnell [17]. Stearic acid derivatives with the nitroxide-containing ring in the C-5 or C-16 positions the (12, 3) and (1, 14) fatty acids respectively) were prepared by minor modification of the general procedures outlined in ref. 17. Galactosyl psychosine prepared from beef brain cerebroside was acylated with the various fatty acid spin labels as described earlier [18]. Corresponding phospholipid spin labels were prepared by acylation of palmitoyl lyso-phosphatidylcholine with the appropriate fatty acid spin label [17, 19].

Preparation of lipid samples. Lipid samples were prepared by first mixing appropriate amounts of each component dissolved in chloroform/methanol and pumping extensively under vacuum to remove traces of solvent. Dried lipids were suspended in buffer by vortexing.

Oriented spectra. Lipids were deposited from chloroform/methanol as films on selected sections of glass microscope slides (total lipid about 2 mg). The plates were dried under vacuum for 90 min and hydrated overnight in a small, sealed container. A second plate was then pressed onto the first and the bilayers were ordered by shearing gently. Plates could be oriented in the EPR spectrometer using a teflon holder. Spectra were recorded on a Varian E 12 EPR spectrometer.

Electron microscopy, freeze-etching. Liposomes were prepared from dried lipid mixtures (total about 1 mg) by vortexing in pH 7.0 phosphate buffered saline at 45 °C. In general, the larger liposomes were harvested by centrifugation at $750 \times g$ for 10 min. Typically these large liposomes were incubated at 4 °C in 250 μl of phosphate buffered saline with 750 HAU of influenza virus. After 45 min the sample

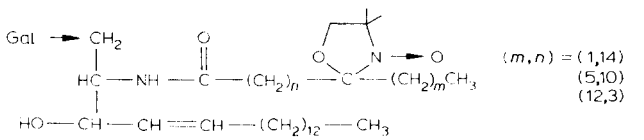
was pelleted at $750 \times g$ for 10 min and washed with 0.5 ml of cold buffer. Samples were rapidly quenched from 4 °C on 3 mm copper discs and freeze-etched on a Balzers BA 510 Freeze-Fracture device. Replicas were cleaned in commercial bleach and ethanol prior to examination on a Phillips EM 200.

Inhibition of hemagglutination. The following lipid samples were prepared: (i) 1.5 mg egg phosphatidylcholine+0.77 mg cholesterol; (ii) 1.5 mg egg phosphatidylcholine+0.77 mg cholesterol+0.114 mg phosphatidylserine; (iii) 1.5 mg egg phosphatidylcholine+0.77 mg cholesterol+0.7 mg beef brain gangliosides.

The dried lipids were vortexed vigorously in 100 μl of phosphate buffered saline. Three sets of serial two-fold dilutions of influenza virus were made in 0.1 ml of the same buffer. 10 μl of the appropriate liposome mixture was added to each series of dilutions, and incubated at 4 °C for 30 min to allow adsorption of virus. 0.1 ml of fresh 1 % human erythrocytes in phosphate buffered saline was added, and the micro-titre plate was allowed to stand overnight at 4 °C. The end point was read as the highest dilution of virus at which erythrocytes were not completely agglutinated. (Identical method for Sendai virus).

RESULTS AND DISCUSSION

We have previously [18] reported the synthesis of galactosyl ceramide with a spin label attached near the methyl end of its fatty acid chain. Using the same general procedures we have prepared two other derivatives with labels closer to the head group.



The divalent cation, Ca^{2+}, in cation/lipid molar ratios of 0.4 : 1 to 4 : 1 showed no special tendency to cluster or immobilize the (1, 14)-labelled glycolipid [18]. Clustering would be expected to lead to spin-exchange broadening of the EPR signal [20], and immobilization to other well-characterized [21, 22] spectral changes. These observations have also been borne out with the glycolipids labelled near the head group region and would argue against a strong glycolipid-Ca^{2+} interaction.

Like phospholipids [10, 23 24], the neutral glycolipid galactosyl ceramide tends to be excluded [18] from rigid, phosphatidylserine-enriched domains formed by headgroup crosslinking in the presence of Ca^{2+}.

Having a family of glycolipids labelled at various positions along the fatty acid chain makes it possible to measure order parameters, S [17, 25, 26], as a function of distance from the polar head group region of the lipid bilayer. The resulting fluidity gradient found using glycolipid spin labels has been compared to that found using phospholipid labels. For this purpose glycolipid or phospholipid labels were included at a concentration of 1 % in fully hydrated bilayers of egg phosphatidylcholine. For each label the order parameter, S, was determined in the conventional manner [17, 25, 26] using the formula

$$S = \left(\frac{T_{\parallel} - T_{\perp}}{T_{zz} - T_{xx}}\right) \cdot \left(\frac{a}{a'}\right)$$

TABLE I

SPECTRAL DATA AND ORDER PARAMETERS FOR PHOSPHOLIPID AND GLYCOLIPID SPIN LABELS IN LIPID BILAYERS

Values of $T_{\parallel}$, $T_{\perp}$ and the calculated order parameter S for various spin labelled galactosyl ceramides and phosphatidylcholines in bilayers of egg phosphatidylcholine. Both phosphatidylcholine and galactosyl ceramide values are averages of two different experiments. EPR spectra were recorded at 23 °C.

	$T_{\parallel}$ gauss	$T_{\perp}$ gauss	S
(12,3) Phosphatidylcholine	25.85	8.85	0.649
(5,10) Phosphatidylcholine	20.75	10.55	0.405
(1,14) Phosphatidylcholine	17.05	12.35	0.188
(12,3) Galactosyl ceramide	27.53	8.30	0.723
(5,10) Galactosyl ceramide	22.10	10.00	0.477
(1,14) Galactosyl ceramide	17.15	12.15	0.200

where $T_{\parallel}$ and $T_{\perp}$ are the measured spectral parameters, T_{zz} and T_{xx} are crystal parameters for the nitroxide radical and a/a' is a correction for polarity. For the (12, 3) labels $T_{\parallel}$ and $T_{\perp}$ may be measured directly from the isotropic spectra. For the (5, 10) and (1, 14) labels these parameters were obtained by orienting the hydrated lipid bilayers perpendicular and parallel respectively to the magnetic field of the spectrometer. A variety of methods has evolved for the preparation of oriented multibilayers [27]; the method employed here was gentle shearing of hydrated lipid films formed on glass slides. Measured values of $T_{\parallel}$ and $T_{\perp}$ and calculated order parameters are given in Table I and shown plotted in Fig. 1. The values found for phospholipids in egg phosphatidylcholine are in good agreement with those reported previously by McConnell and McFarland [26].

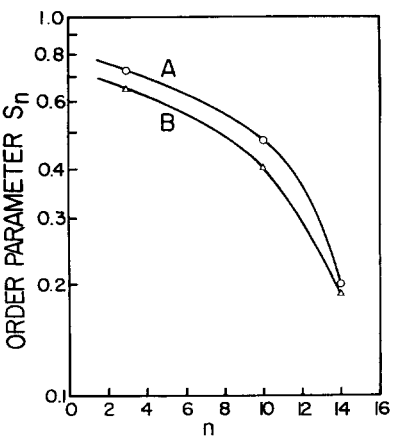


Fig. 1. The order parameter, S, plotted as a function of n, where n is the number of methylene carbons between the spin-label ring and the carboxyl function of the fatty acid. The data (from Table I) is for fully hydrated bilayers of egg phosphatidylcholine containing 1% spin labelled (A) galactosyl ceramide and (B) phosphatidylcholine. Temperature of all spectra, 23 °C.

Since both the labelled glycolipids and labelled phospholipids were incorporated at low concentrations their spectra should reflect conditions characteristic of the egg phosphatidylcholine bilayer immediately surrounding the label in question. Clearly, as was first shown with spin labels [28], there is a fluidity gradient toward the hydrophobic centre of the lipid bilayer. The gradient around the glycolipid is qualitatively very similar to that seen by a phospholipid. However, for each position in the fatty acid chain the order parameter sensed by the glycolipid label is higher than that sensed by the corresponding phospholipid label. In fact, the glycolipid values correspond to those expected for a phospholipid spin label if (a) 15–20 mol % cholesterol were included in the bilayers [26] or (b) the spin label were 1–3 carbons closer to the head group region. This observation could be explained by a tighter packing of phospholipids around glycolipids than around other phospholipids. In this regard Tinker et al. [29] have recently remarked on the ability of high concentrations of the glycolipid, globoside, to rigidify phosphatidylcholine-containing bilayers. Alternatively, the spin labelled fatty acid in galactosyl ceramide might occupy a position 1–3 carbons higher in the membrane than the fatty acid in phosphatidylcholine. This latter possibility seemed quite feasible on (PCK) model building grounds since the bulky (and likely extensively hydrogen-bonded) galactose residue may not pack down amongst the choline head groups.

It is possible to test these explanations. Firstly, the difference between glycolipid and phospholipid label order parameters is least pronounced at the methyl end of the fatty acid chain. This would be expected in the case of tighter packing resulting from head group differences. Secondly, in separate experiments we have shown that addition of unlabelled glycolipids in various concentrations increases the bulk rigidity of fluid lipids such as egg phosphatidylcholine or dioleoyl phosphatidylcholine. One of the primary reasons for synthesizing spin labelled glycolipids was to study their behaviour at the low concentrations in which they generally occur in membranes. Nevertheless, if it is true that phospholipids pack more tightly around glycolipids than around other phospholipids, one would expect a bulk effect at reasonably high glycolipid concentrations (10–40 %) and this is observed. For instance at 23 °C the inclusion of 40 % (unlabelled) galactosyl ceramide in vesicles of egg phosphatidylcholine increases the (12, 3) glycolipid label order parameter by 0.021 units and the corresponding phospholipid label order parameter by 0.045 units. Analogous results have been obtained with the (5, 10) labels. The magnitude of the effect depends directly on the amount of added ceramide. Beef brain gangliosides have a similar effect on fluid lipid bilayers (Sharom and Grant, unpublished). A detailed interpretation of the relative effects of glycolipids on glycolipid labels vs. phospholipid labels may be complicated by the fact that the order parameters are different to start with.

It is known that spin labels can represent a finite perturbation on the system being probed. Recently there has been some interest in using NMR spectroscopy to measure the (unperturbed) fluidity gradient in membranes and model systems [30–35], and in several cases the results have been compared to those found using spin labels. Where free fatty acids have been used as probes the general conclusion seems to be that NMR and EPR measurements can give qualitatively similar results but that order parameters determined by the two methods are quantitatively different [30, 33, 35]. Unfortunately, it is not known how much of this order parameter differ-

ence is due to spin label perturbation and how much is due to the different spectrometer frequencies employed (NMR and EPR order parameters may well not be equally sensitive to certain motional processes occurring in lipid acyl chains). The fluidity gradient data reported here is essentially internally controlled so that differences between phospholipid and glycolipid should be at least qualitatively correct. We have seen the same effect in bilayers of dioleoyl phosphatidylcholine (unpublished) results).

The glycolipid spin labels described here are not limited to use in model membranes. We have found it possible to incorporate them into myelin membranes, erythrocyte ghosts and intact BHK-21 cells (in the latter case the nitroxide label had to be protected against reduction by the presence of ferricyanide in the external medium). In each case incorporation was achieved by adding sonicated vesicles of dipalmitoyl phosphatidylcholine containing 33 % galactosyl ceramide spin label to a suspension of washed cells or membranes. The actual mechanism of incorporation in these experiments has not been investigated.

The gangliosides, a class of glycolipids which bear sialic acid residues, have gained considerable attention in recent years. Little is known about their fuction as receptors but certain aspects of their behaviour [36, 37] should lend themselves well to investigation in model systems. It has already been shown by A.M. Haywood [38–40] that beef brain gangliosides are effective Sendai virus receptors in lipid liposomes. We have confirmed this and shown that such liposomes also specifically bind

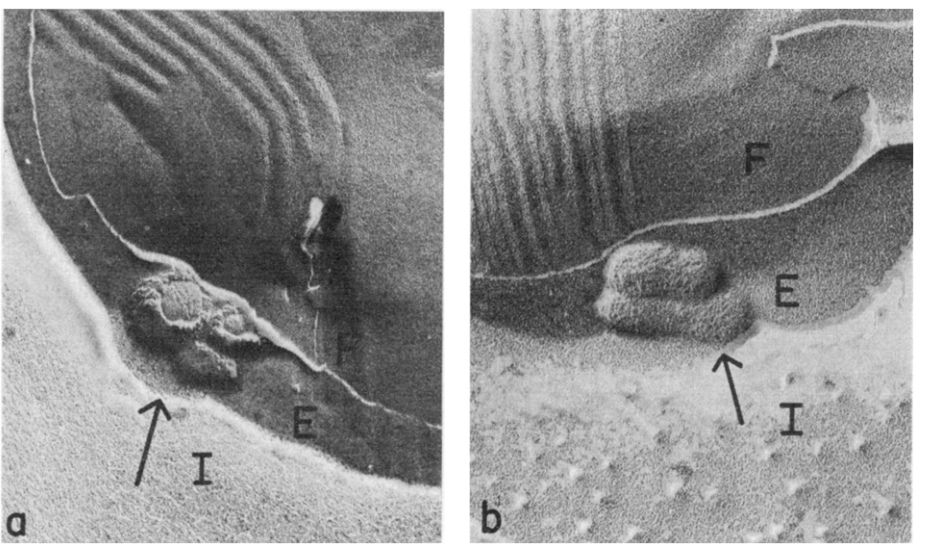


Fig. 2. Freeze-etch electron micrographs of two different influenza virus strains bound to liposomes of 75/25/5 mole fraction dipalmitoyl phosphatidylcholine/dioleoyl phosphatidylcholine/beef brain gangliosides. Samples were incubated at 4 °C, a temperature at which the lipid mixture has coexisting rigid and fluid domains. Arrows point to the virus: (a) strain A/PR8/34 etched 1 min; (b) Port Chalmers strain etched 2 min, (both replicated at −100 °C). Direction of shadow is from bottom to top. I, ice; E, liposome etch face; F, liposome fracture face. Magnification, × 79 000.

influenza virus (presumably by virtue of the ganglioside sialic acid residues) [41]. In hemagglutination inhibition tests such as those described in the Experimental section liposomes of 2:1 egg phosphatidylcholine/cholesterol or of egg phosphatidylcholine/cholesterol containing phosphatidylserine did not compete effectively with human erythrocytes for influenza virus. When 10 mol % beef brain ganglioside was added viral hemagglutination was dramatically inhibited.

Fig. 2 shows freeze-etch electron micrographs of two strains of influenza virus bound to the surface of lipid vesicles containing beef brain gangliosides as receptors (lipid mixture, 75 : 25 : 5 mole ratio of dipalmitoyl/dioleoyl phosphatidylcholine/ganglioside. We have been using influenza virus as markers in our work with model membranes containing gangliosides. Influenza virus are readily recognizable in freeze-etch micrographs (in our experience Sendai virus tend to be difficult to distinguish from small vesicles) and do not show a marked tendency to fuse with membranes. In each case the virus particles can be seen binding to the liposome etch face. Note that the older (PR8) strain is rounded while the more recent (Port Chalmers) variety is still peanut shaped.

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REFERENCES

1 Gulik-Krzywicki, T. (1975) Biochim. Biophys. Acta 415, 1–28
2 Hong, K. and Hubbell, W. L. (1973) Biochemistry 12, 4517–4523
3 MacLennan, D. H., Seeman, P., Iles, G. H. and Yip, C. C. (1971) J. Biol. Chem. 246, 2702–2710
4 Hazelbauer, G. L. and Changeux, J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1479–1483
5 Michaelson, D. M. and Raftery, M. A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4768–4772
6 Racker, E. and Fisher, L. W. (1975) Biochem. Biophys. Res. Commun. 67, 1144–1150
7 Bretscher, M. S. (1972) J. Mol. Biol. 71, 523–528
8 Zwaal, R. F. A., Roelofsen, B., Comfurius, P. and Van Deenen, L. L. M. (1975) Biochim. Biophys. Acta 406, 83–96
9 Lenard, J. and Rothman, J. E. (1976) 73, 391–395
10 Jacobson, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152–161
11 Grant, C. W. M. (1975) Biophysical J. 15, 949–952
12 Shimshick, E. J., Kleemann, W., Hubbell, W. L. and McConnell, H. M. (1973) J. Supra. Mol. Struct. 285–294
13 Chapman, D. (1975) Quart. Rev. Biophys. 8, 185–235
14 Ververgaert, P. H. J. Th., Verkleij, A. J., Elbers, P. F. and Van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 311, 320–329
15 Kanfer, J. N. (1969) Methods in Enzymology 14, 660–664
16 Waggoner, A. S., Kingzett, T. J., Rottschaefer, S., Griffith, O. M. and Keith, A. D. (1969) Chem. Phys. Lipids 3, 245–253
17 Hubbell, W. L. and McConnell, H. M. (1971) J. Amer. Chem. Soc. 93, 314–326
18 Sharom, F. J. and Grant, C. W. M. (1975) Biochem. Biophys. Res. Commun. 67, 1501–1506
19 Robles, E. C. and Van den Berg, D. (1969) Biochim. Biophys. Acta 187, 520–526
20 Devaux, P., Scandella, C. J. and McConnell, H. M. (1973) J. Mag. Res. 9, 474–485

21 McConnell, H. M. and McFarland, B. G. (1970) Quart. Rev. Biophys. 3, 91–136
22 Jost, P., Waggoner, A. S. and Griffith, O. H. (1971) in Structure and Function of Biological Membranes, Academic Press
23 Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881–887
24 Ito, T., Ohnishi, S., Ishinaga, M. and Kito, M. (1975) Biochemistry 14, 3062–3069
25 Seelig, J. (1970) J. Amer. Chem. Soc. 92, 3881–3887
26 McConnell, H. M. and McFarland, B. G. (1972) Ann. N.Y. Acad. Sci. 195, 207–217
27 Smith, I. C. P. and Butler, K. W. (1975) in Spin Labelling (L. J. Berliner ed.), Academic Press
28 Hubbell, W. L. and McConnell, H. M. (1969) Proc. Natl. Acad. Sci. 64, 20–27
29 Tinker, D. O., Pinteric, L., Hsia, J. C. and Rand, R. P. (1976) Can. J. Biochem. 54, 209–218
30 Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839–4845
31 Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P. and Smith, I. C. P. (1974) Biochem. Biophys. Res. Commun. 60, 844–850
32 Stockton, G. W., Johnson, K. G., Butler, K. W., Polnaszek, C. F., Cyr, R. and Smith, I. C. P. (1975) Biochim. Biophys. Acta 401, 535–539
33 Seelig, J. and Niederberger, W. (1974) Biochemistry 13, 1585–1588
34 Metcalfe, J. C., Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K. and Partington, P. (1971) Nature 233, 199–201
35 Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasen, F. and Smith, I. C. P. (1976) Biochemistry 15, 954–966
36 Craig, S. W. and Cuatrecasas, P. (1975) Proc. Natl. Acad. Sci. 72, 3844–3848
37 Hakomori, S. (1975) Biochim. Biophys. Acta 417, 55–89
38 Haywood, A. M. (1974) J. Mol. Biol. 83, 427–436
39 Haywood, A. M. (1974) J. Mol. Biol. 87, 625–628
40 Haywood, A. M. (1975) J. Gen. Virol. 29, 63–68
41 Haff, R. F. and Stewart, R. C. (1965) J. Immunol. 94, 842–851