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GLYCOLIPID CRYPTICITY IN MEMBRANES — NOT A SIMPLE SHIELDING EFFECT OF MACROMOLECULES

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The potential of membrane-bound macromolecules for shielding glycolipids from involvement in specific binding events was considered in model membranes. Serum albumin and several Dextrans were covalently derivatized with oleic acid so that they adsorbed irreversibly to lipid bilayers. This provided a means of generating bilayer membranes with a considerable layer of attached material. Gangliosides dispersed in such membranes were subjected to attack by the enzyme, neuraminidase, in order to assess their 'accessibility'. We were surprised to find that we could not demonstrate any significant reduction in ganglioside hydrolysis in phosphatidylcholine bilayers bearing extensive surface coats of protein or polysaccharide. We conclude that non-specific, physical shielding by macromolecules is an unlikely source of the often-observed 'crypticity' of glycolipids at the cell surface. Consistent with this interpretation was a relative lack of headgroup motional restriction seen for spin-labelled ganglioside headgroups in the same bilayers and in cell membranes.

Introduction

The surface-exposed carbohydrate headgroups of glycolipids have been considered to act as points of specific attachment for exogenous agents, including antibodies, lectins, toxins, polypeptide hormones, viruses and other cells. Furthermore, the carbohydrate phenotype expressed has been shown to bear some relation to cell cycle and cell transformation. Hence, it is thought by many that attachment sites provided by glycolipids (and glycoproteins) have a role in mediation of many aspects of cellular interaction with the surrounding environment. Regulation of the 'availability' of a given oligosaccharide headgroup at the membrane surface thus becomes an important consideration.

One major mechanism of receptor regulation is metabolic control: control of the biochemical pathways for receptor synthesis, and control of membrane turnover. There also exists, however, the concept that the simple presence of a given receptor in a membrane is not all-important: that its successful involvement in a binding event may depend critically upon its arrangement relative to other membrane structures. Receptors that are present in the membrane and yet fail to interact with external ligands are said to be cryptic. One of the earliest indications of receptor crypticity was the observation in Hakomori's laboratory that anti-globoside antibodies bind much more extensively to fetal erythrocytes than to those of later developmental stages (in spite of their possessing similar quantities of globoside) ([1], and reviewed in Ref. 2). It was pointed out by the same workers that certain glycolipids in transformed cells react more strongly with their specific antisera than do those in the parent cell line [3]. A seemingly related phenomenon has been the observation by a number of groups that certain enzymes exhibit a variable activity on membrane-bound glycolipids,

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depending upon the cell type. For instance, Weinstein et al. [4] demonstrated that gangliosides of L cells were resistant to neuraminidase. Gahmberg and Hakomori [5] reported that glycolipid accessibility to galactose oxidase in 3T3 and NIL cells depended upon cell transformation and cell cycle (see also Refs. 6 and 7). An interesting additional observation has been that pretreatment of cells with proteolytic enzymes, such as pronase or trypsin, typically enhances the extent of glycolipid enzymatic hydrolysis by 100% or more (see, e.g., Refs. 1, 8–11). It is also noteworthy that bound, exogenously added gangliosides in several cell lines have been shown to have a different accessibility than their native counterparts in the same membranes [12].

Certainly observations such as those above are consistent with the concept that glycocalyx material, including proteins and oligosaccharides, can physically 'bury' glycolipids — and thus make them less accessible to approaching macromolecules. However, the same observations might alternatively be explained by differences in glycolipid distribution, orientation, mobility or specific interactions. Model membrane studies have clearly demonstrated that factors other than steric interference by macromolecules can determine the course of a specific binding event. For instance extensive work from McConnell's [13] and Alving's [14] laboratories has emphasized that lipid hapten involvement in antibody binding and complement fixation are exquisitely sensitive to lipid composition and fluidity of the host membrane ([13] and references therein, and reviewed in Ref. 14). Recent studies of glycosidase attack on gangliosides in various model membranes have also noted a strong dependence upon host membrane lipid composition ([15,16], but see Ref. 17). With regard to lectin binding, Surolia et al. [18] have considered the possible influence of glycolipid dynamics and topographic distribution; and Curatolo et al. [19] have emphasized the need for the glycolipid headgroup to protrude sufficiently from the membrane surface.

In this manuscript we have attempted to focus specifically on the possibility that glycolipids may be physically shielded from contact with approaching macromolecules by membrane (glyco)proteins.

Materials and Methods

Sources of phospholipids, gangliosides and cholesterol were as described previously [20]. Lipid purity was checked by thin-layer chromatography on silica gel GF_{254} (Stahl). Dextran T500 was from Pharmacia, and dicyclohexylcarbodiimide was from Eastman Kodak. Oleic acid, *N*-hydroxysuccinimide, globulin-free bovine serum albumin and M_r 90 000 Dextran were from Sigma. *Vibrio cholerae* neuraminidase (1 I.U./ml) was purchased from Calbiochem.

Derivatization of serum albumin with fatty acids was carried out along the general lines of the procedure of Lapidot et al. [21]. Thus, in a typical preparation, oleic acid (containing a small amount of tritiated material from New England Nuclear) was converted to the *N*-hydroxysuccinimide ester. 18 mg of this were then combined with 100 mg of bovine serum albumin in 2% aqueous sodium deoxycholate. Gentle stirring for 2 h at 37°C and 4 days at 22°C yielded a final product with an average of 15–16 fatty acids per albumin. The soluble fraction was dialysed exhaustively against 4°C distilled water containing 0.025% sodium azide, and then lyophilized.

The basic method employed for linking fatty acids to Dextran was that described by Wolf et al. [22] for stearic acid. Typically, 40 mg oleic acid chloride (with a trace amount of tritiated material) were incubated for 4 days at 22°C with 200 mg of Dextran in 24 ml of dimethylsulfoxide/pyridine (5 : 1). The product was dialysed against distilled water and precipitated with ethanol. The precipitate was redissolved in phosphate-buffered saline and dialysed exhaustively against 4°C distilled water containing 0.025% sodium azide before being lyophilized.

Preparation of model membranes and assay for N-acetylneuraminic acid release by neuraminidase

Vesicles of egg phosphatidylcholine containing 5 mol% of bovine brain ganglioside were prepared by hydration of thin films dried down in a test tube from chloroform/methanol (1 : 1). Typically, 18.9 mg of total lipid were hydrated with 600 μl of phosphate-buffered saline, pH 7.4, (containing 2 mM, Ca^{2+} and Mg^{2+}) by vigorous vortexing with glass beads. Vesicles prepared in this way have a

high percentage of single bilayer structures. Each sample consisted of 30 μl of the above suspension of lipid vesicles, to which was added either 10 μl of saline (blank) or 10 μl of saline containing 0.5 mg of Dextran or albumin surface-coat material. The samples were incubated with surface-coat material for at least 30 min at 22°C prior to assaying for sialic acid release by neuraminidase. Where noted, some samples were incubated with much larger quantities of surface coat. Subsequent to incubation with surface-coat material, to each sample was added 10 μl of neuraminidase (0.01 I.U.) or 10 μl of saline (control). The mixture was allowed to react at 37°C for 15 min.

Sialic acid assay

We used a scaled-down version of the thiobarbiturate assay described by Warren [23]. All volumes were reduced by a factor of 4, except that we used 200 μl arsenite solution rather than 250 μl. Interfering chromophores absorbing maximally at 232 nm were controlled for by simultaneously assaying coated and uncoated liposomes that had not been treated with neuraminidase.

Results

For the purpose of the experiments described here we wished to have protein and oligosaccharide macro-structures which could be forced to occupy the lipid/water interface of our bilayer membranes. Serum albumin provided a convenient M_r 67 000 globular protein; while Dextrans offered suitable massive polysaccharides. Both of these species adhere measurably to lipid membranes in their own right [24–28]. However, it seemed desirable to ensure that the binding was essentially irreversible so that, like membrane (glyco)proteins,

TABLE I

CHARACTERISTICS OF TYPICAL FATTY ACID DERIVATIVES OF DEXTRAN AND SERUM ALBUMIN

Surface coat material binding assays described in this table and in Figs. 1–4 were carried out in phosphate-buffered saline, pH 7.4, (containing Ca^{2+} and Mg^{2+}) using large, stable liposomes which could be washed repeatedly by low-speed differential centrifugation. These were made from dimyristoylphosphatidylcholine/cholesterol in a 4 : 1.5 mol ratio, although similar, but less accurate, results were obtained using liposomes of pure egg phosphatidylcholine. Lipid mixtures in $CHCl_3/CH_3OH$ (1 : 1) were dried down under a stream of N_2 gas, and then placed in a vacuum desiccator for 2 h to remove traces of solvent. Dry films were hydrated at 37°C by gently shaking with glass beads in a small volume of saline. Liposomes so produced were washed twice at 22°C by differential centrifugation at 350 × *g* for 15 min, and supernatants discarded (total lipid loss in supernatants, 50–60%). For binding assays, liposomes produced as above were incubated with surface-coat material at 22°C for 60 min, and then washed three times by repeated sedimentation at 350 × *g* for 15 min and gentle resuspension in fresh buffer. After the first such wash only insignificant amounts of labelled surface-coat material were removed by subsequent washes, even after incubating overnight in fresh buffer as was done for the data here and in Fig. 1.

Derivative (with average No. of associated fatty acids)	Percentage of fatty acid extractable by organic solvent (i.e., non-covalently bound)	mg of derivative essentially irreversibly bound subsequent to incubating 4.6 mg lipid (as liposomes) with 2 mg derivative
M_r 90 000 Dextran with oleic acid (1.8)	1.6% [a]	0.27
M_r 500 000 Dextran with oleic acid (9)	0.6% [a]	0.35
Serum albumin with oleic acid (15.3)	5.5% [b]	1.07

[a] Lyophilized derivative was extracted with hexane

[b] Lyophilized derivative was extracted with tetrahydrofuran

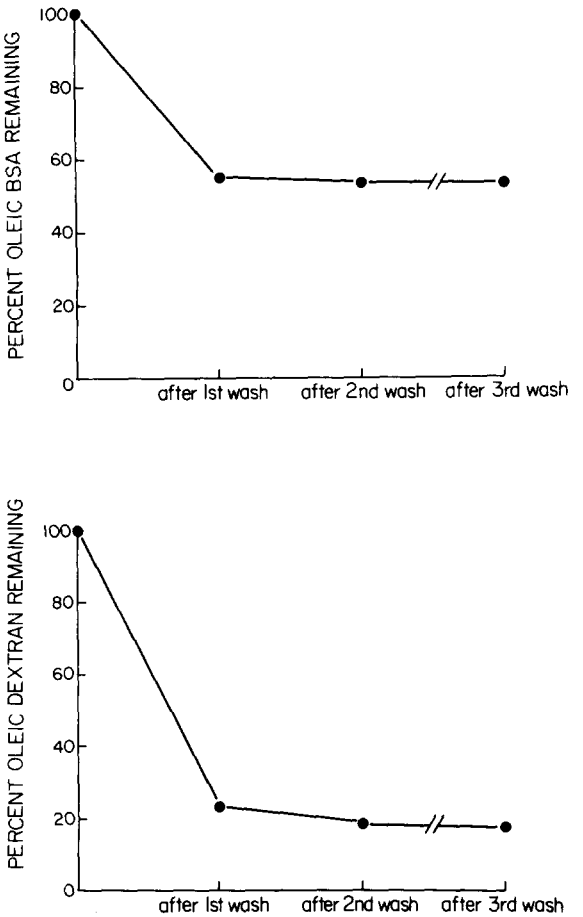

Fig. 1. Washing curves for surface-coated liposomes. Upper: using fatty acid-conjugated bovine serum albumin (BSA). Lower: using fatty acid-conjugated dextran T500. See Table I for details.

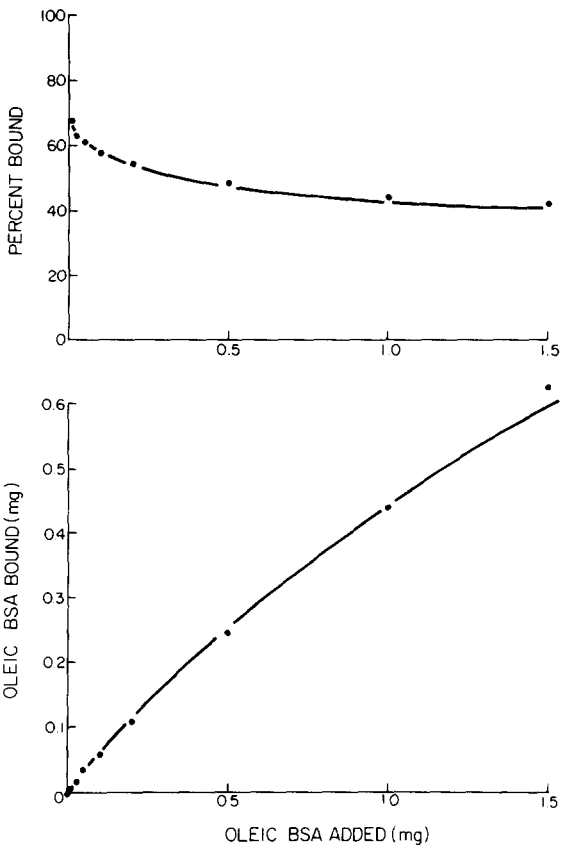

Fig. 2. Lipid bilayer binding curves for fatty acid-conjugated serum albumin. See Table I for binding assay details. 250-μl aliquots of a large liposome suspension comprising 9.2 mg of lipid in 2250 μl of phosphate-buffered saline were exposed to surface-coat material for 60 min at 22°C, and then washed by differential centrifugation. BSA, bovine serum albumin.

the attached material was a permanent membrane feature. To this end, we covalently linked fatty acids to each albumin or dextran. By including some radioactively labelled fatty acid in the procedure we were able to keep track of the extent of derivatization, and also, at a later stage, to keep track of the amount of albumin or dextran associated with our model membranes. Table I lists the characteristics of typical preparations used in this work. Each preparation was tested by solvent extraction of the freeze-dried material to ensure that fatty acids were indeed covalently attached to albumin or dextran. Ability of the derivatized material to bind to lipid bilayers was measured by exposure to a known amount of lipid, followed by repeated differential centrifugation.

Covalent attachment of stearic acid has been used by other workers where there was a requirement to induce adhesion of Dextran [22] and serum albumin [21] to lipid bilayers. Technically, however, we have had cleaner results using the more fluid oleic acid. The results of experiments to characterize the surface-attaching properties of this material are shown in Table I and Figs. 1–4. Attachment was measured by exposing liposomes to derivatized albumin or Dextran, and then subjecting them to differential centrifugation: bound material was quantitated by scintillation counting. Multilamellar liposomes are particularly suitable for accurate, repeated differential centrifugation.

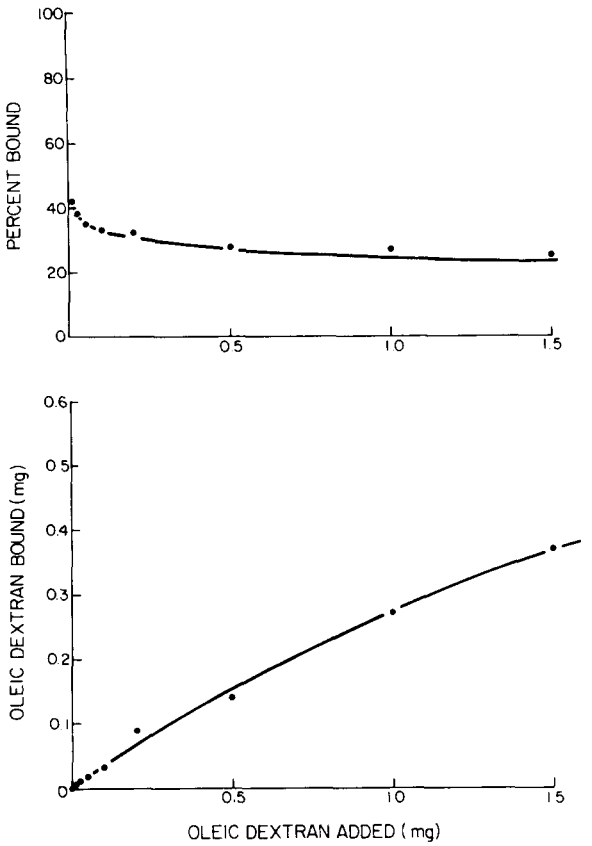

Fig. 3. Lipid bilayer binding curves for fatty acid-conjugated Dextran T500. See Table I for binding assay details. 250-μl aliquots of a large liposome suspension comprising 9.2 mg of lipid in 2250 μl of phosphate-buffered saline were exposed to surface-coat material for 60 min at 22°C, and then washed by differential centrifugation.

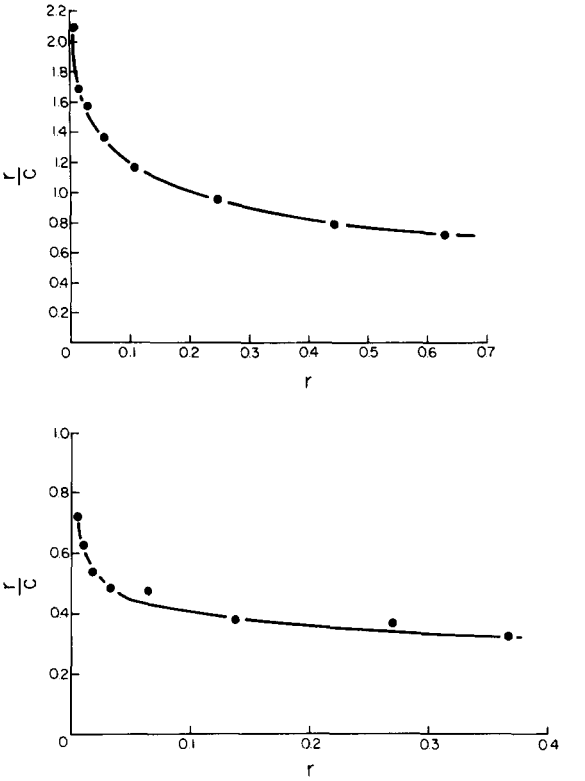

Fig. 4. Scatchard type plots for the data in Figs. 2 and 3. Upper plot: fatty acid-conjugated bovine serum albumin binding. Lower plot: fatty acid-conjugated dextran binding. r, μg of bound surface-coat material per assay tube; c, μg of remaining free surface-coat material per assay tube.

Note that, even after sitting for many hours in wash buffer, the surface-attached (fatty acid-conjugated) Dextran or albumin does not fall off (Table I and Fig. 1). Normal serum albumin and Dextran have been found to adhere extensively to cell membranes and lipid bilayers at concentrations of a few mg/ml [24–28]. Wolf et al. [22] have reported essentially complete surface coverage of bilayer membranes by stearic acid-derivatized Dextran at 0.04 mg/ml. Experiments described in this paper have typically been carried out using 10 mg/ml of conjugated albumin or Dextran — a concentration which at a very conservative estimate should be adequate to produce monolayer coverage.

Figs. 2 and 3 show the results of experiments intended to titrate the level of surface coverage with conjugated albumin and Dextran. Note that there is no obvious plateau to the binding curves: it appears possible to achieve considerably more than single monolayer coverage since, within limits, as more material is added, more binds. Scatchard plots of such data (Fig. 4) show that there is a wide range of binding affinity, and that the affinity of attachment decreases as the extent of surface coverage increases.

Effect of surface macromolecules on glycolipid accessibility to neuraminidase

Fig. 5 is a scale drawing, the major features of which were taken from a previous article by one of us (C.W.M.G., Ref. 29), representing a section from the membrane of the human erythrocyte. It

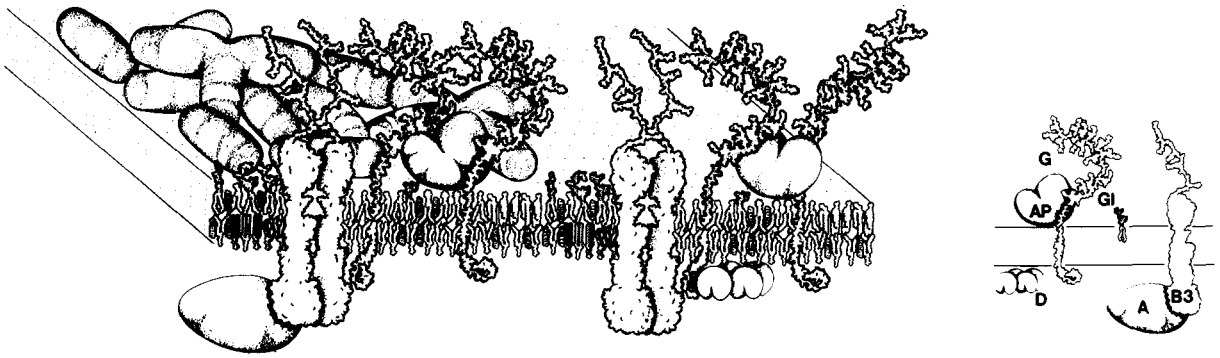

Fig. 5. Scale drawing of a section of the erythrocyte membrane. The features of this figure have been described in detail by one of us (C.W.M.G.) previously [29]. Components shown and their ratio and arrangement correspond to literature descriptions of red cell membrane architecture (see references to right-hand figure). Where known, structural details were derived from Corey-Pauling-Koltun space-filling molecular models, while other species are shown only as globes of appropriate dimensions. The left-hand outer surface of the membrane section shown has been drawn with adsorbed serum albumin (oblong, 67000 molecular weight [30] and IgG (T-shaped, 160000 molecular weight [31]. B3, band 3 [32,33]. G, glycophorin [33,34]. A, ankyrin [35]. AP and D, peripheral proteins such as alkaline phosphatase and glucose-6-phosphate dehydrogenase, respectively [33]. G1, glycolipid (ganglioside).

TABLE II

GANGLIOSIDE SUSCEPTIBILITY TO NEURAMINIDASE ATTACK: COMPARISON BETWEEN LIPID BILAYERS WITH AND WITHOUT A SURFACE LAYER OF SERUM ALBUMIN (a) OR DEXTRAN T500 (b)

This table lists the results of seven experiments carried out on different occasions. Values of A_{549} varied somewhat from one experiment to the next, depending presumably upon factors such as variability in the lipid preparations. Each separate experiment was carried out in quintuplicate (S.D. shown). Each sample was done with a paired blank, which was identical except for having no enzyme added. Technical details are described in Materials and Methods. The average over all these experiments for the ratio of neuraminic acid release from coated vs. uncoated bilayers was 1.22 ± 0.30 (a) or 1.02 ± 0.26 (b). According to a pair-wise Student's *t*-test, this shows no significant shielding of ganglioside headgroups by surface bound protein. The concentration of fatty acid-conjugated serum albumin (a) or Dextran T500 (b) used in the experiments covered in the table was 10 mg/ml. However, even extremely high concentrations (e.g., 80 (a) or 50 (b) mg/ml) failed to significantly inhibit enzymatic hydrolysis of membrane bound gangliosides.

Experiment	Neuraminic acid released (A_{549}) ± S.D.		
	With surface coat	Without surface coat	Ratio (coated/uncoated)
(a)			
1	0.381 ± 0.122	0.283 ± 0.098	1.35
2	0.238 ± 0.021	0.279 ± 0.013	0.85
3	0.321 ± 0.069	0.281 ± 0.072	1.14
4	0.275 ± 0.091	0.215 ± 0.034	1.28
5	0.343 ± 0.112	0.353 ± 0.137	0.97
6	0.427 ± 0.086	0.243 ± 0.084	1.76
7	0.356 ± 0.046	0.368 ± 0.061	0.97
(b)			
1	0.266 ± 0.054	0.309 ± 0.037	0.86
2	0.191 ± 0.049	0.221 ± 0.025	0.86
3	0.258 ± 0.078	0.281 ± 0.072	0.92
4	0.241 ± 0.058	0.215 ± 0.034	1.12
5	0.318 ± 0.073	0.353 ± 0.137	0.90
6	0.383 ± 0.070	0.243 ± 0.084	1.58
7	0.410 ± 0.019	0.368 ± 0.061	1.11

attempts to put glycolipids in perspective relative to conditions encountered in the cell membrane. Component ratios and features have been duplicated as accurately as possible. Clearly membrane glycoproteins would appear to have the potential to physically interfere with macromolecule access to neighbouring glycolipid headgroups. Note that one portion of the membrane has been shown with a modest layer of adsorbed serum albumin (and IgG) at the outer surface — and that these too might be expected intuitively to reduce the accessibility of surface receptors.

The actual results of experiments in which neuraminidase was allowed to act on bilayer membranes bearing beef brain gangliosides are reproduced in Table IIa and b. The release of *N*-acetylneuraminic acid was monitored, using a colourimetric assay, for membranes with no additional surface material, and for the same membranes with adsorbed layers of albumin or Dextran. In several cases exceedingly large amounts of surface-coat material were used with the same result. Typically samples were run in quintuplicate, and Table IIa and b summarizes a number of such experiments. In spite of the fact that the experimental technique employed should readily show up a difference of 20% in neuraminic acid release between samples, no such difference is apparent from the tables. Note that surface-coat material had no direct effect on the enzyme, as demonstrated by assaying neuraminic acid release from a standard substrate, *N*-acetylneuraminyl lactose (Table III). It would thus appear that non-specific shielding of glycolipids by surface-bound globular proteins or polysaccharide does not occur in our model system.

Effect of surface macromolecules on headgroup dynamics of spin-labelled glycolipids

The question of possible surface material interference with glycolipid headgroups may also be addressed using spectroscopic probes. We have previously synthesized a variety of glycolipids with spin labels covalently attached to headgroup sugar residues (reviewed in Ref. 20). The nitroxide-containing ring is similar in size and attachment to a hexose, and seems to approximate adequately headgroup sugar motional freedom. We have incorporated such probe-labelled glycolipids into bilayer membranes identical to those used in the experiments described in the previous section. In the presence of derivatised albumin or Dextran, the correlation time for headgroup label reorientation remained within experimental error of that found in uncoated membranes ($(14.6 \pm 0.5) \cdot 10^{-10}$ sec^{-1} for gangliosides and $(12.7 \pm 0.5) \cdot 10^{-10}$ sec^{-1} for globoside) [36].

Hence, the surface-bound macromolecules appeared not to reduce intrinsic oligosaccharide headgroup motional freedom. This result is in agreement with a previous observation from our laboratory [20] that, although headgroup mobility was found to be measurably different in several cultured cell lines, trypsinization did not appreciably alter the observed values.

TABLE III

NEURAMINIDASE ACTIVITY ON THE SOLUBLE SUBSTRATE, NEURAMINYL LACTOSE, IN THE PRESENCE AND ABSENCE OF SURFACE-COAT MATERIAL

The concentration of surface coat material was 10 mg/ml. In Expt. 2 the samples were diluted ×5 relative to those in Expt. 1. For this assay 40-μl samples containing 500 μg of surface-coat material and 240 μg neuraminyl lactose were incubated for 15 min at 37°C with 10 μl of a neuraminidase stock solution (24 mg substrate per unit enzyme).

Experiment	Neuraminic acid released (A_{594}) ±S.D.		
	Derivatized serum albumin	Derivatized Dextran T500	No albumin or Dextran
1	0.886±0.031	0.893±0.052	0.952±0.029
2	0.198±0.011	0.182±0.008	0.181±0.006

Discussion

It is noteworthy that we have been unable in these experiments to demonstrate any reduction in ganglioside 'accessibility' to neuraminidase in the presence of extensive surface coverage by polypeptides or polysaccharide. This result contrasts sharply with the marked effect of host lipid matrix composition on neuraminic acid release from glycolipids [15,16]. Lack of surface macromolecule influence may reflect a high mobility on the part of the latter, such that enzyme is able to diffuse in rapidly. In this regard, however, we have found that prior cross-linking of a serum albumin surface coat with glutaraldehyde did not alter our basic result (unpublished data). Also, as already indicated, the attached surface material employed here does not desorb at an appreciable rate.

Our suggestion is that, in fact, non-specific interaction with surface macromolecules may play a relatively minor role in determining glycolipid headgroup 'accessibility' at the cell surface. This conclusion is consistent with our observation that surface macromolecules have little influence on glycolipid headgroup dynamics. Such a view would demand a greater emphasis on the alternative factors: host lipid matrix, plus glycolipid dynamics, distribution and specific interactions.

Acknowledgements

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