Relationship between Lipid Fluidity and Water Permeability of Bovine Tracheal Epithelial Cell Apical Membranes†

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Received July 23, 1985

ABSTRACT: Apical membrane vesicles were prepared from bovine tracheal epithelial cells. These membranes were enriched in alkaline phosphatase specific activity 35-fold compared to cellular homogenates. Steady-state fluorescence polarization studies of these membranes, using three fluorophores, demonstrated that they possessed a relatively low fluidity. Studies using the probe 1,6-diphenyl-1,3,5-hexatriene detected thermotropic transitions at 25.7 ± 0.4 and 26.8 ± 0.6 °C in these membranes and their liposomes, respectively. Analysis of the composition of these membranes revealed a fatty acyl saturation index of 0.59 ± 0.02, a protein/lipid ratio (w/w) of 0.60 ± 0.06, a cholesterol/phospholipid ratio (mol/mol) of 0.83 ± 0.11, and a sphingomyelin/lecithin ratio (mol/mol) of 0.64 ± 0.10. Membrane vesicles were osmotically active when studied by a stopped-flow nephelometric technique. Arrhenius plots of rates of osmotic water efflux demonstrated break points at approximately 28 and 18 °C, with activation energies of 16.7 ± 0.2 kcal mol^{-1} from 35 to 28 °C, 8.3 ± 0.5 kcal mol^{-1} from 28 to 18 °C, and approximately 3.0 kcal mol^{-1} below 18 °C. Treatment of membrane vesicles with benzyl alcohol, a known fluidizer, decreased lipid order (increased fluidity) and increased the rate of osmotic water efflux. The present results suggest that water crosses tracheal epithelial cell apical membranes by solubility–diffusion across the lipid domain and that increases in fluidity correlate with increases in the water permeability of these membranes.

The epithelium of the mammalian respiratory tract is covered by a protective layer of mucous and a layer of water surrounding the cilia between the mucous layer and cell surfaces (Yoneda, 1976). Airway mucous is produced by the submucosal glands and goblet cells (Wanner, 1977), while the periciliary fluid most likely results from active ion secretion by the surface epithelial cells (Nadel et al., 1979). In the canine trachea, net fluid secretion is isotonic with respect to blood, and fluid movement parallels active chloride secretion (Welsh et al., 1980). Measurements of water and ion secretion in bovine trachea suggest that it is a "leaky" epithelium with a relatively high hydraulic conductivity (Durand et al., 1981). In the most extensively tested model of transepithelial water transport, water movement occurs osmotically secondary to active ion transport (Curran & MacIntosh, 1962; Diamond, 1964, 1979). Furthermore, recent investigations suggest that, even in leaky epithelium, osmotic water flow occurs primarily via a transcellular pathway (Van Os et al., 1979; Persson & Spring, 1982).

Prior studies in our laboratories (Brasitus & Schachter, 1980, 1982, 1984; Brasitus et al., 1980; Worman & Field, 1984) have described the lipid composition and lipid fluidity[1] as well as the osmotic water permeability of apical (brush border) membranes prepared from small intestinal enterocytes. Recently, Langridge-Smith et al. (1983) have purified apical membrane vesicles from epithelial cells of the bovine trachea. Since water flow across the apical membranes of tracheal epithelial cells may be an important step in airway fluid secretion and water permeability may be influenced by the lipid composition and fluidity of biological membranes (Bittman & Blau, 1972; Finkelstein & Cass, 1967), we decided to analyze the lipid composition and fluidity of tracheal apical membranes and examine the relationship between lipid fluidity and the water permeability of these membranes. The results of these studies serve as the basis for the present report.

Materials and Methods

Materials. Fatty acid methyl esters, GLC columns, and lipid standards were obtained from Applied Science Corp. (State College, PA) and/or Supelco (BelleFonte, PA). All other materials, unless otherwise indicated, were purchased from either Fisher Chemical Co. (Fairlawn, NJ) or Sigma Chemical Co. (St. Louis, MO).

Membrane Vesicle Preparation. Bovine trachea were obtained from a local slaughterhouse and kept on ice after removal from the animal until use less than 1 h later. Epithelial cells were isolated and apical membranes purified as previously described (Langridge-Smith et al., 1983). The final apical

†Supported in part by National Institutes of Health Grant HL-26421-04. H.J.W. is a recipient of the Calvin Fentress Research Fellowship of the Pritzker School of Medicine of The University of Chicago.

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[1] The term "lipid fluidity" as applied to anisotropic bilayer membranes is used to denote the relative motional freedom of the lipid molecules or substituents thereof. A more detailed description of the sense in which the term is used has been published (Brasitus & Schachter, 1982). Briefly, as evaluated by steady-state fluorescence polarization of lipid fluorophores, fluidity is assessed via the parameters of the modified Perrin equation described under Materials and Methods. An increase in fluidity corresponds to a decrease either in the correlation time, T_c, or in the hindered anisotropy, r_∞, of the fluorophore. Hence, the term combines the concepts of the dynamic and static (lipid order) components of fluidity.

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membrane pellet was suspended in 100 mM mannitol/1 mM tris(hydroxymethyl)aminomethane–*N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid (Tris–Hepes) (pH 7.4). Protein was determined by the method of Lowry et al. (1951). Alkaline phosphatase (*p*-nitrophenylphosphatase) activity was measured as previously described (Brasitus & Schachter, 1980). In osmotic water transport experiments, freshly prepared membrane vesicles as well as vesicles stored frozen in buffer at −70 °C and subsequently thawed (once) gave identical results. For fluorescence polarization studies, membrane vesicles were stored at 0–4 °C and used within 24 h of preparation.

Fluorescence Polarization Studies. Three fluorophores were used: 1,6 diphenyl-1,3,5-hexatriene (DPH), DL-2-(9-anthroyl)stearic acid (2-AS), and DL-12-(9-anthroyl)stearic acid (12-AS). All compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Molecular Probes Inc. (Junction City, OR). Steady-state fluorescence polarization studies were performed with a Perkin-Elmer 650-40 spectrofluorometer adapted for fluorescence polarization. Liposomes were prepared from lipid extracts of membranes as previously described (Brasitus & Schachter, 1980). The methods used to load the membranes and liposomes and the quantification of the polarization of fluorescence have been described (Brasitus & Schachter, 1984). The content of each fluorophore in the membranes and liposomes was estimated fluorometrically as described by Cogan and Schachter (1981). Final molar ratios of probe/lipid ranged from 0.001 to 0.002, and the anisotropy differences noted in these studies could not be ascribed to differences in probe concentrations in the membranes or liposomes. Corrections for light scattering (membrane and liposome suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the preparations after each estimation) were made routinely, and the combined corrections were less than 3% of the total fluorescence intensity observed for DPH-loaded preparations and less than 5% of that observed for anthroyloxystearate-loaded suspensions. The results were obtained according to the modified Perrin relationship (Heyn, 1979; Jahnig, 1979; Van Blitterswijk et al., 1981) $r = r_\infty + (r_0 - r_\infty)[T_c/T_c + T_f)]$, where r is the fluorescence anisotropy, r_0 is the maximal limiting anisotropy, taken as 0.365 for DPH (Shinitzky & Barenholz, 1974) and 0.285 for the anthroyloxy probes (Schachter & Shinitzky, 1977), r_∞ is the limiting hindered anisotropy, T_c is the correlation time, and T_f is the mean lifetime of the excited state. The lifetime, T_f, was estimated by the total fluorescence intensity as previously described (Brasitus & Schachter, 1984). No changes in the excited-state lifetimes were demonstrated by using each probe in each membrane or liposome preparation (Brasitus & Schachter, 1984). Values of r_∞ for DPH were calculated from r values as previously described by Van Blitterswijk et al. (1981). The static component of membrane fluidity was assessed by an order parameter, S, where $S = (r_\infty/r_0)^{1/2}$ as described previously (Van Blitterswijk et al., 1981). The polarization of fluorescence expressed as the fluorescence anisotropy, r, was determined over the range of 10–40 °C for apical membranes and liposomes using DPH. The logarithm of the lipid order, S, determined from r_∞ was plotted against $1/T$ (K^{-1}) as described (Brasitus et al., 1980), to detect thermotropic transitions.

Composition Studies. Total lipids were extracted from the membranes by the method of Folch et al. (1957). Cholesterol and phospholipids were measured by the methods of Zlatkis et al. (1953) and Ames and Dubin (1960), respectively. The phospholipid composition of the extracts was further examined by thin-layer chromatography according to the procedure of Katz et al. (1976). Fatty acids of the total lipid extracts were derivatized as described by Gartner and Vahouny (1972). Fatty acid methyl esters were determined on a Hewlett-Packard 5790A gas–liquid chromatograph (GLC) equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator, using authentic fatty acid methyl esters to identify retention times (Gartner & Vahouny, 1972.

Table I: Steady-State Fluorescence Polarization Studies on Tracheal Epithelial Cell Apical Membranes and Liposomes[a]

probe	preparations	anisotropy, r	hindered anisotropy, r_∞	order parameter, S
DPH	membranes	0.23 ± 0.01	0.21 ± 0.01	0.77 ± 0.02
	liposomes	0.18 ± 0.01	0.14 ± 0.01	0.62 ± 0.02
2-AS	membranes	0.14 ± 0.01		
	liposomes	0.11 ± 0.01		
12-AS	membranes	0.15 ± 0.01		
	liposomes	0.12 ± 0.01		

[a] Values represent means ± SE of six separate determinations of three different membranes preparations and four separate determinations of two different liposome preparations at 25 °C.

Stopped-Flow Nephelometric Measurements. The construction of the home-built stopped-flow apparatus used in the present studies has been described elsewhere (Johnson & Taylor, 1978). The apparatus was set to function as a nephelometer by using 500-nm filters (Omega Optical Co., Battleboro, VT) for both excitation and 90° emission. Methods used to measure light scattering and the rate of change of scattered light caused by osmotic shrinkage of tracheal apical membrane vesicles have been described (Worman & Field, 1984, 1985). Briefly, one drive syringe of the stopped-flow apparatus was filled with 0.1–0.4 mg mL^{-1} membrane protein suspended in 100 mM mannitol/1 mM Tris–Hepes (pH 7.4); the other was filled with hyperosmotic mannitol/1 mM Tris–Hepes (pH 7.4). The contents of the syringes were mixed in a 1 to 1 ratio. Scattered-light intensity vs. time was stored in digital form on a Digital PDP 11/10 computer (Digital Equipment Corp., Maynard, MA) and recorded on a Hewlett-Packard 7015 X-Y recorder (Hewlett-Packard Co., Palo Alto, CA). Temperature was kept constant with a circulating water bath and monitored by a thermocouple probe close to the reaction cuvette. In the experiments using benzyl alcohol and methyl alcohol, the solutions were the same as above but contained the desired alcohol concentrations in both syringes.

Statistical Methods. All values are expressed as mean ± SE. Paired or unpaired t tests were used for all statistical analyses. A P value less than 0.05 was considered significant.

RESULTS

Apical Membrane Purification. Apical membranes [1.10 ± 0.16 μmol (mg of protein)$^{-1}$ min^{-1}] showed approximately a 35-fold enrichment in the specific activity of the marker enzyme alkaline phosphatase compared to the activity in the starting homogenate [0.03 ± 0.01 μmol (mg of protein)$^{-1}$ min^{-1}] ($N = 4$). As shown previously (Langridge-Smith et al., 1983), these plasma membranes were also minimally contaminated by mitochondrial, microsomal, and basolateral membranes (data not shown).

Fluorescence Polarization Studies. The apical membranes prepared from tracheal epithelial cells were found to possess a relatively low fluidity, as assessed by fluorescence polarization techniques using all three fluorophores (Table I). It should be noted that these probes differ in a number of respects

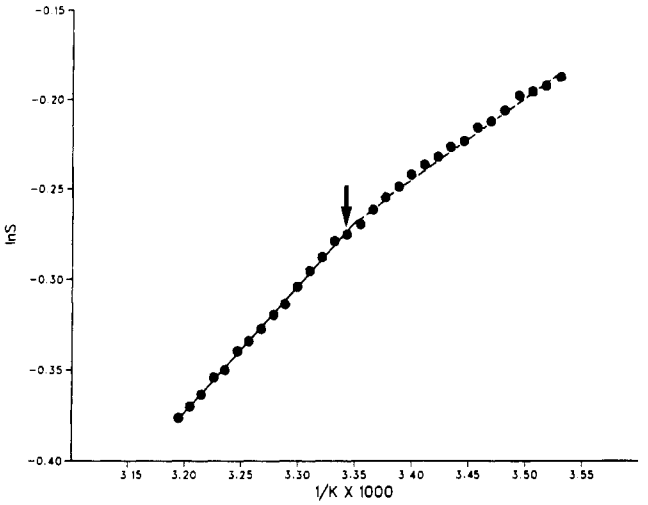

FIGURE 1: Arrhenius plot of lipid structural order parameter, S, for tracheal apical membranes and liposomes determined by DPH steady-state fluorescence polarization. Each data point in the figure is a mean value for three membrane preparations and two liposome preparations. Standard errors are omitted for clarity, since all were less than 3% of the mean values.

(Aldrich & Vanderkooi, 1976; Lentz et al., 1976; Thulborn et al., 1978, 1979; Thulborn & Sawyer, 1978; Cadenhead et al., 1977; Bashford et al., 1976). DPH molecules are rod shaped (Aldrich & Vanderkooi, 1976), localize deep in the lipid bilayer (Lentz et al., 1976), and are aligned relatively parallel to the phospholipid acyl chains (Aldrich & Vanderkooi, 1976). The transition moments for absorption and emission of this probe are along its long axis (Thulborn et al., 1976). The anthroyloxy fatty acid probes (2-AS and 12-AS) assume a more spherical shape in bilayers than DPH (Thulborn et al., 1979) and localize at various depths in the bilayer; i.e., 2-AS localizes in the bilayer closer to the aqueous interface than 12-AS (Bashford et al., 1976; Thulborn et al., 1978, 1979). Their emission moment is in the plane of the anthracene ring and at 30° to their short molecular axis (Thulborn et al., 1979). In biological and artificial membranes, the structural organization of the lipid bilayer appears to limit the extent of rotation of DPH; therefore, r_∞ values for this probe are high and largely determine r (Van Blitterswijk et al., 1981). Other probes such as 2-AS and 12-AS yield relatively low values of r_∞ in bilayer membranes, and their r values reflect mainly T_c, i.e., the speed of rotation (Vincent et al., 1982; Schachter et al., 1982). In the present studies (see Table I), both the static and dynamic components of membrane lipid fluidity, as assessed by r_∞ and S of DPH and r values of 2-AS and 12-AS, respectively, were found to be relatively low in these apical membranes when compared to a number of other membranes (Schachter & Shinitzky, 1977; Schachter, 1984) including rat colonic basolateral plasma membranes (Brasitus & Keresztes, 1984), rat enterocyte basolateral plasma membranes (Brasitus & Schachter, 1980; Schachter, 1984), and hepatic sinusoidal plasma membranes (Storch et al., 1983).

As also shown in Table I, the values of r for all three probes and r_∞ and S for DPH of the membrane preparations are approximately 20–30% higher than those for their liposome preparations. This indicates that both the static and dynamic components of fluidity are considerably greater in the liposomes than in the membranes, presumably secondary to the presence of proteins in the membrane preparations.

The effect of temperatures on the order parameter, S, of diphenylhexatriene in apical membranes is illustrated by the Arrhenius plot in Figure 1. A distinct break in the plots of these membranes and their liposomes (not shown) was seen at 25.7 ± 0.4 and 26.8 ± 0.6 °C, respectively (N = 3).

Table II: Composition of Neutral Lipids and Phospholipids of Tracheal Epithelial Cell Apical Membranes[a]

component	% of total lipid extract (w/w)
cholesterol esters	3.7 ± 0.5
triglycerides	3.7 ± 0.5
free fatty acids	16.3 ± 1.5
cholesterol	22.1 ± 2.2
phosphatidylethanolamine	16.5 ± 0.6
phosphatidylcholine	22.4 ± 1.5
sphingomyelin	14.3 ± 0.9
lysophosphatidylcholine	1.1 ± 0.5

[a] Values represent means ± SE for four separate preparations.

Table III: Composition of Total Fatty Acids of Tracheal Epithelial Cell Apical Membranes[a]

fatty acid	% by weight	fatty acid	% by weight
14:0	0.50 ± 0.15	20:0	0.59 ± 0.03
14:1	0.26 ± 0.06	20:1	2.15 ± 0.34
16:0	19.30 ± 1.20	20:2	1.18 ± 0.16
16:1	1.27 ± 0.15	20:4	8.65 ± 0.47
18:0	22.80 ± 0.15	24:0	8.22 ± 0.98
18:1	23.70 ± 0.36	22:6	0.25 ± 0.02
18:2	11.50 ± 0.37		

[a] Values are means ± SE for four separate preparations of apical membranes.

Compositional Studies. The neutral and phospholipid compositions of tracheal apical membranes were analyzed by thin-layer chromatography. Expressed as percent weight of total lipid (Table II), the major neutral lipids were cholesterol (22.1%) and free fatty acids (16.3%). The major phospholipids were phosphatidylcholine (22.4%), phosphatidylethanolamine (16.5%), and sphingomyelin (14.3%). Analysis of the total fatty acids of this membrane by GLC (Table III) revealed that the major fatty acids were oleic (18:1), stearic (18:0), and palmitic (16:0) acids (23.7, 22.8, and 19.3%, respectively).

As assessed by steady-state fluorescence polarization, the apical membranes appeared to possess a relatively low lipid fluidity. Prior studies in model bilayers and natural membrane have correlated lipid fluidity values with membrane lipid and protein composition (Chapman & Penkett, 1966; Oldfield & Chapman, 1971; Hubbell & McConnell, 1971; Shinitzky & Inbar, 1976; Shinitzky & Barenholz, 1978). It was therefore of interest to examine these parameters in this plasma membrane. Tracheal apical membranes possessed relatively high values for the molar ratios of cholesterol/phospholipid (0.83 ± 0.11) and sphingomyelin/lecithin (0.64 ± 0.10) (N = 4). The protein/lipid ratio (w/w) (0.66 ± 0.06) and saturation index (0.56 ± 0.02) (N = 4) were also high in this membrane (Schachter & Shinitzky, 1977; Brasitus & Keresztes, 1984). It would appear that, at least in part, these compositional parameters were responsible for the low fluidity of these membranes.

Osmotic Properties and Water Transport. The osmotic responsiveness of tracheal epithelial cell apical membrane vesicles was examined by measuring the scattered light intensity of vesicle suspensions of media varying in osmolarity (mannitol concentration was varied). A plot of the scattered light intensity vs. the reciprocal of the suspending medium osmolarity showed a nearly linear relationship (not shown). The increases in scattered light intensity (more negative values) seen at higher osmolarities indicated that as vesicles shrink in hyperosmotic medium, scattered light intensity increased. The nearly linear relationship between these two parameters

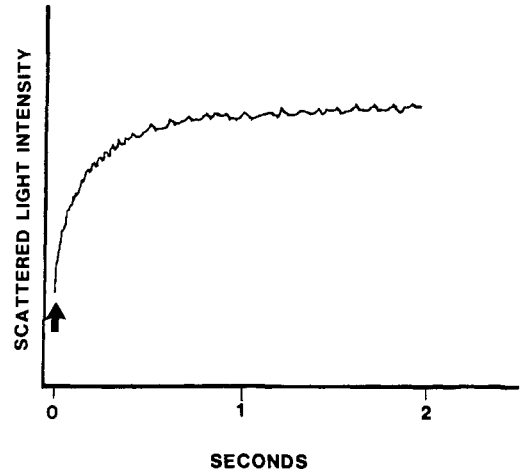

FIGURE 2: Change in scattered light intensity vs. time for tracheal apical membrane vesicles exposed to a hyperosmotic mannitol solution. Mixing occurred at the arrow. The intitial conditions were the following: 0.125 mg mL^{-1} membrane vesicle protein in reaction cuvette; intravesicular solution, 100 mM mannitol/1 mM Tris–Hepes (pH 7.4); extravesicular medium, 275 mM mannitol/1 mM Tris–Hepes (pH 7.4). Temperature was kept constant at 30 °C.

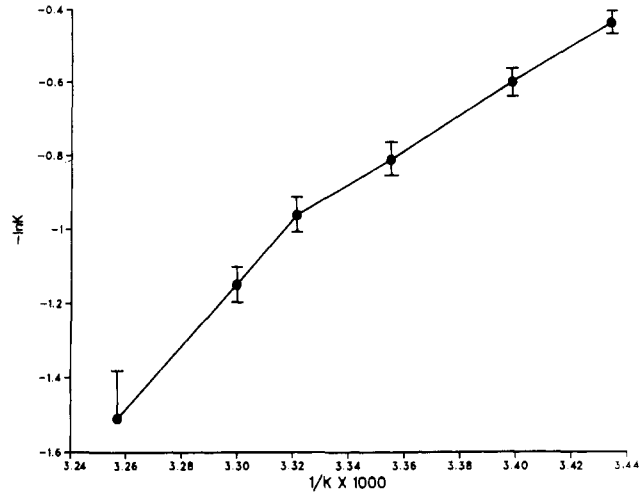

FIGURE 3: Arrhenius plot of rate constant for osmotic shrinkage of tracheal epithelial cell apical membrane vesicles. Conditions for experiments were as described in Figure 2 except temperature was varied as desired. Values in figure are means ± SE for four preparations. With each preparation, at least three trials were run at each temperature.

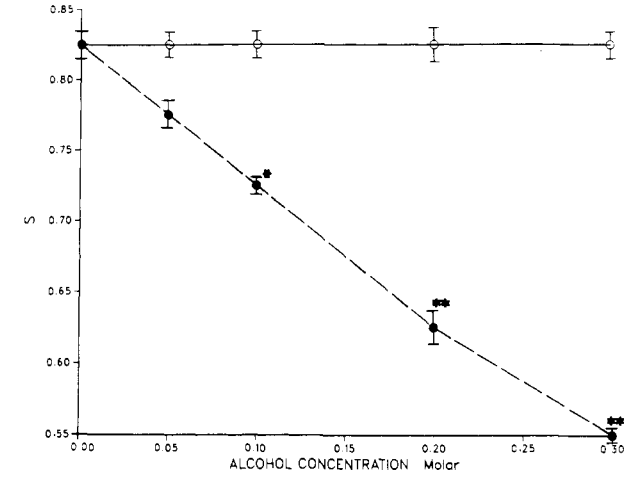

FIGURE 4: Effects of various concentrations of methyl alcohol (○) and benzyl alcohol (●) on tracheal epithelial cell apical membrane lipid order determined by DPH fluorescence polarization at 25 °C. Values are means ± SE for three preparations. (One asterisk) Different from 0 mM alcohol and corresponding dose of methyl alcohol at $p < 0.05$; (two asterisks) different from 0 mM alcohol and corresponding dose of methyl alcohol at $p < 0.01$.

also shows that scattered light intensity was a good indication of vesicle volume over the osmolarity range examined (i.e., negative scattered light intensity can be substituted for volume in the Boyle van't Hoff equation). In control experiments without membranes present, the contribution of various concentrations of mannitol to the scattered light intensity was negligible.

The time course of the change in vesicle volume, when exposed to hyperosmotic mannitol, was resolved with stopped-flow kinetics by measuring scattered light intensity vs. time immediately after mixing. Changes in volume vs. time for vesicles exposed to an osmotic gradient with a nonpermeating solute ($\sigma = 1$) can be related to the osmotic water permeability by

$$dv/dt = P_w A(\Delta P - \Delta\pi)$$

where dv/dt is the change in volume with time, P_w, is the osmotic permeability coefficient, A is the vesicle surface area, $\Delta\pi$ is the osmotic pressure difference, and ΔP is the hydrostatic pressure difference (Kedem & Katchalsky, 1958). Since scattered light intensity is a good indicator of vesicle volume of apical membrane vesicles, changes in scattered light intensity vs. time reflect dv/dt in the equation.

Figure 2 shows the change in scattered light intensity vs. time for tracheal epithelial apical membrane vesicles exposed to a mannitol concentration difference in 175 mOsm (higher concentration on the outside). Tracings such as those in Figure 2 could be fit fairly well to a single-exponential function, the exponential rate constant being proportional to the rate of volume change, dv/dt. These rate constants can therefore be considered as relative indicators of the osmotic permeability, P_w, when $\Delta P = 0$ at specific initial osmotic gradients and vesicle sizes.

Figure 3 shows an Arrhenius plot of the rate constants for osmotic shirnkage of tracheal apical membrane vesicles. This plot demonstrates a break at approximately 28 °C, i.e., within the vicinity of the break in the Arrhenius plots of lipid order (Figure 1). From the slopes of the Arrhenius plot, the activation energies of osmotic water flow can be determined. Different activation energies were obtained above (16.7 ± 0.2 kcal mol^{-1}) and below (8.3 ± 0.5 kcal mol^{-1}) 28 °C; at temperatures below 18 °C (not shown in Figure 3), the slope again changed, giving an activation energy of 2.9 ± 0.2 kcal mol^{-1} ($N = 4$) between 18 and 11 °C.

Effects of Benzyl Alcohol on Fluidity and Water Permeability. To further examine the relationship between water permeability and the lipid order of tracheal epithelial cell apical membranes, the lipid order (fluidity) was altered with the local anesthetic benzyl alcohol (Seeman, 1972). The decrease in lipid order caused by various concentrations of benzyl alcohol is shown in Figure 4. Methyl alcohol, however, did not alter lipid order at concentrations as high as 300 mM. Osmotic shrinkage was then examined in tracheal epithelial cell apical membranes exposed to various mixtures of the two alcohols. The total alcohol concentration (benzyl alcohol plus methyl alcohol) in all experiments was kept at 300 mM to ensure identical initial vesicle volumes and surface areas and thereby preclude factors other than the osmotic permeability, P_w, that might possibly contribute to different rate constants for osmotic shrinkage. Figure 5 shows the effect of increasing concentrations of benzyl alcohol on membrane water permeability. Compared to 300 mM methyl alcohol, membrane water

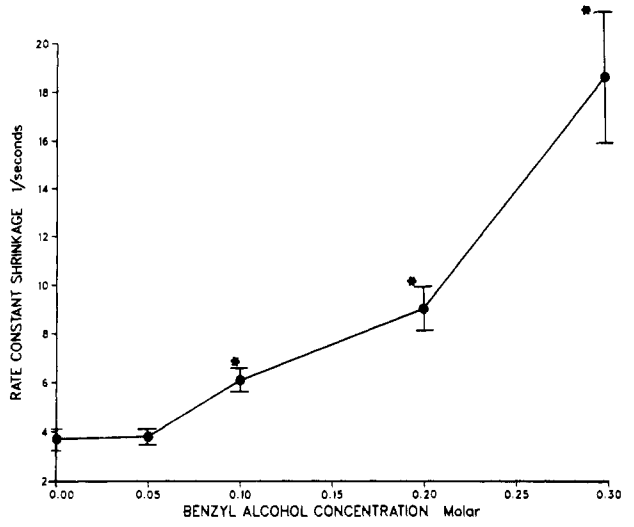

FIGURE 5: Effects of various alcohol mixtures on tracheal epithelial cell membrane water permeability at 25 °C. Values given are means ± SE for the rate constants of vesicles shrinkage for four preparations at 0 and 100 mM benzyl alcohol and for three preparations at 50, 200, and 300 mM benzyl alcohol. Total alcohol concentration (methyl plus benzyl) was always 300 mM. (Asterisk) Different from 0 mM benzyl alcohol at $p < 0.05$.

permeability began to increase at a benzyl alcohol concentration between 50 and 100 mM. As seen in Figure 4, the lipid order parameter also decreased over a similar range of concentrations of benzyl alcohol. These results demonstrate that the observed increases in water permeability correlate with decreases in lipid order.

Discussion

In the present report, steady-state fluorescence polarization studies utilizing the fluorophores DPH, 2-AS, and 12-AS demonstrated anisotropy values of 0.23 ± 0.01, 0.14 ± 0.01, and 0.15 ± 0.01, respectively, at 25 °C for tracheal epithelial cell apical membranes. These values are similar to those previously obtained for rat enterocyte (Brasitus et al., 1980; Brasitus & Schachter, 1980) and colonocyte (Brasitus & Keresztes, 1984) apical membranes and are among the highest values reported, to date, for plasma membranes studied by fluorescence polarization techniques (Schachter & Shinitzky, 1977). In this regard, high anisotropy values, i.e., low fluidities, have been correlated with high molar ratios of cholesterol/phospholipid and sphingomyelin/lecithin, high ratios of protein/lipid (w/w), and also a greater number of saturated acyl chains in phospholipids (Chapman & Penkett, 1966; Oldfield & Chapman, 1971; Hubbell & McConnell, 1971; Shinitzky & Inbar, 1976; Shinitzky & Barenholz, 1978). The relatively low fluidity of bovine tracheal apical membranes appears to result from high values for these parameters in this membrane. The present results also demonstrate that a lipid thermotropic transition can be detected at approximately 26 °C in these apical membranes and their liposomes by steady-state fluorescence polarization using the fluorophore diphenylhexatriene. Earlier studies have shown that this technique detects only the lower critical temperature of broad transitions observed by differential scanning calorimetry in rat enterocyte (Brasitus et al., 1980) and hepatocyte (Livingstone & Schachter, 1980) plasma membranes. In the present studies, the transition temperature detected in bovine tracheal apical membranes and liposomes, therefore, parobably represents the lower critical temperature of the transition.

The present experiments on water transport suggest that osmotic water flow across tracheal apical membranes most likely occurs by solubility–diffusion across the membrane lipid domain. Except for temperatures below 18 °C, aqueous pores do not appear to be a significant pathway for transmembrane water flow. Support for this contention comes from the values of the activation energies of transmembrane water flow, which are similar to those seen in lipid bilayers and liposomes where aqueous pores do not exit (Price & Thompson, 1969; Reeves & Dowben, 1970). These activation energies are consistent with the "solubility–diffusion model" of transmembrane water flow (Price & Thompson, 1969; Reeves & Dowben, 1970) in which water molecules must "dissolve" in the lipid bilayer and diffuse across it. When aqueous pores are present for transmembrane flow, the activation energy of the process is usually around 4 kcal mol^{-1}, which is similar to that for the diffusion and viscosity of bulk water (Wang et al., 1953). Activation energies in this range have been measured in mammalian red cells (Macey, 1984) and in liposomes (Boehler et al., 1978) and brush border membrane vesicles (Worman & Field, 1985) treated with gramicidin A, an antibiotic which forms transmembrane aqueous pores (Rosenberg & Finkelstein, 1978). We cannot, however, be certain as to why at temperatures below 18 °C the activation energy for water flow decreases to 3 kcal mol^{-1} in tracheal apical membrane vesicles. Perhaps at low temperatures the membrane lipids assume a highly ordered structure and become impermeable to water, and aqueous defects in the lipid bilayer or a small number of existing aqueous protein pores become the primary pathway for water flow.

If at physiological temperatures transmembrane water flow in tracheal apical membranes occurs by diffusion across the lipid bilayer, one would expect a possible relationship between lipid fluidity and water permeability. Several lines of evidence from the present and previous studies suggest that such a relationship, indeed, exists. First, as shown in the present studies, the break point seen in the Arrhenius plot at 28 °C for water efflux from tracheal apical membrane vesicles is in the viciinity of this membrane's lipid thermotropic transition temperature of 26 °C. Similar relationships between changes in lipid order at phase transition temperatures and water permeability have also been demonstrated in small intestinal brush border membrane vesicles (Worman & Field, 1985), synthetic liposomes (Boehler et al., 1978), and V-79 Chinese hamster lung fibroblasts (Rule et al., 1980). Second, prior studies in model planar bilayers and liposomes have also suggested a relationship between water permeability and fluidity. Cholesterol significantly decreases water permeability above phase transition temperatures of planar lipid bilayers (Finkelstein & Cass, 1967) and liposomes (Bittman & Blau, 1972; Jain et al., 1973) and increases it below the transition temperatures (Bittman & Blau, 1972). These observations are consistent with cholesterol's known effects on lipid order (Chapman & Penkett, 1966). Additionally, an increase in the ratio of unsaturated to saturated fatty acyl chains in phospholipid liposomes (Bittman & Blau, 1972) as well as treatment of liposomes with lipid-soluble alkanols (Jain et al., 1973) has been shown to increase water permeability, also consistent with the effects of these manipulations on lipid order (Seeman, 1972; Shinitzky & Barenholz, 1978). Third, the relationship between membrane lipid order and water permeability in tracheal membranes is strongly supported by the present experiments using benzyl alcohol to alter lipid order. Anesthetic alcohols such as benzyl alcohol are known to decrease the lipid order of model and biological membranes (Seeman, 1972; Colley & Metcalfe, 1972). The ability of alcohols to decrease lipid order of biological membranes correlates well with their

lipid/water partition coefficient (Fernadez et al., 1984), hence the lack of effect of methyl alcohol on apical membrane fluidity. Finally, prior studies by Borochov and Borochov (1979) which demonstrated an increase in membrane fluidity in liposomes and plant protoplasts upon osmotic swelling also lend theoretical support to the correlation between membrane fluidity and water permeability. The data in the present study demonstrate that as the lipid order of tracheal epithelial cell apical membranes is decreased with benzyl alcohol, membrane water permeability increases.

In contrast to the relationship between lipid order and water permeability in model membranes and tracheal apical membranes are the observations reported for mammalian red blood cells. Treatment of mammalian red cells with anesthetic *n*-alkanols decreases lipid order but also decreases water permeability (Kutchai et al., 1980). Furthermore, membrane cholesterol content does not appear to have a profound effect on mammalian red cell water permeability (Kutchai et al., 1980; Sha'afi et al., 1969). These findings are consistent with the hypothesis that mammalian red cell membranes have aqueous pores for transmembrane water flow, whereas the tracheal apical membranes do not. The state of the membrane lipids may not greatly affect red cell water permeability since water bypasses the lipid domain by flow through aqueous transmembrane protein pores. The inhibitory action of *n*-alkanols on red cell water permeability might actually be caused by denaturation of the protein pores, which would exert an overall inhibitory effect on water permeability, similar to that seen with sulfhydryl-reactive agents (Macey, 1984). In tracheal apical membranes, where aqueous pores are apparently absent, anesthetic alcohols increase lipid fluidity and cause an increase in water permeability similar to that observed in synthetic liposomes (Jain et al., 1973). Thus, the findings in the present study suggest that lipid fluidity and lipid composition may be important determinants of membrane water permeability in cells that do not contain transmembrane aqueous pores.

ACKNOWLEDGMENTS

We are indebted to Dr. Edwin W. Taylor for the use of the stopped-flow apparatus. We thank Jiyong Ahn and Kimberli Coleman for technical assistance and Dolores Gordon for secretarial support.

Registry No. 14:0, 544-63-8; 14:1, 28039-98-7; 16:0, 57-10-3; 16:1, 28039-99-8; 18:0, 57-11-4; 18:1, 112-80-1; cholesterol, 57-88-5; methyl alcohol, 67-56-1; benzyl alcohol, 100-51-6; water, 7732-18-5.

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Reconstitution of Membrane Proteins: Catalysis by Cholesterol of Insertion of Integral Membrane Proteins into Preformed Lipid Bilayers†

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Received August 13, 1985

ABSTRACT: The presence of cholesterol in small unilamellar vesicles (ULV) of dimyristoylphosphatidylcholine (DMPC) catalyzes fusion of the vesicles at temperatures below the upper limit for the gel to liquid-crystalline phase transition of the DMPC. The extent to which ULV grow depends on the concentration of cholesterol in the vesicles and on temperature. Maximum growth occurs at 21 °C. It decreases as the temperature is lowered below 21 °C. Growth does not occur at temperatures above the phase transition. In addition, the presence of cholesterol in ULV of DMPC catalyzes the insertion of integral membrane proteins into the vesicles. Thus, bacteriorhodopsin from *Halobacterium halobrium*, UDPglucuronosyltransferase (EC 2.4.1.17) from pig liver microsomes, and cytochrome oxidase from beef heart mitochondria formed stable lipid–protein complexes spontaneously when added to ULV containing cholesterol at temperatures under which these vesicles would fuse. Incorporation of these proteins into the ULV of DMPC did not occur in the absence of cholesterol or in the presence of cholesterol when the temperature of the system was above that for the phase transition. It appears that cholesterol lowers the energy barrier for fusion of ULV of DMPC and for insertion of integral membrane proteins into these bilayers. Studies with bacteriorhodopsin suggest that the energy barrier for insertion of proteins into ULV containing cholesterol is smaller than the energy barrier for fusion of the ULV with each other.

We have described recently a simple method for reconstituting pure integral membrane proteins into preformed unilamellar bilayers (ULV)[1] of phosphatidylcholine (Scotto & Zakim, 1985). Microsomal UDPglucuronosyltransferase, mitochondrial cytochrome oxidase, and bacteriorhodopsin were incorporated into preformed ULV of dimyristoylphosphatidylcholine (DMPC), for example, when relatively small amounts of myristate (as little as 0.1 mol %) were present in the ULV and the temperature of the system was below that for the gel to liquid-crystalline phase transition. The importance of myristate in the bilayers of DMPC was its promotion of fusion of the ULV (Kantor & Prestegard, 1975), although incorporation of proteins into the ULV appeared to be an event separate from fusion of the ULV (Scotto & Zakim, 1985). We concluded that conditions for fusion of ULV also allowed for the facile insertion into bilayers of large, integral membrane proteins independently of fusion of the ULV. These obser-

† This work was supported by a grant from the National Science Foundation (PCM 8311967) and in part by a grant from the National Institutes of Health (BRSG S07 RR05396).

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[1] Abbreviations: ULV, unilamellar vesicles or bilayers; DMPC, dimyristoylphosphatidylcholine; UDP, uridine 5′-diphosphate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; TCA, trichloroacetic acid.

0006-2960/86/0425-1555$01.50/0