Effect of Cholesterol Oxidase Treatment on Physical State of Renal Brush Border Membranes: Evidence for a Cholesterol Pool Interacting Weakly with Membrane Lipids

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ABSTRACT: Treatment with cholesterol oxidases has shown that cholesterol is heterogeneously distributed in brush borders isolated from the apical membrane domain of the renal and intestinal epithelial cells [Bloj, B., & Zilversmit, D. B. (1982) *J. Biol. Chem. 257*, 7608–7614; El Yandouzi, E. H., & Le Grimellec, C. (1992) *Biochemistry 31*, 547–551]. To better understand the origin of cholesterol heterogeneity, the effects of cholesterol oxidation by *Brevibacterium* sp. cholesterol oxidase on the physical state of renal brush border membrane vesicles were determined using steady-state fluorescence polarization and differential phase fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH). Selective quenching by trinitrobenzenesulfonate indicated that DPH distributes equally between outer and inner membrane leaflets. Oxidation of 90% of the cholesterol decreased the steady-state anisotropy of DPH, determined at 37 °C, by 14%. This modification corresponded to a change in the lipid order, the rotational rate of the probe being unaffected. Oxidation of the cholesterol corresponding to the readily accessible pool (30% of the total cholesterol), on the other hand, had a very limited effect on the membrane physical state. This contrasted with the linear decrease in both anisotropy and fluorescence lifetime of DPH obtained when cholesterol was replaced by cholestenone in liposomes made of phosphatidylcholine/sterol (1/1 molar ratio). These results indicate that, in brush border membranes, the cholesterol readily accessible to cholesterol oxidase interacts only weakly with the other membrane lipids.

Fully polarized epithelial cells such as those found in the kidney and in the intestine have apical and basolateral plasma membrane macrodomains whose lipid composition and physical state markedly differ (Brasitus & Schachter, 1980; Le Grimellec et al., 1982; Carmel et al., 1985). These differences could play an important role in the control of the vectorial functions of epithelial cells (Le Grimellec et al., 1988a; Brasitus & Dudeja, 1988). Cholesterol is among the lipids involved in the polarity of the plasma membrane. Under various physiological and experimental conditions, the cholesterol content of the apical domain was reported to be regulated independently of the content of the basolateral domain (Brasitus & Schachter, 1982; Molitoris et al., 1985). Moreover, in the kidney, the cholesterol content of the basolateral domain is significantly lower than that of the apical membrane (Molitoris et al., 1985). This raises the controversial question of the existence of various cholesterol pools in the plasma membrane of eucaryotic cells (Yeagle, 1985; Van Meer, 1987; Schroeder et al., 1991; Lange, 1992). In both intestinal and renal cells, isolation of the apical plasma membrane domain gives closed vesicles [brush border membrane vesicles (BBM)[1]], right side oriented (Haase et al., 1978; Vénien & Le Grimellec, 1988). Under control conditions, cholesterol distribution in renal and intestinal BBM was shown to be heterogeneous, with one pool rapidly exchangeable or accessible to cholesterol oxidases, which accounted for 15–30% of the total cholesterol (Bloj & Zilversmit, 1982; El Yandouzi & Le Grimellec, 1992). This suggests that the heterogeneity of cholesterol distribution might be a common feature of the apical membrane of epithelial cells. The origin(s) of such an heterogeneity, however, remains poorly understood.

Cholesterol oxidases, a family of enzymes oxidizing cholesterol to give cholest-4-en-3-one (cholestenone), have been frequently used to probe the distribution of cholesterol in plasma membranes and in whole cells (Moore et al., 1977; Lange & Ramos, 1983; Slotte et al., 1989). Data from model systems have shown that the phospholipid-ordering properties of cholesterol and cholestenone differ (Vincent & Gallay, 1983; Ben-Yashar & Barenholz, 1989). Because, in most experiments, membranes were fixed with glutaraldehyde or treated with phospholipases prior to cholesterol oxidase addition [see Lange (1992)], the modifications in membrane physical state that accompany cholesterol oxidase treatment of biological membranes remain poorly documented. We hypothesize that knowledge of these modifications in a system such as renal BBM, for which no fixation is required for cholesterol oxidase attack and whose tightness and orientation are maintained during the treatment (El Yandouzi & Le Grimellec, 1992), may help in the understanding of the existence of cholesterol pools in biological membranes.

In the present experiments, variations in the fluorescence polarization, lifetime, and differential tangent (Lakowicz et al., 1979) of 1,6-diphenyl-1,3,5-hexatriene (DPH) were used to follow the effects on the physical state of renal BBM of the *Brevibacterium* sp. enzyme, which oxidizes more than 90% of the cholesterol according to a biphasic process.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol oxidase (E.C. 1.1.3.6) from *Brevibacterium* sp. was obtained from Beckman Instruments (Nyon, Switzerland). DPH was purchased from Molecular Probes

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; TNBS, trinitrobenzenesulfonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; BBM, brush border membrane vesicles.

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(Junction City, OR). Color-free 2,4,6-trinitrobenzenesulfonate (TNBS) was obtained from Eastman Kodak. All other reagents were of analytical grade.

Membrane Preparation. Brush border membrane vesicles (BBM) from the kidney cortex of male New Zealand white rabbits (2–2.5 kg body weight) were isolated as previously described (Le Grimellec et al., 1982), using an $MgCl_2$ precipitation method (Booth & Kenny, 1974), which gave right-side-out sealed vesicles (Vénien et al., 1988). Membranes were suspended (4 mg of protein/mL) in 150 mM NaCl/20 mM Hepes (pH 7.4) and either used the same day or kept at −80 °C until used (within 1 week). In BBM preparations, the activity of alkaline phosphatase was enriched 13-fold over that of cortical homogenate, compared to less than 0.7-fold for Na^+/K^+-ATPase and less than 0.5-fold for glucose-6-phosphatase.

Treatment of BBM with Cholesterol Oxidase. The day of the experiment, 1 volume of the BBM preparation was added to 5 volumes of a 50 mM mannitol/2 mM Tris (pH 7.0) solution (final NaCl concentration 25 mM). After a 10-min preincubation at 37 °C, cholesterol oxidase was added to a final concentration of 1 IU/mL. After various times of incubation (1–240 min) at 37 °C, two aliquots (200 μg of protein) were taken. The reaction was stopped by adding 12 mL of ice-cooled saline solution (0.9% NaCl) (Lange et al., 1984). BBM were collected by centrifugation (20 min, 40000*g*, 5 °C), washed once using the same procedure and suspended in 0.9% NaCl. Aliquots were taken for lipid extraction, fluorescence experiments, and protein determination.

Lipid Analysis. Lipids were extracted by using the method of Bligh and Dyer (1959). Extraction was repeated three times to obtain a maximum recovery (Carmel et al., 1985). Extracts were pooled, dried under nitrogen, and solubilized in a chloroform/methanol mixture (2:1 v/v). Separation of neutral lipids was performed by thin-layer chromatography on precoated silica gel plates (Merck), using hexane/diethyl ether/acetic acid (130:30:1.5 v/v/v) as developing solvent. After brief exposure to iodine vapor, the spots were identified by comparison with authentic standards, and lipids were extracted from silica by addition of 12 mL of chloroform/methanol (1:1 v/v). The solvent was evaporated under nitrogen and the lipids were solubilized in 1 mL of 2-propanol. The amount of cholesterol oxidized, corrected for the recovery of extraction, was determined by measuring the remaining cholesterol (Omodéo-Salé et al., 1984).

Fluorescence Experiments. Labeling with DPH of control and cholesterol oxidase-treated vesicles was achieved as previously described, using 90-min incubations at 37 °C to obtain maximum intensity (Le Grimellec et al., 1982). Final concentrations of fluorophore and BBM protein were 0.5 μM and 75 μg/mL, respectively.

Fluorescence intensity, fluorescence polarization, and lifetime measurements were performed on a SLM 4800 S spectrofluorometer as previously described (Le Grimellec et al., 1988b). Briefly, the probe was excited at 362 nm while the emission was measured at 430 nm. Light scattering was reduced to very low levels (less than 1%) by the use of cutoff filters. Steady-state fluorescence anisotropy, r_{ss}, was determined according to

$$r_{ss} = (I_{\parallel} - I_{\perp}G)/(I_{\parallel} + 2I_{\perp}G)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed with the analyzing polarizer parallel and perpendicular to the polarized vertical excitation beam. G, the g-factor, was used to correct for the unequal transmission of differently polarized light.

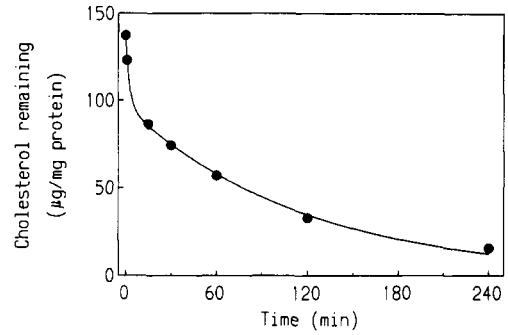

FIGURE 1: Oxidation of cholesterol in renal brush border membranes by *Brevibacterium* sp. cholesterol oxidase. Representative experiment showing the time-dependent effect of 1 IU/mL cholesterol oxidase on the cholesterol content of unfixed BBM.

Fluorescence lifetimes were measured by the phase and modulation technique (Spencer & Weber, 1970). Both excitation and emission polarizers were left in place with the excitation polarizer vertically aligned and the emission polarizer at 54° from the vertical. Apparent lifetimes were determined at 30 and 18 MHz using DPH in heptane as isochronal reference standard (Barrow & Lentz, 1983). Heterogeneity analysis was performed using the software provided by the SLM Company. Unless specified, the measurements were performed at 37 °C.

Differential polarized phase fluorometry (Lakowicz et al., 1979) was used to determine the rotational rate (R) and limiting anisotropy r_∞ [$r_\infty = r_{ss} + (r_{ss} - r_0)/6R\tau$] of DPH. Differential tangent (tan Δ) was measured at 30 MHz. An r_0 value of 0.390 was used for DPH (Lakowicz et al., 1979).

Liposomes. Multilamellar liposomes used for steady-state anisotropy, and lifetime determinations were formed as previously described (Le Grimellec et al., 1982) by adding double-distilled water at 60 °C to a dry film constituted of dipalmitoylphosphatidylcholine (DPPC), sterol (cholesterol and/or cholestenone), and fluorescent probe (molar ratio 1/1/0.002). Several cycles of vortexing in the presence of glass beads provided homogeneous suspensions.

Labeling by TNBS. Chemical labeling of amino groups of control and cholesterol oxidase-treated BBM was done as previously described (El Tandouzi & Le Grimellec, 1992), using neutralized color-free TNBS. Unreacted TNBS was eliminated by centrifugation and washing.

Enzyme and Protein Determination. Activities of the marker enzymes were determined as previously described (Le Grimellec et al., 1982). Protein concentration was determined by the method of Lowry et al., (1951) after precipitation with 10% trichloroacetic acid, using bovine serum albumin as a standard.

RESULTS

Oxidation of Cholesterol in BBM. As previously reported (El Yandouzi & Le Grimellec, 1992), addition of cholesterol oxidase to native, right-side-out renal brush border membrane vesicles results in a biphasic decrease in membrane cholesterol such as, after a 4-h treatment, 90% of the cholesterol is oxidized (Figure 1). For these preparations, whose membrane cholesterol concentration was in the usual range (139 ± 4 μg/mg of protein; cholesterol/phospholipid ratio 0.61), curve fitting by the sum of two exponentials (goodness of fit $R^2 = 0.999$) followed by kinetic analysis using the model of Bloj and Zilversmit (1976) confirmed the presence of a readily

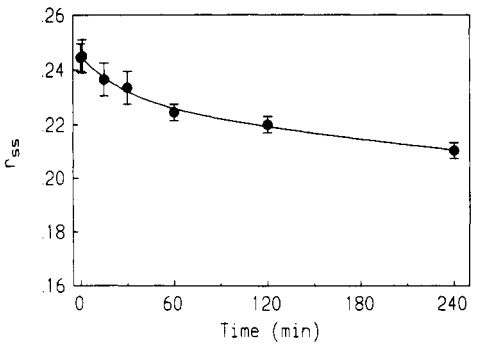

FIGURE 2: Effect of cholesterol oxidase treatment on steady-state anisotropy of DPH in BBM. After various times of incubation with 1 IU/mL cholesterol oxidase, aliquots of the BBM suspension were taken, washed, and labeled with 0.5 μM DPH as described under Experimental Procedures. Steady-state anisotropy was determined at 37 °C. Data are presented as mean ± SD of three different BBM preparations.

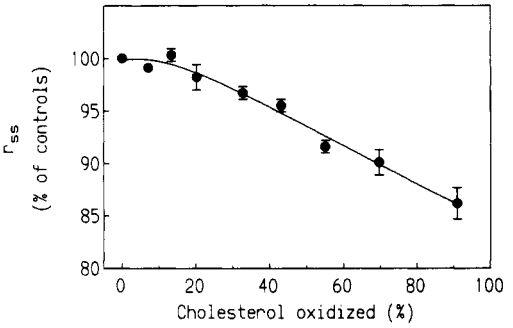

FIGURE 3: Relationship between DPH steady-state anisotropy and the fraction of oxidized cholesterol in BBM. The fraction of cholesterol oxidized in BBM samples was determined from the amount of remaining cholesterol.

accessible cholesterol pool that accounts for 30% ± 3% of the total cholesterol. The rate constant of cholesterol oxidation (K_0) was 40 ± 12 h^{-1}, an order of magnitude higher than that of cholesterol movement from the low to the readily accessible pool (K_{ab} = 0.5 ± 0.07 h^{-1}) and from the readily to the less accessible pool (K_{ba} = 1.24 ± 0.22 h^{-1}; $t_{1/2}$ = 24 min).

Localization of DPH in BBM. Trinitrophenylation of the primary amino groups exposed on the external leaflet of membranes results in a selective quenching of DPH in this leaflet [see Schroeder (1988)]. The fluorescence intensity of DPH in brush border membranes prelabeled by TNBS under nonpermeant conditions (4 °C) was half (2.91 ± 0.08 units of fluorescence/mg of protein) that of controls (5.93 ± 0.04 units/mg of protein). This value was not significantly modified by a 4-h cholesterol oxidase treatment. On the other hand, incubation at 37 °C resulted in a nearly total (94%) quenching of the fluorescence (0.38 ± 0.05 unit/mg of protein).

Steady-State Anisotropy of Cholesterol Oxidase-Treated Membranes. In control brush border membranes, steady-state anisotropy values, determined at 37 °C, were r_{ss} = 0.245 ± 0.007 for DPH. This value was not significantly modified by a 4-h incubation at 37 °C. As shown by Figure 2, addition of cholesterol oxidase to the vesicles resulted in a time-dependent decrease in fluorescence anisotropy of the fluorophore. Oxidation of 90% of membrane cholesterol resulted in a 14% drop in fluorescence anisotropy of DPH (r_{ss} = 0.211 ± 0.005). The effect of cholesterol oxidation on fluorescence anisotropy was not linear (Figure 3): oxidation of up to 30% of membrane cholesterol had only a limited effect on anisotropy (r_{ss} = 0.237 ± 0.006). This contrasted with the data obtained from DPPC/cholesterol/cholestenone (phospholipid/sterol molar ratio 1/1) liposomes where the percentage of cholestenone was varied (Figure 4). It is noteworthy that cholestenone exerted a significant ordering effect for DPPC in the liquid crystal phase with r_{ss} = 0.074 for pure DPPC as compared to r_{ss} = 0.189 for DPPC/cholestenone (1/1) at 48 °C.

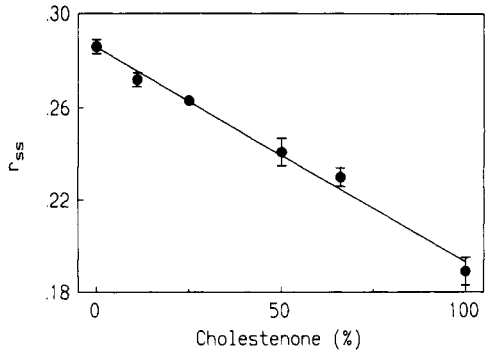

FIGURE 4: Effect of cholesterol replacement by cholestenone on steady-state anisotropy of DPH in dipalmitoylphosphatidylcholine/cholesterol liposomes. Anisotropy of multilamellar vesicles constituted of DPPC, cholesterol and/or cholestenone, and DPH (molar ratio 1/1/0.002) was determined at 48 °C. Data are from three series of experiments.

Fluorescence Lifetimes and Dynamic Depolarization. In accordance with literature (Dale et al., 1977; Parasassi et al., 1984; Fiorini et al., 1987; Lentz, 1989), determination of DPH fluorescence lifetimes at 30 and 18 MHz revealed the presence of a small fraction of a short-lifetime component in controls (Table I). Treatment of renal brush border membranes with cholesterol oxidase resulted in a time-dependent biphasic decrease in the apparent fluorescence lifetimes of DPH (Figure 5). Heterogeneity analysis for 50% and 70% cholesterol oxidation indicated that this decrease was essentially attributable to a decrease in the long-lifetime component. Curve fitting by nonlinear regression confirmed that the decrease in lifetime was best described (R^2 = 0.998) by the sum of two exponentials and a constant, $Y = 0.93 \exp(-55X) + 2.47 \exp(-0.68X) + 6.13$. Entering these data in the two-pools kinetic model (Bloj & Zilversmit, 1976) led to values of the cholesterol-accessible pool (29% ± 3%) and rate constants close to those obtained by chemical determination (K_0 = 53 ± 16, K_{ab} = 0.51 ± 0.12, K_{ba} = 1.18 ± 0.25 h^{-1}; $t_{1/2}$ = 25 min). In accordance with this analysis, plot of DPH lifetime against the fraction of cholesterol oxidized in brush border membranes gave a linear relationship (Figure 6). A comparable linear relationship was observed, at a temperature higher than the phase transition, for multilamellar liposomes made of DPPC and cholesterol/cholestenone in varying amounts (Figure 7).

Limiting anisotropy, rotational rate, and order parameter of DPH calculated from the dynamic depolarization experiments on brush border membranes are presented in Table II. Limiting anisotropy decreased from 0.230 in controls to 0.182 when 90% of the cholesterol was oxidized. Again, oxidation of cholesterol up to 30% had a limited effect on r_∞. On the other hand, the rotational rates were not significantly affected by cholesterol oxidation.

DISCUSSION

The present experiments demonstrate that oxidation of brush border membrane cholesterol has a significant but limited effect on their physical state at 37 °C. Variations in DPH steady-state anisotropy and fluorescence lifetime as a function of the fraction of cholesterol oxidized confirmed the existence of a heterogeneous distribution of cholesterol in these membranes. They indicate that the pool readily accessible to the

Table I: Fluorescence Lifetime of DPH in Control and Cholesterol Oxidase-Treated BBM[a]

cholesterol oxidized (%)	lifetime (ns)							
	30 MHz		18 MHz					
	τ_p	τ_m	τ_p	τ_m	τ_1	α_1	τ_2	α_2
0	8.36 ± 0.27	9.58 ● 0.14	8.79 ± 0.27	9.81 ± 0.08	9.34	0.94	1.11	0.06
49 ● 2	6.50 ± 0.54	7.60 ± 0.35	6.90 ± 0.47	7.85 ± 0.31	7.59	0.91	1.38	0.09
70 ± 6	5.61 ● 0.29	6.82 ± 0.27	6.03 ± 0.26	7.08 ± 0.24	6.90	0.88	1.14	0.12

[a] Mean values ± SE of four preparations are given. Measurements were performed at 37 °C.

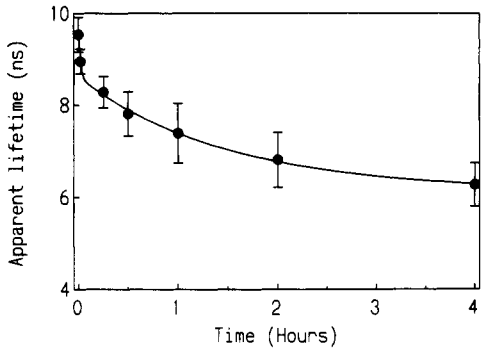

FIGURE 5: Effect of cholesterol oxidase treatment on DPH fluorescence lifetime in BBM. Experimental conditions are those described in Figure 2. Lifetimes by modulation (τ_m) for a modulation frequency of 30 MHz are illustrated. Curve fitting by nonlinear regression indicated that lifetime was best described by the sum of two exponentials plus a constant [$y = 0.93 \exp(-55x) + 2.47 \times \exp(-0.68x) + 6.13$] (goodness of fit $R^2 = 0.998$).

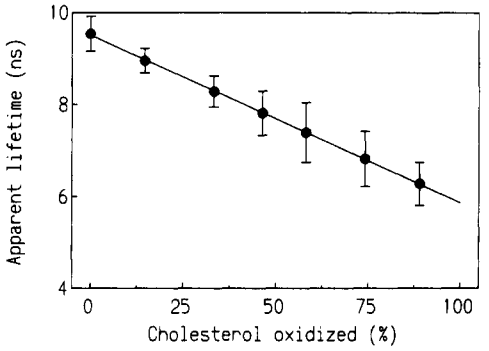

FIGURE 6: Relationship between DPH lifetime and cholesterol oxidation in BBM. Apparent lifetimes (τ_m, 30 MHz) are plotted against the corresponding percentages of BBM cholesterol oxidized.

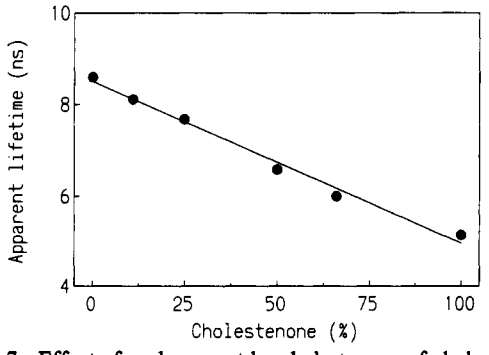

FIGURE 7: Effect of replacement by cholestenone of cholesterol on apparent lifetime of DPH in DPPC/cholesterol liposomes. Apparent lifetime (τ_m, 30 MHz) of liposomes made as in Figure 4 were determined at 48 °C.

oxidase is constituted by cholesterol interacting only weakly with the other membrane lipids.

As previously reported (Le Grimellec et al., 1982; Molitoris et al., 1985; Levi et al., 1989), high values of DPH steady-state anisotropy were measured in brush border membranes

Table II: Limiting Anisotropy, Rotational Rate, and Order Parameter of DPH in Control and Cholesterol Oxidase-Treated BBM[a]

cholesterol oxidized (%)	r_{ss}	r_∞	S^b	R (10^8 s)
0	0.245 ● 0.10	0.230 ± 0.013	0.77 ± 0.03	1.71 ± 0.32
13.4	0.245 ± 0.011	0.229 ± 0.013	0.76 ± 0.02	1.71 ± 0.33
32.8	0.237 ± 0.011	0.220 ± 0.012	0.75 ± 0.02	1.81 ± 0.34
43.1	0.234 ± 0.012	0.214 ± 0.013	0.74 ± 0.02	1.70 ± 0.30
55.0	0.225 ± 0.008[c]	0.205 ● 0.007[c]	0.72 ± 0.01[c]	1.91 ± 0.27
69.6	0.220 ± 0.005[c]	0.197 ± 0.006[c]	0.71 ± 0.01[c]	1.83 ± 0.23
90.9	0.211 ± 0.004[c]	0.182 ± 0.008[c]	0.68 ± 0.01[c]	1.66 ± 0.15

[a] Results are means ● SD of three experiments on different membrane preparations. [b] S $(r_\infty/r_0)^{1/2}$. [c] $P < 0.05$ when compared to controls (0% oxidation).

at 37 °C. Results of dynamic depolarization led to an order parameter of 0.77, which confirmed that membrane lipids are highly ordered in renal brush borders under physiological conditions. Upon cholesterol oxidase treatment, we observed (a) a decrease in DPH steady-state anisotropy and (b) that the decrease in anisotropy obtained for 90% cholesterol oxidation was less than that resulting from omission of cholesterol in liposomes made of brush border membrane lipids (Carmel et al., 1985; Molitoris & Hoilen, 1987). Also, anisotropy of renal brush borders treated by cholesterol oxidase for 4 h remained higher than that of the corresponding basolateral membrane (Le Grimellec et al., 1982; Molitoris & Hoilen, 1987). Previous studies on liposomes have shown that, although less pronounced than that exerted by corresponding amounts of cholesterol, the ordering effect of cholestenone on membrane lipids is still highly significant (Vincent & Gallay, 1983; Ben-Yashar & Barenholz, 1989). In accordance with these experiments, we observed that for DPPC multilamellar vesicles at 48 °C, i.e., above the phase transition temperature, addition of cholestenone (DPPC/sterol 1:1 molar ratio) raised the anisotropy value from 0.074 to 0.189, as compared to 0.286 when cholesterol was used. These values are very close to those reported for small unilamellar vesicles of similar composition (Vincent & Gallay, 1983). Accordingly, this cholestenone ordering effect can, at least partly, account for the limited effect of cholesterol oxidation on the physical state of BBM. This might be correlated with the observation that oxidation of 90% of brush border cholesterol does not affect the integrity of the vesicles (El Yandouzi & Le Grimellec, 1992). Steady-state anisotropy data also indicated that the relationship between cholesterol oxidation and decrease in anisotropy was not linear in renal BBM: oxidation of up to 30% of membrane cholesterol, which corresponded to the readily accessible pool, affected only marginally r_{ss}. On the other hand, r_{ss} decreased linearly as a function of cholestenone concentration when cholesterol was replaced by cholestenone in DPPC/sterol (1:1 molar ratio) liposomes. Taken together, these data strongly suggested that, in the BBM pool readily accessible to cholesterol oxidase, cholesterol interacts weakly with the other membrane constituents. By using different cholesterol oxidases, we previously

proposed that, in the pool readily accessible, cholesterol was interacting only weakly with some lipid species (El Yandouzi & Le Grimellec, 1992) such as phosphatidylethanolamine, whose cholesterol ordering susceptibility is low (van Blitterswijk et al., 1987). Alternatively, data from several laboratories have suggested the existence of cholesterol/cholesterol interactions in liposomes (Rogers et al., 1979; Smutzer & Yeagle, 1985; Nemecz & Schroeder, 1988; Mouritsen, 1991). Evidence for clustering of cholesterol in biological plasma membranes was previously reported (van Blitterswijck et al., 1982; Gordon et al., 1983; Gordon & Mobley, 1984). Existence of cholesterol "enriched" domains where cholesterol/cholesterol interactions are predominant might also lead to the same small effect of oxidation on DPH anisotropy.

Determination of fluorescence lifetime showed that cholesterol oxidase treatment resulted in a time-dependent biphasic decrease in the long-lifetime component of DPH, whose kinetic parameters were similar to those obtained when considering the amount of cholesterol oxidized in the membrane. This observation was explained by the linear relationship that linked the DPH lifetime and the fraction of oxidized cholesterol in the brush borders. Despite this linear relationship, precision of the measurements did not allow resolution of this long lifetime into a sum of components corresponding to the respective contributions of cholesterol/DPH and cholestenone/DPH environments. Lifetime determinations using three modulation frequencies (30, 18, and 6 MHz) failed to improve the resolution (not shown). Precision of the measurements was comparable to that reported for biological membranes when using a limited-frequency phase modulation dynamic spectrofluorometer (Klausner et al., 1980; Verkman & Ives, 1986; Dudeja et al., 1991). A similar linear relationship between lifetime and cholestenone concentration was also obtained for DPPC/sterol liposomes. Such results are consistent with the decrease in DPH lifetime observed when cholesterol was replaced by cholestenone in DPPC/sterol (1:1) liposomes (Vincent & Gallay, 1983). This decrease likely occurs via a modification of the dielectric constant of the probe environment (Zannoni et al., 1983), related to the increase in water penetrability into the bilayer which should result from the increase in the area occupied by the cholestenone molecule (Gallay & DeKruijff, 1982; Slotte, 1992). Additionally, in contrast with cholesterol, cholestenone seems to be randomly distributed in the bilayer plane (Ben-Yashar & Barenholz, 1989). Accordingly, lifetime experiments also demonstrated the heterogeneity of cholesterol distribution in BBM, with the presence of a readily accessible pool accounting for 30% of total membrane cholesterol.

Determination of fluorophore localization by the TNBS technique [see Schroeder (1988)], revealed that, as in other biological membranes including intestinal brush border membranes (Schroeder, 1988; Dudeja et al., 1991), DPH distributes equally between the two leaflet of renal brush border membranes. Accordingly, the changes (δ) in anisotropy or lifetime recorded during the experiments corresponded to $0.5\delta_{ol} + 0.5\delta_{il}$ where δ_{ol} and δ_{il} represent the variation in the outer and inner leaflet, respectively, which precluded any localization of the variation.

Data from dynamic depolarization experiments further indicated that oxidation of cholesterol to cholestenone only affected the limiting anisotropy of DPH, leaving the rotational rate unchanged. Contradictory data were reported about the effect of cholesterol on rotational dynamics of DPH or DPH derivatives in phosphatidylcholine/cholesterol liposomes (Veatch & Styer, 1977; Kinosita & Ikegami, 1984; Straume & Litman, 1987; Yeagle et al., 1990). However, even in lipid systems where substantial cholesterol-dependent acceleration of DPH rotational dynamics was observed, the presence of a small amount (1%) of an intrinsic protein abolished the cholesterol effect (Straume & Litman, 1988). Accordingly, both the fact that cholesterol is not simply withdrawn but replaced by a sterol still acting on the physical state of membrane lipids and the fact that brush border membranes contain large amounts of protein (Carmel et al., 1985; Molitoris et al., 1985), could have limited the effect of cholesterol oxidation on DPH rotational rate. Different empirical relations have been proposed to deduce the limiting hindered anisotropy from r_{ss} (Jähnig, 1979; Fulford & Peel, 1980; Heyn, 1979; van Blitterswijk et al., 1981; Van der Meer et al., 1986). For the limited range of anisotropy variation obtained in our experiments, a linear relation between the two parameters was obtained ($r_\infty = 1.38r_{ss} - 0.108$; correlation coefficient = 0.997). This relation is very similar to the one proposed by Van Blitterswijk et al. (1981). Moreover, the calculations, for control BBM, of the hindered limiting anisotropy using the various methods falls within 5% of the value determined from differential tangent. Our data therefore reinforce the view that, in plasma membranes, DPH steady-state anisotropy essentially reflects the structural order of membrane lipids (Van Blitterswijk et al., 1981; Kinosita et al., 1984; Stubbs & Smith, 1984; Van der Meer et al., 1986). Again, oxidation of the readily accessible cholesterol pool had significantly less effect, per cholesterol oxidized, on r_∞ than oxidation of cholesterol from the slowly accessible pool.

In conclusion, these data demonstrate that, in renal brush border membranes where cholesterol is heterogeneously distributed, the part of cholesterol which corresponds to the pool readily accessible to cholesterol oxidase exerts only a minimal effect on the membrane physical state. This reinforces the hypothesis (El Yandouzi & Le Grimellec, 1992) that this cholesterol has only weak interactions with other membrane lipids. As in intestinal cells where the BBM cholesterol is also heterogeneously distributed and accessible in native membrane preparations, the cholesterol content of the renal BBM can be regulated independently of that of the basolateral domain of the plasma membrane. Thus, renal and intestinal BBM appear to provide good models for studies on relationships between cholesterol heterogeneity and membrane function in epithelial cells. Finally, cholesterol present in these BBM can be oxidized without glutaraldehyde or phospholipase pretreatment. This allowed us to demonstrate that, in BBM vesicles, cholesterol oxidase treatment resulting in oxidation of more than 90% of membrane cholesterol has only a limited effect on the physical state of the membrane.

REFERENCES

Barrow, D. A., & Lentz, B. R. (1983) *J. Biochem. Biophys. Methods 7*, 217–234.

Ben-Yashar, V., & Barenholz, Y. (1989) *Biochim. Biophys. Acta 985*, 271–278.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Bloj, B., & Zilversmit, D. B. (1976) *Biochemistry 15*, 1277–1283.

Bloj, B., & Zilversmit, D. B. (1982) *J. Biol. Chem. 257*, 7608–7614.

Booth, A. G., & Kenny, A. J. (1974) *Biochem. J. 142*, 575–581.

Brasitus, T. A., & Schachter, D. (1980) *Biochemistry 19*, 2763–2769.

Brasitus, T. A., & Schachter, D. (1982) *Biochemistry 21*, 2241–2246.

Brasitus, T. A., & Dudeja, P. K. (1988) in *Advances in Membrane Fluidity* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) Vol. 2, pp 227–254, Alan R. Liss, New York.
Carmel, G., Rodrigue, F., Carriére, S., & Le Grimellec, C. (1985) *Biochim. Biophys. Acta 818*, 149–157.
Dale, R. E., Chen, L. A., & Brand, L. (1977) *J. Biol. Chem. 252*, 7500–7510.
Dudeja, P. K., Harig, J. M., Wali, R. K., Knaup, S. M., Ramaswamy, K., & Brasitus, T. A. (1991) *Arch. Biochem. Biophys. 284*, 338–345.
El Yandouzi, E. H., & Le Grimellec, C. (1992) *Biochemistry 31*, 547–551.
Engel, L. W., & Prendergast, F. G. (1981) *Biochemistry 20*, 7338–7345.
Fiorini, R., Valentino, M., Wang, S., Glaser, M., & Gratton, E. (1987) *Biochemistry 26*, 3864–3870.
Fulford, A. J. C., & Peel, W. E. (1980) *Biochim. Biophys. Acta 598*, 237–246.
Gallay, J., & De Kruijff, B. (1982) *FEBS Lett. 143*, 133–136.
Gordon, L. M., & Mobley, P. W. (1984) *J. Membr. Biol. 79*, 75–86.
Gordon, L. M., Mobley, P. W., Esgate, J. A., Hofmann, G., Whetton, A. D., & Houslay, M. D. (1983) *J. Membr. Biol. 76*, 139–149.
Haase, W., Schafer, A., Murer, H., & Kinne, R. (1978) *Biochem. J. 172*, 57–62.
Heyn, M. P. (1979) *FEBS Lett. 108*, 359–364.
Jähnig, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6361–6365.
Kinosita, K., & Ikegami, A. (1984) *Biochim. Biophys. Acta 769*, 523–527.
Klausner, R. D., Kleinfeld, A. M., Hoover, R. L., & Karnovsky, M. J. (1980) *J. Biol. Chem. 255*, 1286–1295.
Lakowicz, J. R., Prendergast, F. G., & Hogen, D. (1979) *Biochemistry 18*, 508–519.
Lange, Y. (1992) *J. Lipid Res. 33*, 315–321.
Lange, Y., & Ramos, B. (1983) *J. Biol. Chem. 258*, 15130–15134.
Lange, Y., Matthies, H., & Steck, T. L. (1984) *Biochim. Biophys. Acta 769*, 551–562.
Le Grimellec, C., Giocondi, M. C., Carriére, B., Carriére, S., & Cardinal, J. (1982) *Am. J. Physiol. 242*, F246–F253.
Le Grimellec, C., Friedlander, G., & Giocondi, M. C. (1988a) *News Physiol. Sci. 3*, 227–229.
Le Grimellec, C., Friedlander, G., & Giocondi, M. C. (1988b) *Am. J. Physiol. 255*, F22–F32.
Lentz, B. R. (1989) *Chem. Phys. Lipids 50*, 171–190.
Levi, M., Jameson, D. M., & van der Meer, B. W. (1989) *Am. J. Physiol. 256*, F85–F94.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Molitoris, B. A., & Hoilien, C. (1987) *J. Membr. Biol. 99*, 165–172.
Molitoris, B. A., Alfrey, A. C., Harris, R. A., & Simon, F. R. (1985) *Am. J. Physiol. 249*, F12–F19.
Moore, N. F., Patzer, E. J., Barenholz, Y., & Wagner, R. R. (1977) *Biochemistry 16*, 4708–4715.
Mouritsen, O. G. (1991) *Chem. Phys. Lipids 57*, 179–194.
Mrsny, R. J., Volwerk, J. J., & Griffith, O. H. (1986) *Chem. Phys. Lipids 39*, 185–191.
Nemecz, G., & Schroeder, F. (1988) *Biochemistry 27*, 7740–7749.
Omodéo-Salè, F., Marchesini, S., Fishman, P. H., & Berra, B. (1984) *Anal. Biochem. 142*, 347–350.
Parasassi, T., Conti, F., Glaser, M., & Gratton, E. (1984) *J. Biol. Chem. 259*, 14011–14017.
Rogers, J., Lee, A. G., & Wilton, D. C. (1979) *Biochim. Biophys. Acta 552*, 23–37.
Schroeder, F. (1988) in *Advances in Membrane Fluidity* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) Vol. 1, pp 193–217, Alan R. Liss, New York.
Schroeder, F., Jefferson, J. R., Kier, A. B., Knittel, J., Scallen, T. J., Wood, W. G., & Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med. 196*, 235–252.
Slotte, J. P. (1992) *Biochim. Biophys. Acta 1123*, 326–333.
Slotte, J. P., Hedström, G., Ranström, S., & Ekman, S. (1989) *Biochim. Biophys. Acta 985*, 90–96.
Smutzer, G., & Yeagle, P. L. (1985) *Biochim. Biophys. Acta 814*, 274–280.
Spencer, R. D., & Weber, G. (1970) *J. Chem. Phys. 52*, 1654–1663.
Straume, M., & Litman, B. J. (1987) *Biochemistry 26*, 5121–5126.
Straume, M., & Litman, B. J. (1988) *Biochemistry 27*, 7723–7733.
Stubbs, C. D., & Smith, A. D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
van Blitterswijk, W. J., van Hoeven, R. P., & van der Meer, B. W. (1981) *Biochim. Biophys. Acta 644*, 323–332.
van Blitterswijk, W. J., De Veer, J., Krol, J. H., & Emmelot, P. (1982) *Biochim. Biophys. Acta 688*, 495–504.
van Blitterswijk, W. J., van der Meer, B. W., & Hilkmann, H. (1987) *Biochemistry 26*, 1746–1756.
Van der Meer, B. W., van Hoeven, R. P., & van Blitterswijk, W. J. (1986) *Biochim. Biophys. Acta 854*, 38–44.
Van Meer, G. (1987) *Trends Biochem. Sci. 12*, 375–376.
Veatch, W. R., & Stryer, L. (1977) *J. Mol. Biol. 117*, 1109–1113.
Vénien, C., Aubry, M., Crine, P., & Le Grimellec, C. (1988) *Anal. Biochem. 174*, 325–330.
Verkman, A. S., & Ives, H. E. (1986) *Am. J. Physiol. 250*, F633–F643.
Vincent, M., & Gallay, J. (1983) *Biochem. Biophys. Res. Commun. 113*, 799–810.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.
Yeagle, P. L., Albert, A. D., Boesze-Battaglia, K., Young, J., & Frye, J. (1990) *Biophys. J. 57*, 413–424.
Zannoni, C., Arcioni, A., & Cavartota, P. (1983) *Chem. Phys. Lipids 32*, 179–250.