RECONSTITUTING THE BARRIER PROPERTIES OF A WATER-TIGHT EPITHELIAL MEMBRANE BY DESIGN OF LEAFLET-SPECIFIC LIPOSOMES

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Running Title: Reconstituting barrier properties of MDCK apical membranes

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SUMMARY

To define aspects of lipid composition and bilayer asymmetry critical to barrier function we examined the permeabilities of liposomes which model individual leaflets of the apical membrane of a barrier epithelium, MDCK Type 1 cells. Using published lipid compositions we prepared exofacial liposomes containing phosphatidylcholine, sphingomyelin, glycosphingolipids and cholesterol; and cytoplasmic liposomes containing phosphatidylethanolamine, phosphatidylserine and cholesterol. The osmotic permeability of cytoplasmic liposomes to water ($P_f$), solutes and $NH_3$ was $18 - 90$-fold higher than for the exofacial liposomes [$P_f(ex) = 2.4 \pm 0.4 \times 10^{-4}$ cm/s, $P_f(cy) = 4.4 \pm 0.3 \times 10^{-3}$ cm/s; $P_{glycerol}(ex) = 2.5 \pm 0.3 \times 10^{-8}$ cm/s, $P_{glycerol}(cy) = 2.2 \pm 0.02 \times 10^{-6}$ cm/s; $P_{NH3}(ex) = 0.13 \pm 0.04$ cm/s, $P_{NH3}(cy) = 7.9 \pm 1.0$ cm/s]. By contrast, the apparent proton permeability of exofacial liposomes was four-fold higher than cytoplasmic liposomes [$P_{H+}(ex) = 1.1 \pm 0.1 \times 10^{-2}$ cm/s, $P_{H+}(cy) = 2.7 \pm 0.6 \times 10^{-3}$ cm/s]. By adding single leaflet permeabilities, we calculated a theoretical $P_f$ for an MDCK apical membrane of $4.6 \times 10^{-4}$ cm/s, which compares favorably with experimentally determined values. In exofacial liposomes lacking glycosphingolipids or sphingomyelin, permeabilities were $2 - 7$-fold higher indicating that both species play a role in barrier function. Removal of cholesterol resulted in $40 - 280$-fold increases in permeability. We conclude: (1) that we have reconstituted the biophysical properties of a barrier membrane, (2) that the barrier resides in the exofacial leaflet, (3) that both sphingomyelin and glycosphingolipids play a role in reducing membrane permeability but that there is an absolute requirement for cholesterol to mediate this effect, (4) that these results further validate the hypothesis that each leaflet offers an independent resistance to permeation, (5) that proton permeation was enhanced by sphingolipid/cholesterol interactions.
INTRODUCTION

The phospholipid bilayers which constitute cellular boundaries and which permit the selective movement of ions, protons and water through embedded proteins, are themselves often surprisingly permeable to a wide range of small molecules like water and urea. In contrast to this there are certain cells and tissues which, for functional reasons, exhibit exceedingly low permeabilities (1). These include the urinary bladder which must maintain strikingly high concentration gradients for urea, NH₃, protons and CO₂, when compared to the blood and surrounding interstitium (2); the stomach whose secretions have an extremely low pH, and the collecting duct in the kidney which separates the urine being formed from the interstitium (1, 3). Failure of the cell to maintain these barriers can lead to significant human pathology including renal tubular disorders, peptic ulcer disease and cystitis (4, 5).

The features of the lumenal surface which confer these remarkable properties are currently unclear, although it is now accepted that the barrier to permeation resides in the apical membrane of the superficial epithelial cells (3, 6-10). Early physiological studies of mammalian bladder demonstrated an extremely high transepithelial resistance of up to 78000 Ωcm² (11) and electrode impalement studies of the various epithelial cell layers demonstrated that the apical membrane of the most superficial layer was the location of the high electrical resistance (8, 11, 12). Creation of that barrier appears to depend on the cell’s ability to synthesize an asymmetric bilayer i.e. to apportion different lipid constituents to the outer or exofacial leaflet compared with the inner or cytoplasmic leaflet, and then to restrict the intermingling of those lipids (13). An earlier study from our laboratory demonstrated the functional consequences of losing bilayer asymmetry, when gastric apical vesicles – which possessed exceptionally low permeabilities to water, protons and non-electrolytes – were made symmetrical by quantitative lipid extraction and reconstitution into liposomes. The newly formed liposomes, although made
from identical apical membrane lipids, exhibited much higher permeabilities than the native membrane (10). Bilayer asymmetry is maintained by tight junctions between cells which not only isolate the basolateral and apical membranes, but also the exofacial leaflet from the cytoplasmic leaflet of the apical membrane (14, 15). Thus by means of lipid sorting in the trans-Golgi network and vectorial delivery of lipids to specific membrane domains, the cell can erect a membrane with low permeability. The precise structural features responsible for barrier function are unclear, however it appears that certain combinations of lipids result in a more highly ordered membrane which has lower permeabilities for water and non-electrolytes. For example, sphingomyelin (SM)\(^1\) and cholesterol have been shown to associate closely due to van der Waals’s forces and hydrogen-bonding and these interactions induce tight packing in the bilayer (16). We, and others have previously shown that MDCK Type 1 cells grown on permeable supports exhibit the permeability properties of a barrier epithelium (17). Because the lipid structure of the MDCK cell apical membrane is well defined we aimed to reconstitute the low permeability properties of the MDCK Type 1 cell, in an effort to understand the determinants that contribute significantly to this membrane’s barrier properties (17). We also wished to further validate our earlier findings that each leaflet in a phospholipid bilayer offers an independent resistance to permeation (18, 19). This hypothesis can be summarized by the following equation, in which the resistance offered by each leaflet is equivalent to the reciprocal of the permeability:

\[
\frac{1}{P_{\text{AB}}} = \frac{1}{P_A} + \frac{1}{P_B} \quad \text{(Eq. 1)}
\]

where \(P_{\text{AB}}\) is the permeability of a bilayer composed of leaflets A and B; \(P_A\) is the permeability of leaflet A and \(P_B\) is the permeability of leaflet B. We have exploited this property in attempting to recreate the MDCK apical membrane – an asymmetric bilayer. Accordingly we constructed
liposomes with particular combinations of lipids in order to mimic the exofacial leaflet and the cytoplasmic leaflet of MDCK cells, and then measured their permeabilities to water, non-electrolytes, protons, and NH₃. The cytoplasmic leaflets had dramatically higher permeabilities than the exofacial liposomes (20 – 90-fold depending on the permeant). By the use of Eq. 1 we derived a theoretical water permeability for the MDCK apical membrane which correlates extremely well with published permeabilities. Further experiments aimed at identifying the lipids responsible for barrier function implicate sphingolipid/cholesterol interactions as a key determinant in the formation of low permeability membranes.
EXPERIMENTAL PROCEDURES

Liposome Preparation

The following lipids from Avanti Polar Lipids Inc (Alabaster, AL) were used to construct liposomes; bovine heart phosphatidylethanolamine (PE, Cat# 830025), brain phosphatidylserine (PS, Cat# 830032) bovine liver phosphatidylinositol (PI, Cat# 830042), bovine heart phosphatidylcholine (PC, Cat# 830052), cholesterol (Cat# 700000), egg sphingomyelin (SM, Cat# 860061), and brain cerebrosides (GSLs, Cat# 131303). Natural lipids from mammalian tissues were chosen so that acyl chain heterogeneity and degree of unsaturation would reflect the likely chain composition of the canine cells we were modeling. In some experiments dipalmitoylphosphatidylcholine (DPPC) and \textit{E. coli} PE were used and this is indicated in the text. Lipids were mixed by weight according to the proportions shown in Table 1, dissolved in chloroform:methanol (2:1), aliquotted by volume into tubes, then dried down in a heating block set to 37ºC under a stream of nitrogen. Lipids were then completely dried in an evacuated chamber for 2 h. before storage at –20ºC. On the day of liposome preparation, lipids were suspended in buffer containing 150 mM NaCl, 20 mM carboxyfluorescein (CF), 10 mM HEPES pH 7.5. Lipids were vortexed for 2 min., heated to 37ºC in a water bath for 10 min., vortexed for 2 min., probe sonicated 3 x 2 min. on ice at a medium power setting (with 2 min. intervals) and then extruded through a 200 nm polycarbonate membrane. Liposomes were allowed to equilibrate on ice for 90 min., then extravesicular CF was removed by passing vesicles over a Sephadex G50 column (Sigma, St Louis, MO). Vesicles were sized by quasi-elastic light scattering using a Nicomp model 270 submicron particle analyzer as described (3).

Water Permeability Measurements

Osmotic water permeability ($P_f$) was measured at 25ºC as described (3, 18, 20). All other permeabilities were measured at 25ºC also. Briefly, permeabilities were determined using a
stopped-flow fluorimeter (SF.17 MV, Applied Photophysics, Leatherhead, UK) with a measurement dead-time of less than 1 ms. Liposomes containing 20 mM CF were rapidly mixed with an equal volume of an identical buffer that had three times the osmolality due to sucrose addition. The rate of water efflux from vesicles was measured as a function of vesicle shrinkage and CF-fluorescence self-quenching. From parameters which included the initial rate of quenching, vesicle diameter and applied osmotic gradient, \( P_f \) was calculated using MathCad software (MathSoft Inc., Cambridge MA) (21).

**Solute Permeability Measurements**

Permeability measurements were performed as described using a stopped-flow fluorimeter (3, 20, 21). Briefly liposomes were equilibrated in buffer (500 mOsm/kg) containing 200 mM solute (glycerol, urea, formamide or acetamide) for 30-60 min. at room temperature before the experiment was commenced and then the liposomes were rapidly mixed with an equal volume of a solution with identical osmolality, containing 100 mM solute. Osmolalities of all solutions were confirmed and adjusted if necessary, by measuring freezing point depression on a Precision Instruments Osmette A osmometer. The concentration gradient results in solute efflux from liposomes followed by water efflux. Vesicle shrinkage can be monitored due to CF self-quenching. By use of parameters from the single exponential curve fit to the data, \( P_{\text{solute}} \) was solved using MathCad software (3, 20-22).

**Proton Permeability**

Apparent proton permeabilities were measured using pH-dependent quenching of fluorescence as described previously (3, 10, 19, 20, 22). Stopped-flow experiments were performed in which the liposomes were pretreated with 1 µM valinomycin and then rapidly mixed with an identical buffer acidified to pH 6.50. Valinomycin, which was used to collapse any potential difference arising as a result of proton influx, did not appear to be necessary as permeability
measurements performed in its absence did not alter the results. Buffer capacity was
determined on an SLM-Aminco 500C spectrofluorimeter by adding 10 mM acetate (final
concentration) to liposomes as described (3). Fluorescence data from the stopped-flow device
were fit to a single exponential curve and fitting parameters were used to solve the following
equation for $P_{H^+}$

$$J_{H^+} = (P_{H^+})(SA)(\Delta C) = (\Delta pH/\tau)(BCV)$$

where $J_{H^+}$ is the flux of protons, $\Delta C$ is the initial difference in concentration of protons between
the inside and the outside of the vesicle, $\Delta pH$ is the change in pH when time equals $\tau$, the time
constant of the single exponential curve describing the initial change in fluorescence as a
function of time, and BCV is the buffer capacity of an individual vesicle (3, 10, 20).

$NH_3$ Permeability

$NH_3$ permeability was determined using stopped-flow fluorimetry by monitoring the pH sensitive
increase in fluorescence when vesicles equilibrated to pH 6.8 were rapidly mixed with the same
buffer containing 20 mM $NH_4Cl$ as described (3, 10, 20). $NH_3$ in solution passes through the
membrane and becomes protonated to $NH_4^+$ in the vesicle interior. By combining values for the
rate of change of intravesicular pH, the final intravesicular pH and the buffer capacity (assessed
in the same way as for proton permeability), $P_{NH_3}$ was calculated (20).

Statistics

Testing for significant differences was performed by the Bonferroni t-test which allows for
multiple comparisons. Differences from the exofacial permeability were tested for and a $P <
0.05$ was considered significant.
RESULTS

**MDCK Apical Membrane Composition**

The predicted phospholipid composition of the MDCK Type 1 apical membrane leaflets was derived from published analyses (15, 23) and by making a number of experimentally supported but simplifying assumptions about the asymmetric distribution of lipids in the bilayer. Explicitly the assumptions were;

i. that the apical:basolateral membrane surface area ratio is 1:4 (24)

ii. that cholesterol and phospholipid in the apical membrane are present in a 1:1 ratio (13)

iii. that GSLs are distributed between apical and basolateral membranes in a 2:1 ratio (25)

iv. that half of the lipid moles are in each leaflet

v. that all GSLs, SM and PC in the apical membrane is in the exofacial leaflet (13)

vi. that all of the PE, PI and PS in the apical membrane is in the cytoplasmic leaflet (13)

Hansson *et al.* (23) performed careful analyses of total lipids extracted from MDCK Type 1 cells and tabulated in nmol/filter the amount of each lipid present. We used these figures along with further data on the specific apical and basolateral membrane compositions found in (15). After allocating lipids on the basis of the published values and the above-listed assumptions, the remaining moles were balanced with cholesterol for which no definitive information exists as to bilayer distribution. Resulting liposome compositions are shown in Table 1.

Liposomes were prepared by suspending dried lipid mixtures in buffer containing 20 mM CF, heating to 37°C, vortexing extensively, probe sonicating and then extruding through a 200 nm polycarbonate membrane using an Avanti mini-extruder (26). Phospholipid and cholesterol quantitation were performed by HPLC analysis (Avanti Polar Lipids Inc., Alabaster, AL) and confirmed that all the constituents in the cytoplasmic liposomes were present in the correct
proportions. HPLC analyses of exofacial liposomes were performed by Avanti Polar Lipids Inc., and confirmed that PC, SM and cholesterol were present in the correct relative ratios, but were unable to provide any information about the presence or concentration of GSLs. To confirm that GSLs were being incorporated at the correct molar ratio in the lipid mixture, liposomes were prepared from lipids in (chloroform:methanol) which had been spiked with BODIPY FL C₅-glucocerebroside (Molecular Probes; Eugene, OR), ¹⁴C-cholesterol (NEN; Boston, MA) and ³H-PC (NEN; Boston, MA) prior to drying down under nitrogen. After the liposomes were prepared, they were washed by repeated centrifugation and then aliquots of the final liposome suspension were either scintillation counted or lysed with 0.025% (w/v) Triton X-100 and fluorimetry performed. From fluorescence standard curves and the known specific activity of the isotopes used we confirmed that each component was present in the correct amount relative to the others.

Water Permeability of Exofacial and Cytoplasmic Leaflet Liposomes

Exofacial and cytoplasmic liposomes were tested for their permeability to water using stopped-flow fluorimetry. Vesicles with entrapped carboxyfluorescein (CF) were rapidly mixed with an identical buffer to which sucrose (an impermeant solute) was added. Vesicles were thus rapidly exposed to an external solution osmolality two-fold higher than inside. Water efflux in response to the imposed osmotic gradient causes vesicle shrinkage and CF self-quenching (Fig. 1c). Cytoplastic liposomes exhibited a permeability to water which was approximately 20-fold higher than that exhibited by exofacial liposomes (Fig. 1c). The rate of vesicle shrinkage displays single exponential decay kinetics and curves have been fitted to all stopped-flow profiles to show this. From parameters which include the initial rate of shrinkage, vesicle diameters, and applied osmotic gradient, the permeability coefficients were calculated. Fig. 1a shows the mean results from three separate liposome preparations. These experiments revealed an extremely low water permeability for exofacial liposomes of 2.4 ± 0.4 x 10⁻⁴ cm/s
while cytoplasmic liposomes were 18.1 times more permeable with a \( P_f = 4.4 \pm 0.3 \times 10^{-3} \text{ cm/s} \) \((P < 0.05)\).

Previous work from our laboratory has shown that the leaflets in a bilayer each offer independent resistances to water and solute permeation (18, 19). From Eq. 1 we can calculate a single leaflet permeability from the exofacial liposomes and a single leaflet permeability for the cytoplasmic liposomes (both symmetric bilayers) and combine them to arrive at a theoretical permeability for an MDCK apical membrane. Performing this calculation for water permeability yields a value of \( 4.6 \times 10^{-4} \text{ cm/s} \) which is in good agreement with published permeabilities for MDCK Type 1 apical membranes which range from \( 1.8 \times 10^{-4} \text{ cm/s} \) to \( 10 \times 10^{-4} \text{ cm/s} \) (17, 27-29).

**Water Permeability of Modified Exofacial Liposomes**

One of the more striking features of the bilayer asymmetry observed in certain barrier membranes is the highly concentrated localization of sphingolipids in the exofacial leaflet (13). We wished to investigate whether GSLs and/or SM contribute significantly to the observed membrane impermeability and therefore carried out stopped-flow experiments on liposomes which lacked these components. We were also interested to explore the role of cholesterol in reducing membrane permeability. Accordingly, we prepared exofacial liposomes minus SM (Exo-SM), minus GSLs (Exo-GSLs) and minus cholesterol (Exo-Chol). In each case the missing component was replaced on a mol\% basis with PC. In Fig. 1d stopped-flow profiles for each of these modifications is shown. When compared to the rate of vesicle shrinkage exhibited by complete exofacial liposomes, removal of either of the sphingolipids resulted in increased water permeability. Removal of cholesterol resulted in extremely fast water fluxes. The combined results from three experiments are shown in Fig. 1b. Removal of GSLs or SM resulted in 2.1-times higher or 2.4-times higher \( P_f \) respectively \((P < 0.05)\). Thus both SM and GSLs contribute to the low permeability properties of this membrane. Unsurprisingly, the
removal of cholesterol also increased the permeability of this membrane. The magnitude of the increase is however, remarkable. Water permeability was increased 37-fold even in the presence of a full complement of sphingolipids.

*Effect of Changing Acyl Chain Composition on Water Permeability*

As we had limited control over the acyl chain composition of these membranes we chose lipids purified from mammalian cells. We anticipated this would yield bilayers with an ‘averaged’ double bond and chain length heterogeneity. To examine further the importance of acyl chain composition on the permeation behavior of these membranes we constructed exofacial liposomes in which we replaced bovine heart PC (predominantly 16:0, 18:1 and 18:2 acylation) with DPPC (16:0-16:0), and cytoplasmic liposomes which had bovine heart PE (predominantly 18:0-20:4) with *E. coli* PE (predominantly 16:0-18:1). The other lipids and their proportions were kept the same. Exofacial liposomes made with DPPC (DPPC/Exo) showed a reduction in $P_f$ from $2.4 \times 10^{-4}$ cm/s to $1.6 \times 10^{-4}$ cm/s while the cytoplasmic liposomes also exhibited a reduction in water permeability from $4.4 \times 10^{-3}$ to $1.5 \times 10^{-3}$ cm/s (Fig. 2b). These membranes exhibited significantly lower water permeability due to a reduction in the number of double bonds ($P < 0.05$). The influence of replacing bovine heart PC with DPPC is particularly interesting, given that this species represents only 9% of the total lipid and yet effects a 1/3 reduction in $P_f$ (Fig. 2a). We further tested these DPPC-exofacial liposomes by removing GSLs and replacing them on a mol for mol basis with DPPC (DPPC/Exo-GSLs in Fig. 2a and 2c). There was no change in permeability (Fig. 2a). As DPPC has a high melting point ($T_m \sim 41^\circ$C) and is structurally analogous to SM, the choice of artificial PC-lipid effectively masks the true effect of removing GSLs from the membrane. When bovine heart PE was replaced with *E. coli* PE the result was a 2/3 reduction in permeability (Fig. 2b and 2d). As PE constitutes 40% of the lipid in this membrane a reduction in double bond complement in PE would be anticipated to have a more profound effect. These data point to the need to carefully consider the
appropriateness of lipids before conducting membrane modeling experiments. The acyl chain content can dramatically affect a membrane’s biophysical properties.

Solute Permeability of Exofacial and Cytoplasmic Leaflet Liposomes
The permeability of inner and outer leaflet liposomes to a range of solutes was examined. By abruptly exposing liposomes pre-loaded with 200 mM solute to an external buffer solution containing 150 mM solute, solute efflux occurs and vesicles shrink leading to CF self-quenching. In Figs. 3 – 6 it can be seen that the permeability of cytoplasmic liposomes to formamide (Fig. 3a,c), acetamide (Fig. 4a,c), urea (Fig. 5a,c) and glycerol (Fig. 6a,c) all exhibit much higher permeabilities than the exofacial liposomes ($P < 0.05$ for all). The permeability of cytoplasmic liposomes to formamide, acetamide, urea and glycerol were 22-fold, 35-fold, 52-fold and 91-fold greater respectively. These differences are greater than that observed for water permeability and demonstrate a rank order that equates with increasing molecular weight of the permeant. The data shows remarkably low permeabilities associated with the exofacial leaflet and indicate that barrier function to solutes can clearly be accounted for by the specialized lipid composition of a single leaflet.

Solute Permeability of Modified Exofacial Leaflet Liposomes
When GSLs, SM and cholesterol were omitted from the exofacial membranes the stopped-flow experiments indicated a higher rate of permeation for all of the solutes occurred. This was especially true when cholesterol was absent and rates were much greater (Figs. 3d - 6d). The results of experiments performed in triplicate are shown in Figs. 3b - 6b. The increases in exofacial permeability when GSLs and SM were removed were fairly constant and did not appear to be solute dependent. For example removal of GSLs resulted in 3.4-times higher (formamide, Fig. 3b), 6.1-times higher (acetamide, Fig. 4b), 4.4-times higher (urea, Fig. 5b) and 5.2-times higher (glycerol, Fig. 6b) permeabilities. For all four solutes the removal of SM
resulted in rates that were somewhat higher than the GSL-lacking liposomes. For formamide, acetamide, urea and glycerol, the increases were 4.3-times, 7.5-times, 5.6-times and 5.9-times respectively. Doing a simple mean on these two sets of numbers shows that the membranes lacking SM were 22% more permeable than those lacking GSLs. As the mole fractions of these two lipids is approximately the same (see Table 1) we conclude that both SM and GSLs contribute to the barrier of the exofacial leaflet, but SM is 20-25% better at reducing permeability. The removal of cholesterol, as for water, resulted in a massive increase in the permeability of all four solutes. The increases for formamide, acetamide, urea and cholesterol were 95-times, 136-times, 191-times and 285-times, which again follows the rank order of molecular weights for these non-electrolytes. These data make it clear that sphingolipids alone will not significantly restrict solute or water permeation.

\textit{NH}_3 \textit{Permeability of Leaflet Liposomes}

The permeation kinetics of \textit{NH}_3 were tested by exposure of liposomes to an \textit{NH}_4\textit{Cl} solution clamped to \textit{pH} 6.8. Free \textit{NH}_3 gas in the buffer permeates across the membrane and upon protonation to \textit{NH}_4^+ effects an increase in intravesicular \textit{pH} which can by measured by fluorescence changes. Fig. 7c shows that \textit{NH}_3 flux is extremely rapid across the membrane of cytoplasmic leaflet liposomes. In contrast, the flux across exofacial membranes is much slower. This illustrates an important physiological phenomenon; namely that ordering a membrane to reduce the permeability of water and small non-electrolytes is also sufficient to hinder the permeability of a gas. Fig. 7a shows the quantitative difference in the permeability of the two membranes to \textit{NH}_3 to be 60-fold. When GSLs were removed from exofacial liposomes the permeability increased 2.2-times and when SM was removed the increase was 4.0-times. Removal of cholesterol increased the \textit{NH}_3 permeability of the membrane 82-fold. These increases are similar to those seen for solutes and imply that \textit{NH}_3 permeation occurs by a similar solubility-diffusion mechanism.
**Apparent Proton Permeability of Leaflet Liposomes**

The proton permeability of the two membranes was assessed by exposing liposomes equilibrated to pH 7.5 to an external buffer with pH 6.9. Therefore what we measure is the rate of dissipation of a pH gradient across the liposomal membrane. While the precise mechanism of proton permeation is unclear, this approach allows us to measure the apparent proton permeability of the membranes in question. It should be noted that this technique does not allow us to necessarily distinguish between proton influx or hydroxide anion efflux. However, because proton flux appears to be independent of pH, there is good reason to believe that protons are in fact the permeating species (for a fuller discussion of this point see (30)). In Fig. 8b typical pH-dependent fluorescence quenching profiles are shown for exofacial and exofacial-lacking-cholesterol liposomes. Cytoplasmic membrane permeability to protons was unexpectedly slower than that of exofacial membranes by a factor of four (Fig. 8a). Membranes with the lowest permeability to protons were cholesterol-depleted exofacial liposomes (Exo-Chol, Fig 8a). This implies that cholesterol may contribute to the proton permeation pathway. The combined permeability data for all liposome variants is shown in Fig. 8a and reveals that the exofacial membranes had the highest proton permeability (0.011 cm/s) and that removal of sphingolipids reduced that permeability approximately two-fold.
DISCUSSION

We have used published data on the lipid composition of MDCK Type 1 apical membranes and in addition made a number of simplifying assumptions regarding its bilayer asymmetry, in order to construct vesicles which mimic each leaflet's composition. This analysis predicts that the apical membrane of these cells is comprised of an asymmetric bilayer which is enriched in SM, GSLs and PC in the exofacial leaflet, while the inner leaflet is composed mostly of the glycerophospholipids, PE, PS and PI (Table 1). Evidence to support these conclusions has come from a number of studies. For instance, in brush border membranes from rabbit intestine it was shown that PE phospholipid was largely inaccessible to reagents from the outside (31). A similar accessibility study of rabbit kidney brush border membranes revealed that PE, PS and PI were localized to the inner leaflet while SM was external (32). In MDCK cells a fluorescent ceramide analogue which was taken up and converted to SM and GSL in the Golgi, was completely extractable by BSA from the apical side, demonstrating a preferential accumulation in the exofacial leaflet (25). The predicted asymmetry also fits with well-established rules which indicate that choline-containing lipids are mostly exofacial while the amino-containing phospholipids localize cytoplasmically (33). Cholesterol is present in both leaflets and was apportioned according to the quantitative analyses described in the reference papers (15, 23).

Because the lipids of the exofacial leaflet of polarized epithelia are laterally constrained by the presence of tight junctions, while the lipids of the cytoplasmic leaflet are not, the inner leaflet of the apical domain is assumed to be the same as that of the basolateral membrane (13, 34). As a consequence it appears likely that the barrier function exhibited by 'water-tight' epithelia resides in the exofacial leaflet. Present studies directly tested this hypothesis and confirmed its validity. For water, small non-electrolytes and NH₃, all substances known to cross phospholipid bilayers by a 'solubility-diffusion' mechanism, the permeability of the cytoplasmic leaflet was one
to two orders of magnitude higher than the exofacial. These data indicated that the presence of appropriate lipids present in defined ratios was sufficient to generate a membrane with extremely low permeability to water, solutes and \( \text{NH}_3 \). Moreover, these experiments have allowed us to quantitate the magnitude of that difference in leaflet permeabilities for a range of biologically relevant permeant molecules.

For water, the cytoplasmic leaflet liposomes were 18-times more permeable than the exofacial with osmotic permeability coefficients of \( 4.4 \times 10^{-3} \) cm/s and \( 2.4 \times 10^{-4} \) cm/s respectively. We have previously shown that each leaflet is able to offer an independent resistance to permeation (18, 19). By analogy with electrical circuit theory, resistance is equal to the reciprocal of the permeability i.e. \( R = 1/P_\ell \) (see Eq. 1). As our exofacial and cytoplasmic liposomes have symmetrical leaflets, knowing the membrane permeability of each bilayer allows us to calculate single leaflet permeabilities according to Eq. 1. Combining the derived value for an exofacial leaflet and a cytoplasmic leaflet gave us a theoretical osmotic permeability coefficient (\( P_\ell \)) of \( 4.6 \times 10^{-4} \) cm/s which correlates well with the measured permeability of this membrane in cultured MDCK Type 1 cells. Such close concordance of measured and theoretical values as determined by our model system argues that the assumptions made and the lipid compositions arrived at are probably reasonable representations of the real membrane and allow us to conclude that the outer leaflet of the MDCK Type 1 apical membrane is probably 20-fold less permeable to water than the inner leaflet. These results also successfully apply Eq. 1 to membranes of real cells for the first time.

Although we based our leaflet compositions on the best evidence available, there is little information known about the acyl chain composition of these various lipid classes in the MDCK apical membrane. Consequently we chose mammalian lipids as the source for these experiments in an attempt to minimize artifacts which may have arisen from the use of bacterial
or other lipid sources. Based on information provided by Avanti Polar Lipids we know the acyl chain composition of our artificial membranes. As expected the saturation ratio (i.e. saturated:unsaturated - S:U) was quite different between the two membranes. Exofacial membranes had an S:U of 2.5 due to the presence of saturated acyl chains on SM and GSL. In contrast, cytoplasmic membranes had an S:U of 0.6 indicating a much higher proportion of double bond-containing fatty acids. Exofacial membranes contained high proportions of 16:0 (35.0%), 18:0 (8.1%) and 24:0 (16.4%) acyl chains. The predominant unsaturated acyl chains were 18:1 and 18:2 (11.1%) and 24:1 (8.4%). In comparison to this the cytoplasmic membranes contained little 16:0 with the predominant saturated species being 18:0 (36.9%). Unsaturated chains were 18:1 (13.3%), 18:2 (12.2%) and 20:4 (29.3%). The acyl chain composition of these membranes is likely to be an important contributor to their permeation properties due to the different packing constraints imposed by the presence of double bonds. Double bonds introduce a rigid bend in an acyl chain, resulting in greater free volume space and higher fluidity in the membrane. It has been shown previously that for substances which cross membranes by a solubility-diffusion mechanism that membrane fluidity and permeability are tightly correlated (20). The results presented here further support this principle.

We investigated the effect of using lipids with different fatty acid substituents. The use of disaturated DPPC in an exofacial membrane reduced the membrane permeability but more surprisingly appeared to substitute for GSLs upon their removal by maintaining low permeability. At 25°C DPPC is in the gel state and with a phosphocholine head group strongly resembles SM. Although there is no amide group for hydrogen-bonding with cholesterol, the tight chain packing induced by cholesterol and saturated PC in the gel state appears sufficient to compensate for loss of GSLs. It is therefore extremely important that the most physiologically relevant lipids are used in membrane modeling studies. Clearly the complement of unsaturated acyl chains is an important determinant in the permeation properties of the membrane.
To further examine the validity of our leaflet-specific liposomes as a model for predicting reconstituted membrane permeabilities it is informative to compare our results with permeabilities found in other barrier epithelia. Our theoretical MDCK membrane permeability to urea (employing Eq. 1) is $6.2 \times 10^{-8}$ cm/s. This compares to values of $2.1 \times 10^{-7}$ cm/s (3-fold higher), $7.8 \times 10^{-7}$ cm/s (13-fold higher) and $5.5 \times 10^{-7}$ cm/s (9-fold higher) for toad bladder apical membrane, mammalian bladder apical membrane and gastric apical membrane respectively (3). Therefore our reconstituted membrane system has a urea permeability approximately an order of magnitude lower than that seen for some other barrier membranes. We have in addition, previously measured the diffusive urea permeability of MDCK cells to be $6.0 \times 10^{-6}$ cm/s (17). This is approximately an order of magnitude higher than is seen for other barrier epithelia, and two orders of magnitude higher than our model liposomes would predict for this membrane. We believe this may indicate some form of facilitated urea uptake by MDCK cells. To the best of our knowledge urea transport activity has not specifically been demonstrated in MDCK’s, however it is possible that cells derived from the distal nephron would possess such an activity. Further support for this conjecture comes from permeability studies performed by Rivers et al. (27) in which they measured the acetamide and formamide permeabilities of MDCK apical membranes to be $4.1 \times 10^{-6}$ and $5.5 \times 10^{-6}$ cm/s respectively. The theoretical permeabilities for these two non-electrolytes as derived from our model liposomes was $3.8 \times 10^{-6}$ and $5.5 \times 10^{-6}$ cm/s respectively which are in very close agreement. Furthermore, liposomes composed of 25% SM, 40% cholesterol and 35% PC, have been shown to have a urea permeability of $4.4 \times 10^{-6}$ cm/s (20) which is close to the value obtained for the exofacial liposomes used in this study ($3.1 \times 10^{-8}$ cm/s with a composition of 25% SM, 39% cholesterol, 36% PC and GSLs). Taken together these results indicate that urea permeability may be facilitated in MDCK cells and not reflect simple membrane permeant
behavior. Measurements of water and other solutes however, appear to validate our hypothesis and the use of Eq. 1 to predict apical membrane permeability properties.

The theoretical MDCK membrane permeability to NH$_3$ is 2.6 x 10$^{-1}$ cm/s, which is approximately an order of magnitude higher than has been measured for other barrier epithelia (3). However, it should be noted that our measurements were performed at 25ºC while the comparative studies made permeability measurements at 20ºC. We have previously shown that NH$_3$ permeability is exquisitely sensitive to membrane fluidity and hence temperature (19) which may explain the difference. The proton permeability for our artificial membrane was 4.3 x 10$^{-3}$ cm/s which compares favorably with values of 8.0 x 10$^{-3}$ cm/s, 3.0 x 10$^{-3}$ cm/s and 19 x 10$^{-3}$ cm/s, obtained for medullary thick ascending limb AM, whole mammalian bladder and gastric AM respectively (3). These comparisons between our *in vitro* reconstituted membranes and real asymmetric barrier membranes, lends support to the conclusion that these artificial liposomes reconstitute the biophysical characteristics of true barrier membranes to a wide range of biologically important permeant molecules.

We performed measurements of proton permeation which relied upon the dissipation of an applied pH gradient across the liposomal membrane with consequent quenching of an intravesicular pH-dependent fluorophore. While this is a well-established technique for monitoring proton permeation processes (3, 10, 19, 20, 22, 30, 37), the exact mechanism is not well understood. Measurements of proton permeability can yield very different values depending on the method used. If small pH gradients are allowed to dissipate around pH 7, values in the range of 10$^{-4}$ cm/s are obtained (35). However, if large pH gradients are applied to liposomes, proton fluxes with values of 10$^{-9}$ cm/s are recorded (36). This phenomenon was explored by Deamer and Nichols (37) and led them to conclude that decay of large pH gradients results in diffusion potentials which limit proton flux. Addition of valinomycin in K$^+$-containing
buffers eliminated the formation of membrane potentials and therefore allowed proton fluxes to become rate-limiting. However, if proton fluxes were measured near pH 7 in the presence of small proton gradients, there was no formation of a limiting diffusion potential (37). Our experiments were carried out under the latter conditions, and as noted by Deamer and Nichols, the presence or absence of valinomycin did not affect the rate of proton flux. While this indicates our measurements were not influenced by the formation of membrane potentials it does appear to contradict rules of electroneutrality. Given that we cannot rule out that our proton flux may be accompanied by some other charge-dissipating process i.e. proton-anion co-permeation, we have chosen to refer to these measurements as demonstrating the apparent proton permeability of the liposomal membranes.

Our measurements of proton permeability in cytoplasmic and exofacial membranes yielded the unusual result that cytoplasmic lipids had four-fold lower proton permeability than the exofacial (Fig. 8). The mechanism by which protons translocate across phospholipid bilayers is not well understood but it has clearly been established that unlike water, solutes and NH₃, membrane fluidity is not a key determinant in permeation (19, 20, 38). In a study which examined the relationship between membrane fluidity and permeability, it was found that liposomes of differing composition and fluidities exhibited water permeabilities which varied up to 70-fold while proton permeability varied no more than three-fold. Moreover, within that three-fold range there was poor correlation between proton permeability and the fluidity of the membrane (20). Another set of experiments performed on DPPC liposomes revealed that protons permeated four times faster when the liposomes were heated above the phase-transition temperature, but that solute fluxes under the same conditions increased by two orders of magnitude (19). Brookes et al. showed that liposomes made from phospholipids extracted from the mitochondrial inner membrane of eight different vertebrate species, with widely varying mitochondrial proton leak and degree of acyl chain unsaturation exhibited no differences in proton permeability (38). They
concluded there was no correlation between liposome proton permeability and phospholipid fatty acid composition. The data presented here further support this. When cholesterol was removed from the exofacial membrane water permeability increased nearly 40-fold. In dramatic contrast to this, proton permeability decreased 6.5-fold.

Proton permeation across membranes has been shown to occur at rates five to six orders of magnitude higher than for other monovalent cations. Several explanations have been proposed to account for this anomalous behavior. The first hypothesizes the presence of fatty acid ‘contaminants’ within the membrane which are available for protonation. The protonated species translocates to the other side, releases a proton and then cycles back as the anionic species (39, 40). The second proposes the formation of transient defects in the interfacial region of the bilayer through which water ‘fingers’ may project briefly and from which hydrated pores carrying protons may be pinched off. Water molecules present within the hydrophobic interior of the bilayer may then form transient hydrogen bonded chains or water wires, along which protons can move, thus allowing sequential water to water translocation across the membrane (30, 39, 41). Evidence for both mechanisms exist and it is likely that both contribute to some degree depending on the nature of the membrane – its composition, thickness, the pH on either side of the bilayer, the relevant buffering agents present and the temperature. Our proton flux data for the modified exofacial membranes reveals an additional intriguing possibility. When either of the sphingolipids was removed, proton permeation was reduced (Fig. 8). When cholesterol was removed entirely the rate fell to one-sixth of that shown by the complete membrane. This phenomenon suggests that there is something specific about the presence of both cholesterol and sphingolipid in this membrane which facilitates proton transfer. One possibility is that the specific hydrogen-bonding interaction which occurs between the 3-OH group of cholesterol and the amide group present on GSLs and SM, provides a suitable early docking site for protons close to the aqueous/lipid headgroup interfacial region.
of this hydrogen-bond may lower the free energy required for protons to partition into the proximal hydrophobic region of the membrane. The slow rate of permeation across cytoplasmic membranes may be consistent with this model. Although cholesterol constitutes 37 mol% of this bilayer, there are no sphingolipids present and therefore the specific hydrogen bonding interaction we speculate may be important, is not present. The effect of cholesterol and cholesterol/sphingolipid interactions on proton transport has not previously been studied. Therefore further work will be needed to confirm this hypothesis.

Our model liposomes appear to recapitulate the barrier properties of the MDCK apical membrane to water, solutes (with the exception of urea) and NH₃, to a degree consistent with that actually observed in these cells. The differences in permeability between the two membrane compositions is most likely due to the reduction in fluidity and increase in chain-ordering which occurs as a consequence of sphingolipid/cholesterol interactions in the exofacial liposomes. Specifically it has been shown that there is likely to be hydrogen bonding interactions between the amide functional group of SM and the 3-OH group of cholesterol (16, 42). In addition there are likely to be strong van der Waal's attractions as a result of the contribution of the planar steroid rings interacting with the saturated hydrocarbon chains of the sphingolipids. These cohesive forces result in greater chain ordering due to a reduction in trans-gauche isomerization about the carbon-carbon bonds and an increase in sphingolipid packing density (42). In addition to these interactions, GSLs are also known to self-associate through hydrogen-bonding of their glycosyl head groups. Therefore, either in isolation or collectively, these molecular interplays may be responsible for reducing the fluidity and consequently the permeability of the exofacial leaflet.

To investigate which of the associative interactions between the lipids used in our exofacial liposomes were of greatest significance we focused on the putative contribution of the
sphingolipids. As GSLs are present in high concentration in the exofacial leaflet of epithelial apical membranes and appear to be almost exclusively localized to this membrane domain we hypothesized that they might play a significant role in reducing membrane permeability. Indeed this proved to be the case with both GSLs and SM contributing to reduced membrane permeability. The removal of approximately 20 mol% of either species and replacement with bovine heart PC resulted in 2 – 3-fold increases in membrane water permeability. For solutes the increases were a little higher (3 – 7-fold). The data suggested that SM was better at reducing membrane permeability than GSLs. This is consistent with findings that show that cholesterol has a greater condensing effect on SM compared to galactosylceramide films when those sphingolipids were acyl chain matched and in similar phase states (43). The greater cross-sectional area-reduction exhibited by SM compared to GSL would certainly lead to tighter packing and hence lower permeability. When cholesterol was removed the water permeability increased by 37-fold. A large increase in permeability was anticipated because of the known ability of cholesterol to condense and rigidify lipid bilayers. Cholesterol with its relatively planar structure orients itself with the polar 3-OH group towards the aqueous phase and its tetracyclic rings positioned between carbons 2 and 10 of the acyl chains (42). This spatial localization tends to increase local ordering of acyl chains in the proximal portion of the leaflet. In addition, as noted, the 3-OH can function as hydrogen bond donor and interact with the amide group of sphingolipids.

It is worth noting that the presence of sphingolipids in high concentrations in the outer leaflet of epithelial apical membranes can trap cholesterol in that leaflet by virtue of hydrogen bonding interactions and therefore may be contributing to the formation and functioning of detergent insoluble glycolipid rafts. These cholesterol and sphingolipid enriched microdomains have been implicated in a range of critical cellular processes including intracellular lipid and protein sorting, caveolae formation and signal transduction (44, 45). The cell therefore, may not only be
erecting a defensive shield at its apical pole but also be assembling functional lipid ensembles necessary for other specific molecular interactions.

These results demonstrate that MDCK Type 1 cells can synthesize a low permeability apical membrane by the selective trafficking of cholesterol, SM and GSLs to the outer leaflet of the plasma membrane. The cytoplasmic leaflet of this membrane, while possessing a relatively high cholesterol content (36 mol%) is nonetheless quite permeable to water and solutes, presumably due to the inability of cholesterol to form hydrogen bonds with glycerophospholipids like PE and PS. The successful application of the additive reciprocal equation for leaflet permeabilities validates our modeling of the MDCK apical membrane. The concordance of derived apical membrane permeability coefficients with actual permeabilities measured by others provides strong evidence that our liposomes had realistic lipid compositions and further validates the hypothesis that leaflets offer independent barriers to the permeation of small uncharged molecules.
ACKNOWLEDGMENTS

We would like to thank Dr. Dexi Liu for use of the particle sizer. This work was supported by a research fellowship from the National Kidney Foundation and by NIH grant DK43955.
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The abbreviations used are: SM, sphingomyelin; MDCK, Madin-Darby canine kidney; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; GSLs, glycosphingolipids; DPPC, dipalmitoylphosphatidylcholine; CF, carboxyfluorescein; HEPES, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; P₀, osmotic water permeability coefficient; Exo-SM, exofacial liposomes minus sphingomyelin; Exo-GSLs, exofacial liposomes minus glycosphingolipids; Exo-Chol., exofacial liposomes minus cholesterol.

For the complete acyl chain composition of these membranes see supplementary material contained in acylcomp.pdf.
Fig. 1. **Water permeability of exofacial, cytoplasmic and modified exofacial liposomes.** Liposomes were prepared as described in Methods with entrapped CF. Stopped-flow experiments were performed in which liposomes were rapidly exposed to a doubling of extravesicular osmolality. Vesicle shrinkage results in CF self-quenching. (A) osmotic water permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes; mean ± SEM for three separate liposome preparations (B) osmotic water permeability of exofacial liposomes modified by the removal of individual lipids; Exo-GSL (Exofacial minus glycosphingolipids); Exo-SM (Exofacial minus sphingomyelin); Exo-Chol (Exofacial minus cholesterol). (C,D) averaged stopped-flow traces (n = 6 - 10) from a single experiment. Fluorescence was converted to relative volume and curves show raw data plus superimposed fitted single exponential decay functions. * $P < 0.05$ compared with Exofacial.

Fig. 2. **Effect of changing acyl chain composition on water permeability.** Liposomes were prepared as described in Methods with entrapped CF. Stopped-flow experiments were performed in which liposomes were rapidly exposed to a doubling of extravesicular osmolality. (A) osmotic water permeability of exofacial liposomes. Exofacial liposomes had bovine heart PC replaced with DPPC (DPPC/Exo), or DPPC/Exo liposomes had GSLs removed and replaced with more DPPC (DPPC/Exo-GSLs); (B) osmotic water permeability of cytoplasmic liposomes. Cytoplasmic liposomes had bovine heart PE replaced with E. coli PE (Ecoli/Cyto); (C, D) averaged stopped-flow traces (n = 6 - 10) from single experiments. Fluorescence was converted to relative volume and curves were fitted with single exponential decay functions. Results shown in A and B are mean ± SEM for three separate liposome preparations. * $P < 0.05$ compared with Cyto.
Fig. 3. Formamide permeability of exofacial, cytoplasmic and modified exofacial liposomes. Liposomes were prepared as described in Methods with entrapped CF. Liposomes were preloaded with 200 mM formamide and then abruptly exposed to a solution of identical osmolality containing 150 mM formamide. Solute efflux and vesicle shrinkage results in CF self-quenching. (A) formamide permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes; mean ± SEM for three separate liposome preparations (B) formamide permeability of exofacial liposomes modified by the removal of individual lipids; Exo-GSL lack glycosphingolipids; Exo-SM lack sphingomyelin; Exo-Chol lack cholesterol (C,D) averaged stopped-flow traces (n = 6 - 10) from a single experiment. Fluorescence was converted to relative volume and curves were fitted with single exponential decay functions. *P < 0.05 compared with Exofacial.

Fig. 4. Acetamide permeability of exofacial, cytoplasmic and modified exofacial liposomes. Liposomes were prepared as described in Methods with entrapped CF. Liposomes were preloaded with 200 mM acetamide and then abruptly exposed to a solution of identical osmolality containing 150 mM acetamide. Solute efflux and vesicle shrinkage results in CF self-quenching. (A) acetamide permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes; mean ± SEM for three separate liposome preparations (B) acetamide permeability of exofacial liposomes modified by the removal of individual lipids; Exo-GSL lack glycosphingolipids; Exo-SM lack sphingomyelin; Exo-Chol lack cholesterol (C,D) averaged stopped-flow traces (n = 6 - 10) from a single experiment. Fluorescence was converted to relative volume and curves were fitted with single exponential decay functions. *P < 0.05 compared with Exofacial.
Fig. 5. **Urea permeability of exofacial, cytoplasmic and modified exofacial liposomes.** Liposomes were prepared as described in Methods with entrapped CF. Liposomes were preloaded with 200 mM urea and then abruptly exposed to a solution of identical osmolality containing 150 mM urea. Solute efflux and vesicle shrinkage results in CF self-quenching. (A) urea permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes; mean ± SEM for three separate liposome preparations (B) urea permeability of exofacial liposomes modified by the removal of individual lipids; Exo-GSL lack glycosphingolipids; Exo-SM lack sphingomyelin; Exo-Chol lack cholesterol (C,D) averaged stopped-flow traces (n = 3 - 8) from a single experiment. Fluorescence was converted to relative volume and curves were fitted with single exponential decay functions. * P < 0.05 compared with Exofacial.

Fig. 6. **Glycerol permeability of exofacial, cytoplasmic and modified exofacial liposomes.** Liposomes were prepared as described in Methods with entrapped CF. Liposomes were preloaded with 200 mM glycerol and then abruptly exposed to a solution of identical osmolality containing 150 mM glycerol. Solute efflux and vesicle shrinkage results in CF self-quenching. (A) glycerol permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes; mean ± SEM for three separate liposome preparations (B) glycerol permeability of exofacial liposomes modified by the removal of individual lipids; Exo-GSL lack glycosphingolipids; Exo-SM lack sphingomyelin; Exo-Chol lack cholesterol (C,D) averaged stopped-flow traces (n = 3 - 8) from a single experiment. Fluorescence was converted to relative volume and curves were fitted with single exponential decay functions. * P < 0.05 compared with Exofacial.
Fig. 7. **NH₃ permeability of exofacial, cytoplasmic and modified exofacial liposomes.** Liposomes were prepared as described in Methods with entrapped CF. Liposomes equilibrated to pH 6.8 were abruptly exposed to a buffer of identical pH containing NH₄Cl. NH₃ entering the liposome becomes protonated to NH₄⁺ and as a result the pH and CF fluorescence increases. (A) NH₃ permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes; mean ± SEM for three separate liposome preparations (B) NH₃ permeability of exofacial liposomes modified by the removal of individual lipids; Exo-GSL lack glycosphingolipids; Exo-SM lack sphingomyelin; Exo-Chol lack cholesterol (C,D) averaged stopped-flow traces (n = 6 - 10) from a single experiment. Fluorescence was converted to pH and curves were fitted with single exponential decay functions. * P < 0.05 compared with Exofacial.

Fig. 8. **Apparent H⁺ permeability of exofacial, cytoplasmic and modified exofacial liposomes.** Liposomes were prepared as described in Methods with entrapped CF. Liposomes were abruptly exposed to a buffer of lower pH such that the extravesicular pH was 6.9. Dissipation of the pH gradient reduces intravesicular pH and CF fluorescence. (A) H⁺ permeability of outer leaflet (Exofacial) and inner leaflet (Cyto) liposomes and of exofacial liposomes modified by the removal of individual lipids; Exo-GSL lack glycosphingolipids; Exo-SM lack sphingomyelin; Exo-Chol lack cholesterol; mean ± SEM for three separate liposome preparations (B) averaged stopped-flow traces (n = 6 - 10) from a single experiment. Fluorescence was converted to pH and curves were fitted with single exponential decay functions. * P < 0.05 compared with Exofacial. The conservativeness of the Bonferroni t-test resulted in only one significant difference. Single comparison testing gave P = 0.034 and P = 0.013 for Exo-GSL and Cyto respectively.
| Lipids   | Exofacial Leaflet Composition | Cytoplasmic Leaflet Composition |
|----------|-------------------------------|---------------------------------|
|          | % by weight | mol% | % by weight | mol% |
| PE       | —           | —    | 48.3        | 40.1 |
| PS       | —           | —    | 25.5        | 20.1 |
| PI       | —           | —    | 3.6         | 2.5  |
| Cholesterol | 37.3       | 52.3 | 22.6        | 37.3 |
| PC       | 12.0        | 8.6  | —           | —    |
| SM       | 23.8        | 18.4 | —           | —    |
| GSL      | 26.8        | 20.7 | —           | —    |
Exofacial DPPC/Exo DPPC/Exo-GSLs

$P_f (\text{cm/s}) \times 10^4$

A

Cyto Ecoli/Cyto

$P_f (\text{cm/s}) \times 10^3$

B

Time (s)

Relative Volume

0.50 0.75 1.00

Exofacial DPPC/Exo DPPC/Exo-GSLs

Cyto Ecoli/Cyto

C

Time (s)

Relative Volume

0.50 0.75 0.90

D

Time (s)

Exofacial DPPC/Exo DPPC/Exo-GSLs

Cyto Ecoli/Cyto

Downloaded from http://www.jbc.org/ on July 9, 2020
## Fatty Acyl Chain Composition of Leaflet Specific Liposomes

| Fatty Acid | Exofacial Membrane (%) | Cytoplasmic Membrane (%) |
|------------|------------------------|--------------------------|
| 16:0       | 35.0                   | 1.1                      |
| 18:0       | 8.1                    | 34.1                     |
| 18:1       | 3.9                    | 12.3                     |
| 18:2       | 7.2                    | 11.3                     |
| 20:0       | 0.8                    |                          |
| 20:1       |                        | 0.6                      |
| 20:3       |                        | 1.9                      |
| 20:4       | 1.4                    | 27.1                     |
| 22:0       | 2.8                    |                          |
| 22:1       | 2.0                    |                          |
| 22:6       | 0.8                    | 4.0                      |
| 23:0       | 1.8                    |                          |
| 23:1       | 1.1                    |                          |
| 24:0       | 16.4                   |                          |
| 24:1       | 8.4                    |                          |
| 25:0       | 2.8                    |                          |
| 25:1       | 1.3                    |                          |
| 26:0       | 2.6                    |                          |
| 26:1       | 1.9                    |                          |
| Other      | 1.7                    | 7.6                      |
