Cholesteryl Ester Absorption by Small Intestinal Brush Border Membrane is Protein-Mediated†

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ABSTRACT: This paper provides unambiguous evidence that brush border membrane vesicles (BBMV) routinely prepared from rabbit small intestine contain a protein that catalyzes the absorption of long-chain cholesteryl ester and ether. The protein is located on the lumenal side of the brush border membrane. The experiments demonstrate that cholesteryl oleate need not be hydrolyzed prior to its incorporation in the BBMV. Unexpectedly and surprisingly, the absorption kinetics of free and esterified cholesterol are very similar in small intestinal BBMV using mixed bile salt micelles and small unilamellar phospholipid vesicles as the donor. The water-soluble form of the protein responsible for this effect is released into the supernatant, probably by autoproteolysis, and catalyzes the exchange of both free and esterified cholesterol between two populations of small unilamellar phospholipid vesicles (SUV). The water-soluble form of the protein was partially purified by a two-step procedure involving gel filtration on Sephadex G-75 and anion-exchange chromatography on Mono Q, yielding a 50-fold increase in the specific activity of the protein. The resulting protein gave two bands on sodium dodecyl sulfate−10% polyacrylamide gel electrophoresis and was used to raise polyclonal antibodies in sheep. The IgG fraction of the sheep antisera blocked the cholesteryl oleate and cholesterol exchange between two populations of SUV mediated by the antigen. The same IgG fraction produced a partial inhibition of cholesterol absorption in small intestinal BBMV. We conclude from the data presented that, contrary to the general belief prevailing in the field of lipid digestion and absorption, long-chain cholesteryl esters may be taken up by the brush border membrane as such and need not be hydrolyzed prior to absorption. The actual contribution of this mechanism to the total absorption of long-chain cholesteryl esters is probably limited by the low solubility of these compounds in mixed bile salt micelles and lipid vesicles.

We reported that the transfer of cholesterol from either small unilamellar phospholipid vesicles (SUV)[1] or mixed bile salt micelles as the donor to small intestinal brush border membrane vesicles (BBMV) as the acceptor is protein-mediated (Thurnhofer & Hauser, 1990a; Thurnhofer et al., 1991). With micelles there is net transfer of cholesterol from the donor to the acceptor, whereas with SUV as the donor there is true mass exchange: at equilibrium cholesterol is evenly distributed between the lipid pools of donor and acceptor. Both cholesterol transfer from micelles and cholesterol exchange between SUV and BBMV are second-order reactions. The reaction mechanism involves collision-induced transfer or exchange of cholesterol between donor and acceptor (Mütsch et al., 1986; Thurnhofer & Hauser, 1990a; Thurnhofer et al., 1991).

After proteolytic treatment of the brush border membrane (BBM), the rate of cholesterol absorption from any donor particle is significantly slowed down. Under these conditions, cholesterol uptake by BBM is a first-order reaction involving the desorption of cholesterol from the donor particle, diffusion of monomeric cholesterol through the aqueous phase and incorporation of the cholesterol molecule into the lipid bilayer of the BBM (Thurnhofer & Hauser, 1990a; Thurnhofer et al., 1991). Comparing the cholesterol absorption before and after proteolytic treatment of the BBM lends support to the notion that this process is protein-mediated.

We reported previously that the rate of exchange of cholesteryl [^{14}C]oleate between SUV of egg phosphatidylcholine (PC) and BBMV is similar to that of free cholesterol (Mütsch et al., 1986). This result is remarkable considering the difference in the chemical structure of cholesterol and long-chain cholesteryl esters and in turn the difference in the physicochemical properties of these two compounds, e.g., the difference in their hydrophobicity and solubility in water and lipid bilayers. A point of concern is that cholesteryl esters might have been hydrolyzed by cholesterol esterase activity present in the BBM and as a result the absorption of free cholesterol and fatty acids was observed rather than that of the intact cholesteryl ester.

The aim of the present study is to shed light on the question of cholesteryl ester absorption and to elucidate the mechanism of cholesteryl ester uptake in the BBM. Evidence is presented which shows that the absorption of long-chain cholesteryl esters by this membrane is a facilitated,

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[1] Abbreviations: BBM, brush border membrane(s); BBMV, brush border membrane vesicle(s); cmc, critical micellar concentration; EDTA, disodium salt of ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SUV, small unilamellar phospholipid vesicle(s).

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protein-mediated process. This finding casts doubt on the generally accepted view that cholesteryl esters have to undergo hydrolysis prior to their absorption by the BBM.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC) and egg phosphatidic acid were purchased from Lipid Products (South Nutfield, Surrey, U.K.), cholesterol and sodium taurocholate from Fluka (Buchs, Switzerland), cholesteryl oleate from Sigma (St. Louis, MO), and lyophilized proteinase K from *Tritirachium album* from Boehringer (Mannheim, Germany). Cholesteryl [1-^{14}C]oleate (60 Ci/mol), [1,2,6,7-$^{3}H_4$(N)]cholesteryl oleate (78 Ci/mmol), [1,2-$^{3}H_2$(N)]cholesterol (50 Ci/mmol), and [24-^{14}C]taurocholic acid (46 Ci/mol) were obtained from DuPont−NEN (Regensdorf, Switzerland), and [1,2-$^{3}H_2$(N)]-cholesteryl oleyl ether (37 Ci/mmol) was from Amersham, U. K. All lipids used in this study were pure by thin-layer chromatography standard.

Methods

Preparation of Brush Border Membrane Vesicles and Various Donor Particles. BBMV were routinely prepared from rabbit duodenum and jejunum according to Hauser et al. (1980). As donor particles, mixed sodium taurocholate micelles containing 0.07 mol % radiolabeled cholesteryl oleate, cholesteryl oleyl ether or cholesterol as well as egg PC SUV containing ~1 mol % of the radiolabeled lipids were used. These particles were made according to published procedures (Thurnhofer et al., 1991; Schulthess et al., 1994).

Lipid Absorption by BBMV. Lipid absorption by BBMV was measured as described previously (Thurnhofer et al., 1991; Schulthess et al., 1994). Mixed bile salt micelles and egg PC SUV containing the radiolabeled lipids were used as donor particles. Donor and acceptor were mixed at appropriate concentrations at time zero and incubated at 23 °C; after timed intervals BBMV were separated from the donor by centrifugation at 115000*g* for 2 min in a Beckman airfuge. Published procedures were used to measure rates of exchange of cholesteryl ester and cholesteryl ether between two populations of SUV (Thurnhofer & Hauser, 1990b; Thurnhofer et al., 1991; Schulthess et al., 1994). SUV of egg PC containing [^{3}H]cholesteryl oleate or [^{3}H]cholesteryl oleyl ether as the donor and SUV of egg PC/egg phosphatidic acid (85:15 mole ratio) as the acceptor, both suspended in buffer A (0.13 M sodium phosphate, pH 7.4) at 1 and 5 mg of total lipid/mL, respectively, were incubated at 23 °C in the absence and presence of supernate proteins (see below). After timed intervals aliquots of 0.1 mL of the incubation mixture were filtered through DEAE-Sepharose Cl-6B columns (2 × 0.6 cm, from Pharmacia) which retained the negatively charged acceptor vesicles. The radioactivity in the donor vesicles eluted from the column was determined in a Beckman LS 7500 liquid scintillation counter.

Proteolytic Treatment of BBMV and Production of Supernate Proteins. The proteolytic treatment of BBMV with proteinase K was carried out as described by Thurnhofer and Hauser (1990b). The liberation of water-soluble lipid exchange proteins from BBMV by autoproteolysis induced by incubating BBMV suspended in buffer B (0.01 M sodium phosphate, pH 7.3, 0.14 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3) at temperatures above 0°C was described previously (Thurnhofer & Hauser, 1990b; Thurnhofer et al., 1991). Proteins thus liberated were separated from BBMV by centrifugation at 115000*g* for 30 min. The pelleted BBMV were resuspended in buffer B and the procedure was repeated twice in order to completely extract supernate proteins. Supernate proteins thus produced were concentrated in dialysis bags (Union Carbide, Type 18/32 with a cutoff molecular mass of 5−8 kDa) using poly(ethylene glycol) externally (M_r = 12 000).

Purification of Water-Soluble Supernate Proteins. Solutions of supernate proteins in buffer B containing about 10 mg of protein/mL were size-fractionated by gel filtration on Sephadex G-75 SF as described before (Thurnhofer et al. 1991). Protein fractions exhibiting cholesteryl oleyl ether exchange activity were pooled, concentrated as described above, dialyzed exhaustively against buffer C (0.020 M piperazine hydrochloride, pH 6.0, and 0.075 M NaCl) and centrifuged at 20000*g* for 5 min. Protein solutions of up to 8 mL thus obtained were subjected to anion-exchange chromatography using Mono Q HR 5/5 FPLC (Pharmacia LKB). All buffers used with this column were degassed and filtered through a Millipore filter of mean pore size 0.45 μm. Proteins with lipid exchange activity bound to the column were eluted with a linear NaCl gradient at a flow rate of 1 mL/min. The NaCl gradient was generated by mixing buffers C and D (0.020 M piperazine hydrochloride, pH 6, and 1 M NaCl). All fractions containing lipid exchange activity were analyzed by SDS−PAGE.

Preparation of Antisera and IgG. Lipid exchange protein-(s) catalyzing the exchange of both cholesteryl ester and ether as well as free cholesterol between two populations of SUV was (were) purified as described above and used to raise polyclonal antibodies in sheep. Sheep preimmune sera and antisera were filtered through sterile filters (0.22 μm, Millex - GS, Millipore, Molsheim, France), and after addition of 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.04% NaN_3 the sera were stored at 4 °C prior to use. The IgG fraction of the antisera was prepared by chromatography on CM Affi-Gel Blue followed by ammonium sulfate precipitation (Gee et al., 1979). The precipitated IgG was dissolved in 0.01 M potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.1 mM PMSF, and 0.04% NaN_3 to protein concentrations of 5−10 mg/mL and dialyzed against this buffer. The resulting IgG solutions were stored at 4 °C and used within 2 weeks after preparation.

Analytical Methods. Lipids from BBMV were extracted according to Hauser et al. (1980). Lipid concentrations were determined by radioactivity measurements counting 3−4 aliquots in a Beckman LS 7500 liquid scintillation counter. Protein concentrations were determined by the bicinchoninic acid method of Pierce. SDS−PAGE was carried out in a Mini-Protean II dual slab cell from Bio-Rad according to the Bio-Rad instruction manual using Bio-Rad molecular mass markers. Protein bands were visualized by Coomassie brilliant blue and/or silver staining. Immunogold staining of BBMV was carried out according to Hyatt (1989) and Behnke et al. (1986).

RESULTS

Cholesteryl Oleate Absorption by Rabbit Jejunal BBMV from Sodium Taurocholate Micelles. Figure 1A shows that

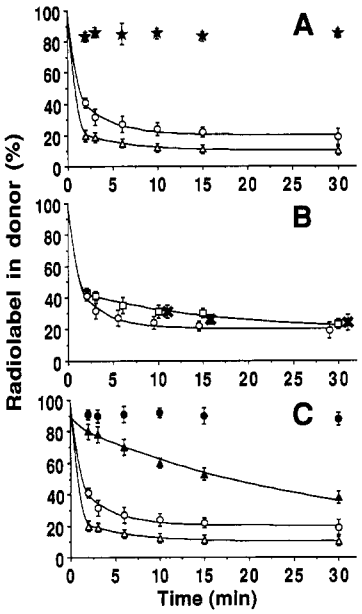

FIGURE 1: Kinetics of the absorption of cholesteryl oleate, cholesteryl oleyl ether and free cholesterol by rabbit jejunal BBMV from mixed taurocholate micelles. Sodium taurocholate mixed micelles (4.8 mM) containing either 3.4 μM cholesteryl [^{14}C]oleate, [^{3}H]cholesteryl oleyl ether, or [^{3}H]cholesterol were dispersed in buffer A (0.13 M sodium phosphate, pH 7.4) and incubated at 23 °C with BBMV at 10 mg of protein/mL. After timed intervals donor and acceptor were separated by centrifugation at 115000g for 2 min at 4 °C. The radioactivity remaining in the donor was determined and plotted as a function of time. The error bars represent the standard deviation of four measurements. The solid lines were obtained by curve-fitting using eq 2 (cf. Appendix). (A) Cholesteryl oleate (○); free cholesterol (△); sodium taurocholate radiolabeled as described in Materials and Methods (★). (B) Cholesteryl oleate (○); cholesteryl oleate corrected for hydrolysis (×); cholesteryl oleyl ether (□). (C) Absorption kinetics of cholesteryl oleate (○) and free cholesterol (△) for native BBMV in comparison to cholesteryl oleate (●) and cholesterol (▲) absorption for proteinase K-treated BBMV. BBMV prepared from rabbit jejunum were treated with proteinase K as described in Methods. The data points for native BBMV are those of panel A.

the absorption of cholesteryl oleate by rabbit brush border membrane vesicles (BBMV) from taurocholate micelles is almost as effective as that of free cholesterol. The kinetic curves shown are biphasic and were fitted using eq 2 of the Appendix (solid lines, Figure 1A). The time resolution of these experiments was limited to about 2 min due to the centrifugation step used to separate donor and acceptor. Therefore meaningful values of the pseudo-first-order rate constants and half-times could not be derived for the initial fast phase of the absorption of cholesteryl oleate, cholesteryl oleyl ether, and free cholesterol. But it is clear from an inspection of Figure 1A that the half-times of the first phase for both esterified and free cholesterol absorption are less than 1 min. Curve-fitting of the second slow phase yielded half-times of 2.4 ± 0.9 and 3.8 ± 0.6 min for the absorption of cholesteryl oleate and free cholesterol, respectively. After the lipid absorption experiments, total lipids were extracted from the BBMV and analyzed by thin-layer chromatography (Figure 2). This analysis showed that the hydrolysis of cholesteryl oleate during absorption was negligible or small, and at any rate it never exceeded 15%. Hence we conclude that more than 85% of cholesteryl oleate was taken up by the BBM as such. The kinetics of cholesteryl oleyl ether absorption by BBMV was identical to that of cholesteryl oleate, particularly if with the latter compound a correction for hydrolysis was introduced (Figure 1B). The ether analog is known to be resistant to cholesterol esterases and other lipases which might be present in the BBM. Thin-layer chromatography of the lipid extract of BBMV which had been incubated with taurocholate micelles containing [^{3}H]-cholesteryl oleyl ether proved that the ether was indeed incorporated in the lipid bilayer of the BBMV. The chromatogram obtained was independent of time and identical to that shown on the left of Figure 2, indicating that the R_f value of cholesteryl oleyl ether is identical to that of cholesteryl oleate (data not shown). After treatment of BBMV with proteinase K, the absorption of cholesteryl oleate was negligible and that of free cholesterol was significantly reduced to half-times of the order of 1 h (Figure 1C), consistent with a previous report (Thurnhofer et al., 1991).

Cholesteryl Oleate Absorption by Rabbit Jejunal BBMV from Egg PC SUV. The absorption of cholesteryl oleate and cholesteryl oleyl ether from egg PC SUV as the donor was markedly slower than that from mixed bile salt micelles (cf. Figures 1 and 3). The absorption kinetics of cholesteryl oleate and cholesteryl oleyl ether were identical within the error of the measurement. For both cholesteryl ester and ether, biphasic kinetics were observed (Figure 3A). Pseudo-first-order rate constants k_1 derived from curve-fitting (cf. eq 4 of the Appendix) of the kinetic data in Figure 3A are summarized in Table 1. The pseudo-first-order rate constants k_1 obtained for the initial fast phase of cholesteryl oleyl ether absorption increased linearly with the BBM (acceptor) concentration (Figure 4 and Table 1). The absorption of cholesteryl oleate and cholesteryl oleyl ether is markedly slowed down in the second phase. The pseudo-first-order rate constant k_1' derived for the second slow phase is independent of the BBM concentration within the relatively large error of this measurement (Figure 4 and Table 1). As a result of this second slow phase of cholesteryl ester and ether absorption, the equilibrium distribution x_∞ of these two lipids between donor and acceptor membranes was reached only after about 24 h. The x_∞ values determined experimentally for cholesteryl oleate and cholesteryl oleyl ether were 31% ± 9% and 23% ± 5%, respectively, as compared to the expected x_∞ of 25% calculated from the lipid composition of donor and acceptor membranes. For comparison, the absorption of free cholesterol from egg PC SUV as the donor is included in Figure 3A. In contrast to cholesteryl ester and ether, the absorption of free cholesterol was monophasic and the expected equilibrium value of x_∞ = 25% was approached after about 3 h. The pseudo-first-order rate constant k_1 for cholesterol absorption derived from curve-fitting agreed well with published values (Thurnhofer & Hauser, 1990a).

As shown in Figure 3B, the treatment of BBMV with proteinase K resulted in a significant reduction of the rate of lipid absorption. The rate of absorption of cholesteryl oleate by proteinase K-treated BBMV was negligible (Figure 3B, Table 1), while the absorption of free cholesterol by these membranes was a true first-order reaction characterized by a rate constant $k_1 = 0.07 \pm 0.02$ h^{-1} corresponding to a half-time of $t_{1/2} = 7.4 \pm 2.1$ h (Table 1). These values are entirely consistent with published data for passive cholesterol exchange between two populations of SUV (McLean & Phillips, 1981).

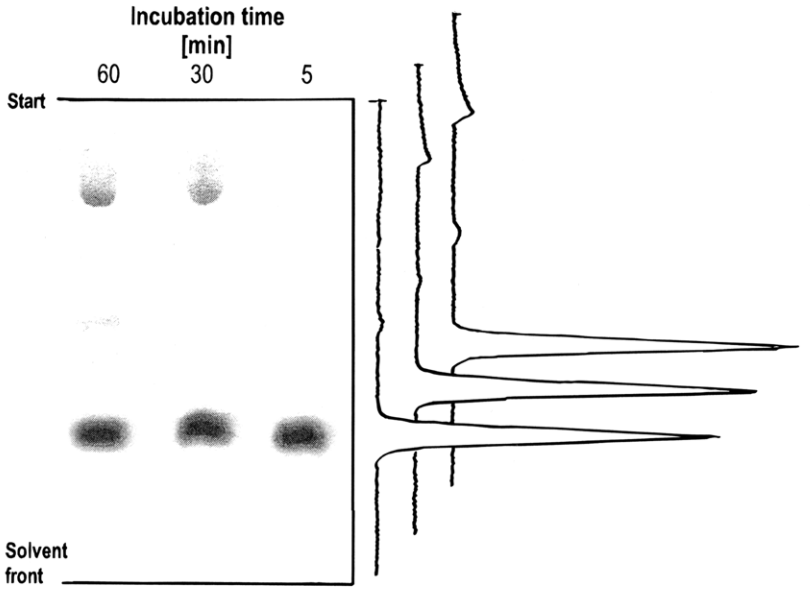

FIGURE 2: Thin-layer chromatograms of total lipids extracted from BBMV after incubation with mixed bile salt micelles of composition as described in Figure 1. An aliquot of the lipid extract (0.5 mg of lipid) in $CHCl_3/CH_3OH$ (2:1 v/v) was applied to the thin-layer plate (20 × 20 cm, silica gel 60 F 254 from Merck, Darmstadt, Germany) as a band of ~1.5-cm width. Cyclohexane/diethylether (1:1 v/v) was used as the solvent, and radiolabeled cholesteryl [1-^{14}C]oleate and its degradation products were made visible by storage phosphor imaging. The R_f values of cholesteryl oleate and oleic acid are ~ 0.7 and ~ 0.15, respectively. The numbers on top of each lane give the time (minutes) BBMV were incubated with mixed bile salt micelles prior to extracting the total lipids. In the panel on the right, densitometer tracings of the three lanes are given showing that 11% and 14% of the cholesteryl oleate were hydrolyzed after incubation of BBMV with mixed bile salt micelles for 30 and 60 min, respectively. No free fatty acid was detected after 5 min of incubation. The degradation product at ~ 7 cm ($R_f \approx 0.42$) was less than 1% and not identified.

As mentioned, for sterol absorption from mixed bile salt micelles, BBMV were extracted at the end of the absorption experiment. Thin-layer chromatography of the lipid extract confirmed the results of Figure 2. This result was substantiated by the finding that identical kinetics were obtained within the experimental error for the absorption of cholesteryl oleate ^{3}H-labeled in the ring and ^{14}C-labeled in the fatty acid. Indeed, the kinetic curve for cholesteryl oleate absorption shown in Figure 3A represents the mean of two series of experiments carried out with ^{3}H- and ^{14}C-labeled cholesteryl oleate.

Cholesteryl Oleyl Ether Exchange between Two Populations of SUV. The passive exchange of [^{3}H]cholesteryl oleyl ether between two populations of SUV in the absence of supernate proteins was too slow to be measurable (solid squares, Figure 5). This is true for incubation times up to 24 h and also agrees with a recent report (Morrison et al., 1994). The addition of supernate proteins induced the exchange of cholesteryl oleyl ether between two populations of SUV. The kinetic curves shown in Figure 5 are monophasic, and the pseudo-first-order rate constants (k_1) derived by curve-fitting (cf. eq 3 of the Appendix) increased approximately linearly with the concentration of supernate proteins (Table 2). The kinetic curves shown in Figure 5 approached the equilibrium x_∞ of about 25% after 4 h, which is somewhat larger than the expected x_∞ of 17% calculated from the lipid composition of the two populations of SUV. When supernate proteins (1.4 mg/mL) were incubated with proteinase K (3.3 mg/mL) at 23 °C for 2 h prior to the lipid exchange experiment, the rate of cholesteryl oleyl ether transfer was reduced to levels of the passive exchange (solid squares, Figure 5).

Purification of a High Molecular Weight Lipid Exchange Protein from Rabbit Jejunal BBMV That Catalyzes Cholesteryl Ester and Ether Exchange between Two Populations of Small Unilamellar Phospholipid Vesicles. Supernate proteins liberated from rabbit jejunal BBMV by autoproteolysis as described in Methods were subjected to gel filtration on Sephadex G-75 (Figure 6). As shown in this figure the cholesteryl oleyl ether exchange activity coeluted with the leading edge of the major protein peak (peak 1, Figure 6A). Consistent with previous publications from this laboratory, the cholesterol exchange activity was eluted in two peaks, the first one in the column void volume coinciding with the cholesteryl ether exchange activity and the second one at an elution volume $v_e = 122$ mL. The first peak (peak 1 in Figure 6) eluted in the column void volume has an apparent molecular mass >70 kDa, while the apparent molecular mass of the second peak (labeled peak 3 in Figure 6B) is 13 kDa (Thurnhofer et al., 1991). Fractions of the main protein peak (peak 1, Figure 6) containing both cholesteryl oleyl ether and cholesterol exchange activity were pooled and further purified by anion-exchange chromatography on Mono Q HR 5/5. About 30−40% of the protein applied to the Mono Q column was eluted in the column pass-through peak (Figure 7). These proteins had no lipid exchange activity. Proteins retained on the column (60−70%) were eluted with a linear NaCl gradient: both the cholesteryl oleyl ether and the cholesterol exchange activity were coeluted as one peak at [NaCl] = 0.18−0.23 M with a shoulder at the trailing edge. The peak exhibiting lipid exchange activity in Figure 7 amounts to 16% ± 6% of the protein retained on the Mono Q column. The purification factor of the anion-exchange chromatography on Mono Q HR 5/5 was 60 ± 10. Fractions eluted from the Mono Q column and active in cholesteryl oleyl ether and cholesterol exchange were analyzed by SDS−10% PAGE. All fractions consisted of two major bands with apparent molecular masses of 57 and 96 kDa. The early fractions eluted from the column showed additional minor bands at 70, 78, and 108

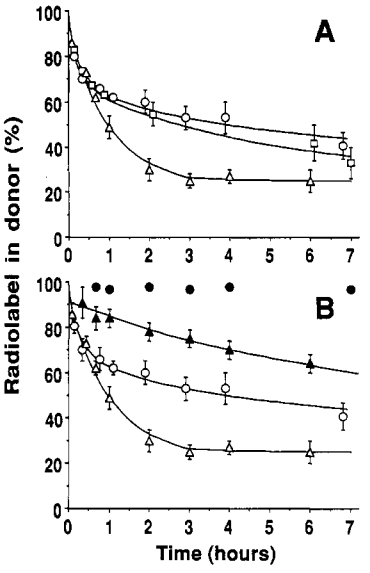

FIGURE 3: (A) Kinetics of cholesteryl oleate (○), cholesteryl oleyl ether (□) and cholesterol (△) absorption by rabbit jejunal BBMV from egg PC SUV. SUV of 1 mg of total lipid/mL containing 1 mol % either cholesteryl [^{14}C]oleate, [^{3}H]cholesteryl oleyl ether, or free [^{3}H]cholesterol were incubated at 23 °C with BBMV (10 mg of protein/mL) in buffer A. After timed intervals, donor and acceptor were separated by centrifugation at 115000*g* for 2 min at 4 °C and the radioactivity remaining in the donor was determined. The error bars represent the standard deviation of 4−6 measurements. The solid lines were obtained by curve-fitting using eqs 3 and 4 of the Appendix for monophasic and biphasic curves, respectively. The decrease in radioactivity in the donor vesicles was biphasic for the absorption of cholesteryl oleate and cholesteryl oleyl ether, while it was monophasic for cholesterol absorption. (B) Kinetics of cholesteryl oleate (○) and cholesterol (△) absorption by native BBMV compared to cholesteryl oleate (●) and cholesterol (▲) absorption by proteinase K-treated BBMV from SUV as the donor. BBMV were treated with proteinase K as described in Methods. The data points for native BBMV are those of panel A.

kDa, while the later ones consisted of the two major bands and occasionally the minor band at 78 kDa was also detected by Coomassie blue staining (Figure 8A). The intensity ratio of the two major bands appeared to be constant in all fractions. Repeating the anion-exchange chromatography did not improve the purity; at best we still obtained two bands at 57 and 96 kDa (Figure 8B). The column fractions which gave two bands on SDS−PAGE were used to raise polyclonal antibodies against the purified protein(s).

Effect of Polyclonal Sheep Antibodies Raised against the Sterol Exchange Protein(s) Purified by the Two-Step Procedure (of Figures 6 and 7). Figure 9 shows the effect of IgG of sheep antisera on cholesteryl oleyl ether and cholesterol exchange between two populations of SUV. In these experiments lipid exchange was mediated either by the antigen used to produce the polyclonal antibodies or by proteins present in peak 1 (cf. Figure 6). Increasing IgG concentrations progressively inhibited both cholesterol and cholesteryl ether exchange between two populations of SUV; the antigen-induced cholesteryl oleyl ether and cholesterol exchange activities were totally blocked at about 0.5 mg of IgG/mL. Higher IgG concentrations were required for blocking the cholesteryl oleyl ether and cholesterol exchange mediated by peak 1 proteins (cf. Figure 9). As shown in Figure 9, the IgG fraction of preimmune serum had no effect on sterol exchange. The IgG fraction of the sheep antiserum also had a significant effect on cholesterol exchange between egg PC SUV containing 1 mol % cholesterol as the donor and rabbit jejunal BBMV as the acceptor. In the presence of IgG at 6 mg of protein/mL the cholesterol exchange was markedly slowed down, and the pseudo-first-order rate constant measured under these conditions was $k_1 = 0.07\ h^{-1}$ corresponding to a half-time $t_{1/2} = 7.4$ h (data not shown). These values are characteristic of passive cholesterol exchange measured between two populations of SUV (McLean & Phillips, 1981; Thurnhofer & Hauser, 1990a). However, in this case the IgG fraction of the preimmune serum had a partial inhibiting effect on cholesterol exchange in that the half-time was doubled to $t_{1/2} \approx 2$ h compared to the control experiment in the absence of IgG (cf. Table 1).

The polyclonal sheep antibody raised against the purified lipid exchange protein(s) was used in immunogold labeling experiments of BBMV. Rabbit small intestinal BBMV incubated with the IgG fraction of sheep antisera gave a positive interaction with protein G−10-nm gold. No immunogold staining was observed with BBMV incubated with sheep preimmune serum and with BBMV treated sequentially with IgG, gold-free protein G, and protein G−10-nm gold (data not shown).

DISCUSSION

We have been using cholesteryl oleyl ether as a model for long-chain cholesteryl esters because ethers are supposed to be resistant to cholesterol esterase and other hydrolases possibly present in the BBM. This assumption is borne out by experiment. The main finding of this work is that the kinetics of cholesteryl ester and ether absorption by BBMV are identical and, more importantly and quite unexpectedly, that these kinetics are similar to the absorption kinetics of free cholesterol. This is true regardless if mixed bile salt micelles or phospholipid SUV are used as the donor. The data in Figures 1 and 3 confirm the conclusion of the previous study by Mütsch et al. (1986) that the absorption of cholesteryl oleate at the BBM closely resembles that of free cholesterol.

Thin-layer chromatography analysis of the total lipids extracted from BBMV after the lipid absorption experiment proves that cholesteryl oleate is essentially incorporated in the BBM as such and therefore hydrolysis of the ester is not a prerequisite for the incorporation of long-chain cholesteryl esters into the BBM. The finding that only a minor fraction of cholesteryl oleate is hydrolyzed by BBMV is not too surprising considering the cholesterol esterase activity present in our BBMV preparation. The residual activity was determined as 1.0 ± 0.3 microunits/mg of protein, whereas BBMV saturated with cholesterol esterase had an activity of about 25 milliunits/mg of protein (Bosner et al., 1988). We conclude that the absorption of cholesteryl oleate and cholesteryl oleyl ether at the BBM level is a facilitated, protein-mediated process, at least in rabbit jejunal BBMV. The data presented suggest but do not prove that cholesterol and cholesteryl ester absorption are catalyzed by the same BBM protein(s).

The conclusion that the absorption of long-chain cholesteryl esters by BBMV is protein-mediated is supported by the following observations: (1) the absorption kinetics of cholesteryl oleate and free cholesterol are very similar and the latter compound has been shown to be taken up by the BBM in a protein-mediated manner (Thurnhofer & Hauser,

Table 1: Cholesteryl Oleate, Cholesteryl Oleyl Ether, and Cholesterol Absorption by Rabbit Jejunal BBMV from Egg PC SUV[a]

		acceptor			first phase		second phase	
absorbed lipid	donor lipid pool b (mg/mL)	BBMV	lipid pool a (mg/mL)	kinetics	k_1 (h^{-1})	half-time $t_{1/2}$ (h)	k_1' (h^{-1})	half-time $t_{1/2}$ (h)
cholesteryl oleate	1	native	3	biphasic	2.4 ± 0.8	0.22 ± 0.07	0.14 ± 0.06	5.0 ± 2.1
cholesteryl oleate	1	proteinase K treated	3		~0	~∞		
	1	native	1.5	biphasic	0.9 ± 0.4	0.46 ± 0.21	0.15 ± 0.13	4.6 ± 4.0
cholesterol oleyl	1	native	3	biphasic	1.8 ± 0.7	0.29 ± 0.11	0.17 ± 0.12	4.1 ± 2.8
ether	1	native	5.5	biphasic	4.6 ± 1.6	0.13 ± 0.05	0.15 ± 0.10	4.6 ± 3.0
	1	native	11	biphasic	11.9 ± 5.1	0.05 ± 0.02	0.47 ± 0.19	1.5 ± 0.6
cholesterol	1	native	3	monophasic	0.75 ± 0.15	0.69 ± 0.14		
cholesterol	1	proteinase K treated	3	monophasic	0.07 ± 0.02	7.4 ± 2.1		

[a] Experimental conditions are given in the legend to Figure 3. Monophasic absorption curves were computer-fitted according to eq 3 and biphasic absorption curves according to eq 4 of the Appendix. k_1 and k_1' represent the pseudo-first-order rate constants of the first and the second kinetic phases, respectively. As a first approximation we assumed the effective lipid pools a and b to be available during the whole time course of the reactions. The effective lipid pool b of the donor particle is identical to the analytical lipid concentration, while the effective lipid pool a of rabbit intestinal BBMV was shown to be given by 0.3[protein concentration] (Mütsch et al., 1986).

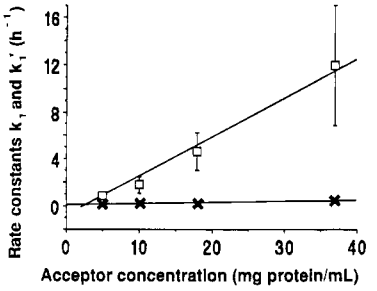

FIGURE 4: Pseudo-first-order rate constants (k_1) of the initial fast phase (□) and k_1' of the second slow phase (×) of [^{3}H]cholesteryl oleyl ether absorption from egg PC SUV as a function of the protein concentration of BBMV. The experimental conditions were described in the legend to Figure 3. The solid lines were fitted to the data by linear regression analysis. The error bars or the size of the symbols represent the standard deviation of the measurements.

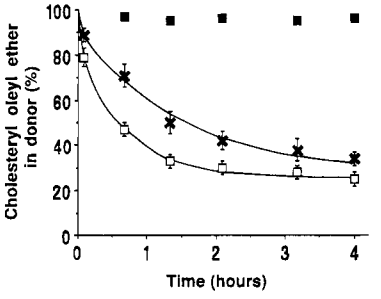

FIGURE 5: [^{3}H]Cholesteryl oleyl ether exchange between two populations of SUV as a function of the supernate protein concentration. Egg PC SUV containing a trace of [^{3}H]cholesteryl oleyl ether dispersed in buffer A at 1 mg of lipid/mL were incubated with SUV of egg PC/egg phosphatidic acid (85:15 mole ratio) at 5 mg of lipid/mL at 23 °C. Cholesteryl oleyl ether exchange was measured in the absence (■) and in the presence of 0.23 mg (×) and 0.7 mg (□) of supernate proteins liberated from BBMV as described under Methods. After timed intervals, the negatively charged acceptor vesicles were separated from the isoelectric donor vesicles by anion exchange chromatography on DEAE-Sepharose Cl-6B and the amount of radiolabeled lipid remaining in the donor was determined. The error bars represent the standard deviation of four measurements. The solid lines were obtained by curve-fitting using eq 3 of the Appendix.

1990a). (2) There is a drastic difference in the absorption of cholesteryl oleyl ether by BBMV before and after proteolytic treatment of the BBM (cf. Figures 1 and 3). (3) Autoproteolysis of BBMV liberates water-soluble proteins that have cholesteryl oleyl ether and cholesterol exchange activity (Figures 6 and 7). (4) Polyclonal antibodies raised against the purified, water-soluble lipid exchange protein(s) liberated from BBMV inhibit the cholesteryl oleyl ether as well as the cholesterol exchange between two populations of SUV mediated by these proteins (Figure 9). (5) The polyclonal antibodies also partially inhibit cholesterol exchange between egg PC SUV and BBMV. (6) Free cholesterol and its ester differ drastically in their physicochemical properties. As expected from these marked differences, the two compounds differ significantly in their passive absorption behavior, e.g., in their passive absorption by proteolytically treated BBMV as shown in Figures 1 and 3 as well as in their passive exchange between two populations of SUV. The passive exchange rate of cholesteryl oleyl ether between two populations of small unilamellar phospholipid vesicles is negligible (Figure 5), whereas the corresponding exchange of free cholesterol is small though measurable. At room temperature the half-time of this exchange reaction is on the order of 10 h (McLean & Phillips, 1981; Thurnhofer & Hauser, 1990a).

Our kinetic data for cholesteryl ester (ether) absorption from lipid SUV indicate that the pseudo-first-order rate constants k_1 of the initial fast phase of absorption depend linearly on the BBM concentration (Figure 4). This suggests that the first phase of cholesteryl ester (ether) absorption is a second-order reaction similar to that of cholesterol (Thurnhofer & Hauser, 1990a). As to the mechanism of cholesteryl ester (ether) absorption we can rule out monomer diffusion based on the results shown in Figure 5 and also fusion of donor particles with BBMV. The latter mechanism is inconsistent with the observation that different components of the SUV used as the donor are transferred to the BBM with distinctly different rates (data not shown). This is also true for mixed bile salt micelles as the donor as demonstrated in Figure 1A: compared to the absorption of cholesteryl oleate and free cholesterol from these micelles, only a limited amount of taurocholate is incorporated in the BBM. Of the remaining possible mechanisms of lipid transfer involving either soluble lipid exchange proteins or collisional contacts between donor and acceptor, we favor the latter. The reason for this is that BBMV were thoroughly washed prior to the absorption measurements in order to remove water-soluble lipid exchange proteins present in the supernatant or possibly adsorbed to the BBM. The collision-induced lipid transfer is also likely to be true for the absorption of cholesteryl ester (ether) from mixed bile salt micelles, although in this case rate constants for the initial fast phase of absorption could not be derived.

Table 2: [^{3}H]Cholesteryl Oleyl Ether Exchange between Two Populations of Small Unilamellar Phospholipid Vesicles at 23 °C as a Function of the Concentration of Rabbit BBMV Supernate Proteins Added to the System[a]

donor lipid pool b (mg/mL)	acceptor lipid pool a (mg/mL)	supernate proteins (mg/mL)	kinetics	k_1 (h^{-1})	half-time $t_{1/2}$ (h)
1	5	0		~0	~∞
1	5	0.23	monophasic	0.57 ± 0.08	1.01 ± 0.14
1	5	0.70	monophasic	1.3 ± 0.2	0.44 ± 0.07

[a] The experimental conditions were described in the legend to Figure 5. Cholesteryl oleyl ether exchange was computer-fitted according to eq 3 of the Appendix. k_1 represents the pseudo-first-order rate constant of the kinetics.

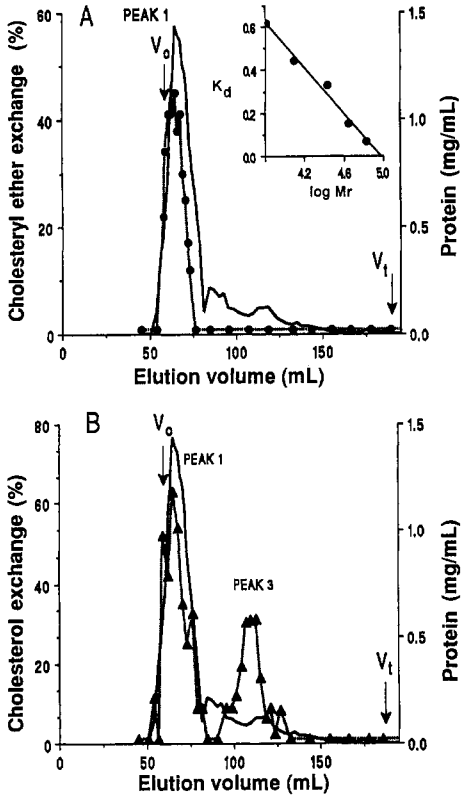

FIGURE 6: Gel filtration patterns of supernate proteins on Sephadex G-75 SF. About 6 mL of supernate proteins in buffer B (0.01 M sodium phosphate, pH 7.3, 0.14 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3) liberated from rabbit small intestinal BBMV by autoproteolysis as described in Methods were applied to a 50 × 2.2 (diameter) cm column equilibrated with the same buffer. The protein (solid line) was eluted in 1.41 mL fractions with the same buffer at a flow rate of 18 mL/h, and cholesteryl oleyl ether (●, panel A) and cholesterol exchange activities (▲, panel B) were determined in these fractions. The exchange activities are given as percent of the total radiolabel transferred from donor to acceptor vesicles within 15 min. The values were corrected for passive exchange activities. The protein recovery was 83% ± 5%. The calibration curve of the column is presented as an inset. K_d values are plotted as a function of log M_r, where M_r is the apparent molecular mass of the marker protein in kilodaltons and $K_d = (V_e - V_0)/(V_t - V_0)$. V_e is the elution volume of the marker proteins; V_0 = 55 mL and V_t = 185 mL are the void and total column volumes, respectively, determined by chromatographing dextran blue and K_3 [$Fe(CN)_6$], respectively. The solid line was fitted to the data points by linear regression analysis ($r^2 = 0.987$). As marker proteins, thyroglobulin (660 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (27 kDa), cytochrome *C* (12.4 kDa), and aprotinin (6.5 kDa) were used.

The assignment of the kinetic pools (derived from the computer analysis of the kinetic curves) to actual lipid pools remains, however, unclear. The protein-mediated cholesteryl ether exchange between two populations of SUV is characterized by a single exponential (Figure 5) with half-times on the order of 1 h (Table 2). In contrast, the cholesteryl

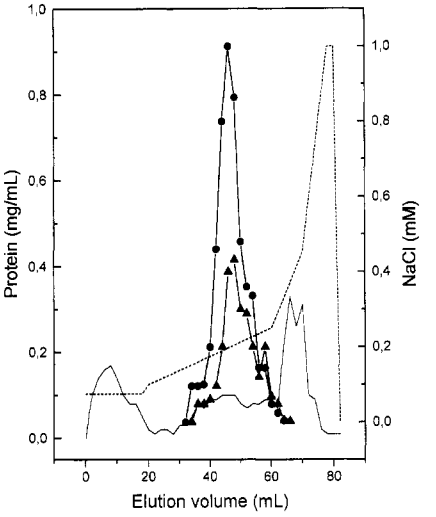

FIGURE 7: Purification of cholesteryl oleyl ether and/or cholesterol exchange protein(s) present in peak 1 (cf. Figure 6) by anion-exchange chromatography using the Mono Q HR 5/5 column and the FPLC system of Pharmacia LKB. Up to 8 mL of peak 1 proteins (1−3 mg of protein/mL) in buffer C were applied to the column equilibrated with the same buffer (0.02 M piperazine hydrochloride, pH 6, and 75 mM NaCl). The protein retained on the column was eluted with a linear NaCl gradient. Protein (solid line); [NaCl] (- - -); cholesteryl oleyl ether exchange (●) and cholesterol exchange activity (▲) in arbitrary units.

ester absorption by BBMV from egg PC SUV as the donor is biphasic with the second slow phase being characterized by half-times of 1.5−5 h (Table 1). Half-times on the order of 1 h or less might be attributed to the transverse or flip-flop movement of long-chain cholesteryl esters in egg PC bilayers, and half-times of several hours might be characteristic of this movement in the brush border membrane. The flip-flop rate of long-chain cholesteryl esters is expected to be significantly lower than that of free cholesterol. Indeed, the half-time of the transverse movement of cholesterol in the red blood cell membrane was reported to be less than 1 min (Jain, 1988). This is consistent with the result that the exchange of free cholesterol between lipid SUV and BBMV is monophasic (cf. Figure 3A and Table 1). Biphasic kinetics were observed for the absorption of both esterified and free cholesterol from mixed bile salt micelles (Figure 1). However, in this case the half-time for the second slow phase of cholesteryl ester absorption is on the order of a few minutes. By the above reasoning this would be inconsistent with the flip-flop motion of long-chain cholesteryl esters. Since all lipid molecules of small mixed bile salt micelles are readily accessible to lipid absorption by BBMV (Thurnhofer et al., 1991), the origin of the second phase of sterol absorption from mixed bile salt micelles remains unclear. We speculate that both phases of sterol absorption from mixed bile salt micelles are protein-mediated.

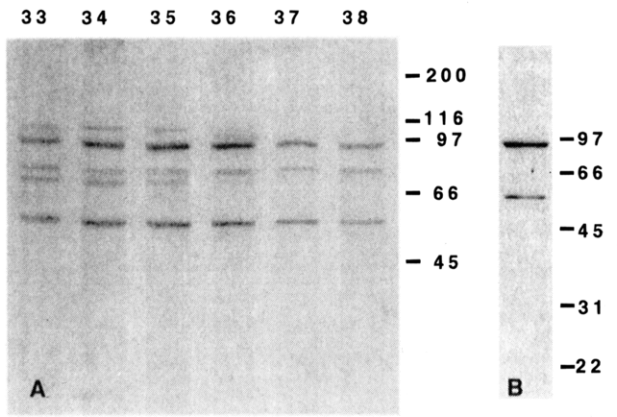

FIGURE 8: (A) SDS−10% PAGE patterns of different fractions eluted with a linear NaCl gradient from the Mono Q HR 5/5 column. An aliquot of each fraction containing 5−10 μg of protein active in the exchange of free and esterified cholesterol (cf. Figure 7) was applied to the polyacrylamide gel. Fraction numbers are given on top of each lane; the sterol exchange activity was maximal in fractions 36 and 37. (B) SDS−10% PAGE pattern characteristic of the sterol exchange protein(s) liberated from the BBM and purified by the two-step procedure described in Figures 6 and 7. The protein(s) purified in this way usually gave two major bands with apparent molecular masses of 57 and 96 kDa. Sometimes an additional minor band at 78 kDa was observed. In both panels gels were stained with Coomassie brilliant blue. The relative positions of the molecular mass markers (from Bio-Rad) expressed in kilodaltons are indicated on the right of each panel.

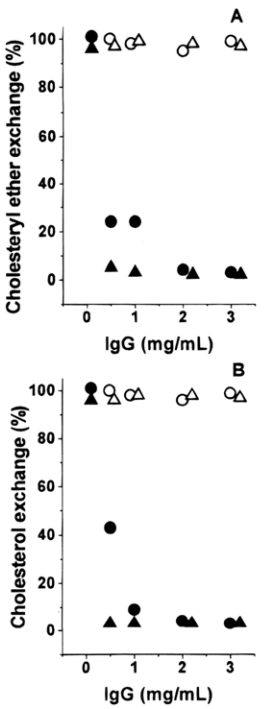

FIGURE 9: Effect of IgG of sheep antisera raised against the purified lipid exchange proteins on cholelesteryl oleyl ether (A) and cholesterol exchange (B) between two populations of SUV (cf. Figure 5). The antigen was purified by a two-step procedure summarized in Figures 6 and 7. Cholesteryl oleyl ether and cholesterol exchange were mediated either by 750 μg of peak 1 protein/mL (●) or by the purified antigen at 35 μg of protein/mL (▲) and the effect of increasing concentrations of IgG of sheep antiserum on lipid excange activity was determined. The open symbols give the effect of IgG of sheep preimmune serum. The size of the symbols represents the standard deviation of four measurements.

The protein(s) responsible for cholesteryl ester (ether) absorption shown in Figures 1 and 3 must be present on the lumenal side of the BBM. Evidence for this conclusion comes from the proteolytic treatment and immunogold labeling of BBMV together with the fact that BBMV are oriented right side out (Haase et al., 1978; Klip et al., 1979). The water-soluble lipid exchange protein(s) liberated from the BBMV has(have) a molecular weight greater than the exclusion limit of Sephadex G 75 (cf. Figure 6). The minimum molecular mass derived from SDS−PAGE is 100 kDa. From the data presented in Figures 6−8 we do not know whether the purified protein is still contaminated with one or several proteins or whether the protein consists of two or several subunits. Work currently in progress in our laboratory will shed light on this question and allow us to differentiate between these possibilities. The protein catalyzes the exchange of both esterified and free cholesterol between two populations of SUV. In all likelihood it is identical to the lipid exchange protein of apparent molecular mass greater than 100 kDa identified previously (Lipka et al., 1995).

All experiments reported here were carried out with rabbit small intestinal BBMV. One important question is whether the absorptive activities of the BBM described here are confined to rabbit small intestine. To shed light on this question the uptake of free and esterified cholesterol was investigated in pig small intestinal BBM and Caco-2 cells. In both systems we were able to verify the results reported here (Schulthess et al., manuscript in preparation), indicating that the cholesteryl ester uptake activity is not a special feature of the rabbit small intestinal BBM.

As to the origin of this protein, one possibility is that it is an integral membrane protein anchored to the external half of the BBM with its active center(s) being exposed to the external or lumenal side. In this way it catalyzes the transfer of dietary lipids from the lipid donor to the BBMV. Consistent with previous results reported from this laboratory, it apparently lacks lipid specificity and has features of a nonspecific lipid transfer protein (Thurnhofer & Hauser, 1990a,b). Its water-soluble form liberated from the BBM is probably the product of autoproteolysis. Alternatively, the protein may be of cytosolic origin or may originate in one of the cell organelles. In preparing BBMV, epithelial cells are lysed by hypotonic and mechanical treatment. As a result, proteins originating in the cytosol or cell organelles may get in contact with the BBM and may be more or less tightly bound to this membrane. In this case the effect reported here could be produced by proteins that cannot be classified as original BBM proteins. However, the question whether adsorption or binding of these proteins to the BBM is an artificial or physiologically meaningful situation is open to debate. The life span of epithelial cells is limited to 48−72 h (Carr & Toner, 1984; Madara & Trier, 1994) and is relatively short compared to those of other cells. As a result of this natural instability, enterocytes are constantly shed off into the intestinal lumen and their contents mix with the intestinal content (Carey & Hernell, 1992). By this shedding of enterocytes which is accompanied by cell lyses and the release of intracellular proteins the same proteins, may get adsorbed or bound to the BBM in vivo that are bound to BBMV in vitro. It is conceivable that in this respect the in vitro situation is quite similar to that encountered in the small intestine. The results obtained with BBMV do not allow us to differentiate between the two possibilities concerning the origin of the protein(s) responsible for cholesteryl ester

absorption in the BBM.

Current work in our laboratory is geared toward the final purification of the sterol exchange protein. Pressing questions that need to be addressed are whether or not the protein consists of a single peptide chain or of several subunits, and more information concerning the size and molecular weight of the protein, its lipid binding properties, and lipid specificity is required. The question whether or not the protein is related to known lipid exchange proteins, e.g., the plasma cholesteryl ester transfer protein (Morton & Zilversmit, 1982; Ihm et al., 1982; Albers et al., 1984; Tall, 1986; Hesler et al., 1987; Drayna et al., 1987; Jarnagin et al., 1987; Jiang et al., 1991; Inazu et al., 1992), the microsomal triglyceride transfer protein (Wetterau et al., 1990; Sharp et al., 1993) or the sterol carrier proteins (Wirtz, 1991) has to await its purification to homogeneity.

Physiological Implications. The molecular mechanism of lipid absorption in the small intestines remains elusive. It is still unknown how lipid molecules dispersed in the lumen of the small intestines gain entrance into enterocytes, the epithelial cells lining the intestinal lumen (Thomson & Dietschy, 1981; Carey et al., 1983; Tso, 1985; Shiau, 1987). A possible role of cholesterol esterase in lipid absorption was first suggested by Treadwell and Vahouney (1968), who observed that intact cholesteryl esters are poorly absorbed. The rates of absorption of different esters of cholesterol were correlated to their rates of hydrolysis by pancreatic cholesterol esterase present in the small intestine (Rudd & Brockman, 1984). Furthermore, the same authors reported that sterically hindered cholesteryl esters, which are resistant to hydrolysis by cholesterol esterase, are inefficiently absorbed in vivo. From these and similar observations a general consensus has evolved that cholesteryl esters are not absorbed per se but have to undergo hydrolysis to free cholesterol and fatty acid (Vahouny & Treadwell, 1964). These lipids together with other products of fat digestion are then emulsified by bile salts and eventually absorbed by the BBM (Thomson & Dietschy, 1981; Carey et al., 1983; Carey & Hernell, 1992). The results presented in this study challenge this generally accepted view. Our data indicate that long-chain cholesteryl esters are almost as efficiently incorporated in the BBM as free cholesterol. Therefore, there would be no need for cholesteryl ester hydrolysis to precede the incorporation (absorption) of this lipid in (to) the BBM. In discussing the physiological significance of this finding it should be borne in mind that the solubility of long-chain cholesteryl esters is limited in lipid bilayers and most likely also in micelles. The solubility of long-chain cholesteryl esters in egg PC bilayers was reported to be about 2 mol % (Janiak et al., 1974, 1979; Hamilton & Small, 1982; Small, 1986). As to the solubility of cholesteryl esters in mixed bile salt micelles, we were unable to find reliable data in the literature. Due to the severe limitation in solubility of long-chain cholesteryl esters in various lipid donors, the actual percentage of dietary cholesterol that is absorbed as free cholesterol by the epithelial cells may therefore greatly outweigh the amount taken up as esterified cholesterol. Nevertheless it is important to note that esterified cholesterol is taken up with similar probability and efficiency as free cholesterol, at least by BBMV and from mixed bile salt micelles as the donor. We conclude that hydrolysis of cholesteryl esters is not a necessary precondition for the absorption of this class of lipids. In vivo, the direct absorption of cholesteryl esters will be accompanied by cholesteryl ester hydrolysis in the presence of bile salt-activated, pancreatic cholesterol esterase and the absorption of the hydrolytic products. The two pathways of cholesteryl ester absorption probably work side-by-side; their relative contributions will depend on the precise conditions prevailing in the lumen of the small intestines.

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APPENDIX

Net transfer of lipids was observed from bile salt micelles as the donor to BBMV as the acceptor. Equations 1 and 2 are valid for monophasic and biphasic absorption kinetics, respectively:

$$x = x_\infty + (x_0 - x_\infty)\, e^{-k_1 t} \quad (1)$$

$$x = x_\infty + (x_0 - x_{1\infty})\, e^{-k_1 t} + (x_{1\infty} - x_\infty)\, e^{-k_1' t} \quad (2)$$

where x_0, x, $x_{1\infty}$, and x_∞ represent the fractions or percentages of radiolabeled lipid in the donor at times 0, t, at an intermediate state, and at the final equilibrium, respectively, and k_1 and k_1' are the pseudo-first-order rate constants of the first and the second phase of lipid absorption, respectively. For true net transfer x_∞ can deviate from zero for reasons discussed before (Schulthess et al., 1994). In the case of net transfer there is a simple relation between the pseudo-first-order rate constant and the half-time: $t_{1/2} = \ln 2/k_1$.

The exchange kinetics of lipids between donor and acceptor membrane and vice versa are described by eqs 3 and 4. The decrease in radioactivity in the donor with time is usually given by either a simple exponential decay (eq 3) or by the sum of two exponentials (eq 4) [cf. McLean and Phillips (1981), Mütsch et al. (1986), and Schulthess et al. (1994)].

$$x = x_\infty + (x_0 - x_\infty)\, e^{-k_1[(a+b)/a]t} \quad (3)$$

$$x = x_\infty + (x_0 - x_{1\infty})\, e^{-k_1[(a_1+b_1)/a_1]t} + (x_{1\infty} - x_\infty)\, e^{-k_1' t} \quad (4)$$

where a and b are the effective lipid pools of acceptor and donor membrane, respectively, and a_1 and b_1 are the effective lipid pools of acceptor and donor, respectively, accesible during the first phase of lipid exchange. Since the lipid pools involved in the second phase of lipid absorption are usually unknown, the lipid pool ratio is included in k_1'.

The half-time for monophasic lipid exchange, i.e., lipid transfer between donor and acceptor membranes with simultaneous back reaction, is $t_{1/2} = (\ln 2/k_1)\ [a/(a + b)]$; for biphasic exchange reactions the half-times for the first and the second phase are $t_{1/2} = (\ln 2/k_1)\ [a_1/(a_1 + b_1)]$ and $t_{1/2} = \ln 2/k_1'$, respectively.

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