Uptake of Cholesterol by Small Intestinal Brush Border Membrane Is Protein-Mediated†

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Received May 9, 1989; Revised Manuscript Received September 20, 1989

ABSTRACT: Absorption of cholesterol by small intestinal brush border membrane from either mixed micelles or small unilamellar vesicles is protein-mediated. It is a second-order reaction. The kinetic data are consistent with a mechanism involving collision-induced transfer of cholesterol. With micelles as the donor particle, there is net transfer of cholesterol while with small unilamellar vesicles as the donor, cholesterol is evenly distributed between the two lipid pools at equilibrium. The cholesterol absorption by brush border membrane from both mixed micelles and small unilamellar vesicles reveals saturation kinetics. Proteolytic treatment of brush border membrane with papain releases about 25% of the total membrane protein. As a result, the cholesterol uptake by brush border membrane changes from a second-order reaction to a first-order one. The reaction mechanism changes from collision-induced cholesterol uptake to a mechanism involving diffusion of monomeric cholesterol through the aqueous phase. The protein(s) released into the supernatant by papain treatment of brush border membrane exhibit(s) cholesterol exchange activity between two populations of small unilamellar vesicles. The supernate–protein(s) bind(s) the spin-labeled cholesterol analogue 3-doxyl-5α-cholestane.

Previously, we reported the transfer of lipids from small unilamellar vesicles of egg phosphatidylcholine (PC)[1] as the donor to intestinal brush border membrane vesicles as the acceptor (Mütsch et al., 1986). The lipids tested were phosphatidylcholine in spin-labeled or radioactive form, spin-labeled cholestane, and radiolabeled cholesteryl oleate. The transfer of these lipids is a second-order reaction, and its mechanism was shown to be collision-induced (Mütsch et al., 1986).

Very little is known about the details of how dietary lipids interact with the absorptive cell surface of the enterocyte, i.e., with the apical or brush border membrane of enterocytes. It is still unknown how the dietary lipids are incorporated into the external layer of the brush border membrane, how they diffuse across this relatively tightly packed brush border membrane, and by which mechanism they are released into the cytosol of the enterocyte. Regarding the first step, the prevailing viewpoint is that the absorption of dietary lipids takes place from mixed micelles consisting of the dietary lipids and bile salts. The site of uptake is the jejunum. Uptake is viewed as an energy-independent, passive diffusion process down a concentration gradient from the intestinal lumen across the brush border membrane to the cytosol of the enterocyte.

Here we provide evidence that the absorption of cholesterol by small intestinal brush border membrane from either small mixed micelles or small unilamellar vesicles is protein-mediated. Contrary to general belief documented in review articles and text books, the absorption of cholesterol is catalyzed by an intrinsic protein of the brush border membrane. This finding may have far-reaching implications in medicine and in the therapeutic approach to obesity and related disorders. It offers the possibility to regulate the cholesterol uptake in small intestine by partial or total inhibition of the protein involved.

Materials and Methods

Egg PC, its lyso compound, and egg phosphatidic acid were purchased from Lipid Products (Nutfield, Surrey, U.K.). Cholesterol was obtained from Merck (Darmstadt, FRG), radiolabeled [1α,2α(N)-^{3}H]cholesterol from Amersham (Buckinghamshire, U.K.), and spin-labeled cholestane, 3-doxyl-5α-cholestane, from Aldrich (Steinheim, FRG). 1,2-[^{3}H]Dipalmitoyl-*sn*-phosphatidylcholine was synthesized as described (Mütsch et al., 1986). The lipids used were shown to be pure by TLC standard. Papain (EC 3.4.22.2) from papaya latex as a suspension of crystals in 50 mM sodium acetate buffer, pH 4.5, and 2,3-dihydroxy-1,4-dithiolbutane were purchased from Sigma (St. Louis, MO). N^{α}-Tosyl-L-lysine chloromethyl ketone hydrochloride was from Fluka, Buchs, Switzerland.

Preparation of Brush Border Membrane Vesicles. Brush border membrane vesicles from rabbit small intestine were prepared essentially according to Hauser et al. (1980) and characterized as described in that reference. The resulting brush border membrane vesicles were dispersed in Hepes–Tris buffer, pH 7.3 (10 mM Hepes–Tris, pH 7.3, 0.3 M D-mannitol, 5 mM EDTA, and 0.02% NaN_3). Contamination with basolateral membranes was assessed by determining Na^+/K^+-ATPase activity, contamination with microsomes was determined by assaying KCN-insensitive NADH–oxidoreductase (Stieger, 1983). On the basis of these marker enzymes, it is concluded that our brush border membrane preparation is essentially free of basolateral and microsomal contamination. Furthermore, there are reports claiming that brush border membranes prepared by the method used here are also free of nuclear, mitochondrial, and cytosolic contaminants (Kessler et al., 1978; Stremmel et al., 1985). For the work presented, it is important to show that the cholesterol uptake activity is associated with the brush border membrane and is not due to cytosolic protein(s). Therefore, the first supernatant containing the cytosol, obtained after centrifuging

† This work was supported by the Swiss National Science Foundation (Grant 3.223.085).

[1] Abbreviations: PC, phosphatidylcholine; 3-doxyl-5α-cholestane, 4,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidine]-*N*-oxyl; ATP, adenosine 5′-triphosphate; NADH, reduced form of nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminoethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TLC, thin-layer chromatography.

0006-2960/90/0429-2142$02.50/0

the brush border membrane vesicles at 48000*g* for 15 min (Hauser et al., 1980), was analyzed for lipid exchange activity (see below). This supernatant exhibited PC exchange consistent with data in the literature (Yamada et al., 1978) and some minor cholesterol exchange activity. The brush border membrane pellet produced by centrifugation at 48000*g* for 15 min was resuspended in 10 mM Hepes–Tris buffer, pH 7.3, the suspension was homogenized with a Potter–Elvehjem homogenizer, and the brush border membrane vesicles were spun down again as described above. The supernatant thus produced contained no lipid exchange activity.

Preparation of Basolateral Membranes from Rabbit Small Intestine. Basolateral membranes were prepared and purified according to Scalera et al. (1980). The crude basolateral membrane suspension in 10 volume % Percoll (from Pharmacia) was subjected to centrifugation at 48000*g* for 60 min at 4 °C. The crude plasma membrane was purified on the Percoll density gradient formed spontaneously upon centrifugation. After centrifugation, the density gradient was fractionated from the top as described by Scalera et al. (1980). Each fraction was analyzed for protein and Na^+/K^+-ATPase activity, which served as a marker for the basolateral membrane. The main fractions, in which the Na^+/K^+-ATPase activity was a maximum, exhibited no cholesterol uptake activity.

Papain Digestion of Brush Border Membrane. Papain was activated by diluting 0.2 mL of the papain suspension (25 mg/mL) with 60 μL of 0.5 M potassium phosphate buffer, pH 6.8, containing 50 mM 2,3-dihydroxy-1,4-dithiolbutane and 10 mM EDTA. The resulting papain solution was saturated with N_2 and incubated at room temperature for 30 min. Brush border membrane vesicles were dispersed in Hepes–Tris buffer, pH 7.3, to a final protein concentration of about 25 mg/mL. Three milliliters of the brush border membrane suspension was mixed with 0.4 mL of the 0.5 M potassium phosphate buffer, pH 6.8, described above and 0.21 mL of the activated papain solution. The resulting brush border membrane suspension (21 mg of membrane protein/mL) contained papain at 1.1 mg/mL (28 units/mL or 1.3 units/mg of brush border membrane protein) and 6.2 mM 2,3-dihydroxy-1,4-dithiolbutane. This suspension was thoroughly flushed with N_2 and incubated at room temperature for 30 min. The reaction was stopped by adding 0.19 mL of a 0.1 M solution of N^{α}-tosyl-L-lysine chloromethyl ketone hydrochloride in H_2O and cooling of the reaction mixture to ~4 °C. The papain-treated brush border membrane vesicles were separated from the supernatant by centrifugation at 120000*g* for 30 min at 4 °C. The supernatant was decanted from the brush border membrane pellet formed by centrifugation. The supernatant contained about 25% of the total brush border membrane protein at a concentration of about 1 mg/mL. The protein present in the supernatant will be referred to as supernate–protein.

Preparation of Donor and Acceptor Particles. Cholesterol absorption by brush border membrane vesicles was measured from either small unilamellar vesicles or mixed micelles as donor particles. Small unilamellar vesicles of egg PC/cholesterol (8:1 weight ratio) and a trace amount of [1α,2α-(N)-^{3}H]cholesterol were made by sonicating 2.5 mL of the mixed lipid dispersion in Hepes–Tris buffer, pH 7.3, with a microtip sonicator (Branson B-30) for 90 min (Mütsch et al., 1986). The small unilamellar donor vesicles used to measure exchange of cholesterol between two populations of small unilamellar lipid vesicles consisted of egg PC/egg phosphatidic acid/cholesterol (6.5:1.5:1.0, weight ratio) and a trace amount of [1α,2α(N)-^{3}H]cholesterol. Small unilamellar vesicles of this composition dispersed in Hepes–Tris buffer, pH 7.3, were produced by sonicating 0.65 mL of the mixed lipid dispersion (1 mg of lipid/mL) at room temperature under Ar in a bath sonicator (Laboratory Supplies, Hicksville, NY) for 2.5 h. Dispersions of mixed micelles consisting of egg lyso-PC/egg PC/cholesterol (60:38:2 weight ratio) in Hepes–Tris buffer, pH 7.3 (total lipid concentration 0.7 mg/mL), were prepared as described previously (Hauser, 1987).

Cholesterol Absorption by Brush Border Membrane Vesicles. Brush border membrane vesicles were incubated at room temperature with either small unilamellar vesicles or mixed micelles labeled with radiolabeled cholesterol or spin-labeled cholestane. After timed intervals, 0.1 mL of the incubation medium was diluted with 0.05 mL of Hepes–Tris buffer, pH 7.3. Donor vesicles were separated from brush border membranes by centrifugation at 80000*g* for 10 min in a Beckman airfuge. The donor vesicles remained in the supernatant under these conditions, and the radioactivity present in donor vesicles was counted in triplicate in a Beckman LS 7500 liquid scintillation counter. Spin-labeled lipid dispersions were made as described before (Mütsch et al., 1986). The concentration of spin-label in the donor particle was chosen such that its ESR spectrum was a simple spin-exchange line. Spin-label transferred to brush border membrane gave rise to an anisotropic ESR spectrum superimposed on the spin exchange line. The intensity of the anisotropic ESR spectrum was used to quantitate the lipid transfer from donor to acceptor (see below). The ESR method of monitoring lipid transfer from donor to acceptor membrane has the advantage that donor and acceptor particles need not be separated prior to determining ESR signal intensities of the label present in the acceptor membrane. Control experiments were carried out in which the brush border membranes were spun down after incubation with donor particles at 80000*g* for 10 min. In this way, the acceptor membranes were separated quantitatively from the donor particles. After resuspension of the brush border membrane pellet in buffer, the ESR spectrum was recorded, and signal intensities were measured by double integration. No difference in results was observed regardless whether or not donor and acceptor particles were separated before recording the ESR spectra.

Determination of Cholesterol Transfer Activity in the Supernatant. The papain treatment of brush border membrane described above released membrane proteins into the supernatant which exhibit cholesterol transfer or exchange activity between two different populations of small unilamellar phospholipid vesicles. The donor vesicles consisted of egg PC/egg phosphatidic acid/cholesterol (6.5:1.5:1.0, weight ratio) and trace amounts of [1α,2α(N)-^{3}H]cholesterol; the acceptor vesicles consisted of egg PC/cholesterol (8:1 weight ratio). Both phospholipid mixtures were dispersed in Hepes–Tris buffer, pH 7.3, and sonicated as described above. The two populations of small unilamellar vesicles thus produced were mixed so that the final total lipid concentration of donor vesicles was 0.1 mg/mL and that of acceptor vesicles 1 mg/mL. The mixture was incubated at room temperature in the absence and presence of supernate–protein (~1 mg/mL), i.e., protein liberated from brush border membrane by papain treatment. After timed intervals, the negatively charged donor vesicles were separated from the isoelectric acceptor vesicles by DEAE-Sepharose CL-6B chromatography (column size 2 × 0.6 cm, from Pharmacia). The negatively charged donor vesicles were retained on the DEAE-Sepharose column while the isoelectric acceptor vesicles were eluted from

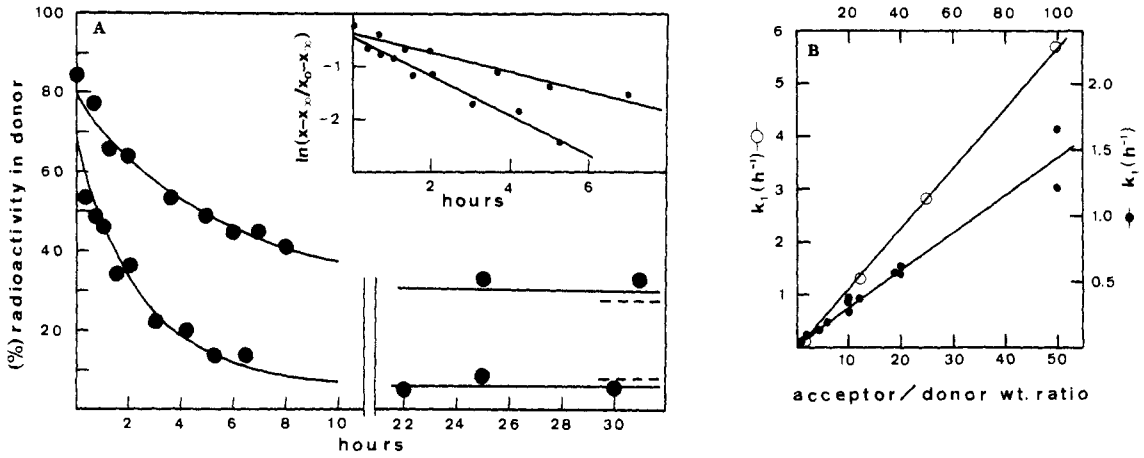

FIGURE 1: (A) Time course of cholesterol absorption from small unilamellar vesicles as the donor to brush border membrane as the acceptor. The donor vesicles consisting of egg PC/cholesterol (8:1 weight ratio) labeled with [1α,2α(N)-^{3}H]cholesterol were prepared and sonicated following standard procedures (Mütsch et al., 1986). The total lipid concentration of the donor vesicle was used as the donor concentration c_D. Brush border membrane vesicles were prepared from rabbit small intestine according to Hauser et al. (1980) and suspended in 0.01 M Hepes–Tris buffer, pH 7.3, containing 0.3 M mannitol, 5 mM EDTA, and 0.02% NaN_3. The resulting brush border membrane vesicles were fairly uniform in diameter, averaging about 200 nm (Perevucnik et al., 1985). 0.85 (0.46) mL of the brush border membrane suspension [23.5 (48.7) mg of protein/mL] was mixed with 0.20 (0.57) mL of the donor vesicles [11.3 (0.99) mg of lipid/mL, 1.8 (7.9) μCi of [^{3}H]cholesterol/mL] and 0.35 (0.47) mL of Hepes–Tris buffer. The values in parentheses refer to the bottom curve. The final concentrations in the incubation medium were [protein] = 14.3 (15.0) mg/mL; the total lipid concentration of brush border membrane lipid was found to be 0.86[protein]; hence, [brush border membrane lipids] = 12.3 (12.9) mg/mL, [donor lipids] = 1.6 (0.38) mg/mL, and [[^{3}H]cholesterol] = 0.26 (3.0) μCi/mL. The acceptor concentration c_A of brush border membrane vesicles was shown to be equal to 0.3[protein] (Mütsch et al., 1986). Hence, the acceptor concentration c_A was 4.3 (4.5) mg of lipid/mL. The calculated equilibria for true mass exchange are therefore 8% and 27% for the bottom and top curve, respectively (see dotted lines). After timed intervals, brush border membranes were separated from the donor vesicles by centrifugation at 80000*g* for 10 min, and the radioactivity (%) present in the supernatant containing the donor vesicles was counted in a Beckman LS 7500 liquid scintillation counter. Insert: Linearization of the curves shown in (A) according to $\ln [(x - x_\infty)/(x_0 - x_\infty)] = -k_1[(c_A + c_D)/c_A]t$ where $x_0 = 100\%$ at $t = 0$ and x and x_∞ represent fractional transfer of radiolabel at time t and at equilibrium ($t \rightarrow \infty$), respectively; k_1 is the pseudo-first-order rate constant; c_A and c_D are the acceptor and donor concentrations, respectively. (B) Pseudo-first-order rate constants k_1 (h^{-1}) as a function of the weight ratio of acceptor to donor lipid. Cholesterol absorption by brush border membrane from either mixed micelles of egg lyso-PC/egg PC/cholesterol (60:38:2 weight ratio; open symbols, top abscissa) or from small unilamellar vesicles of egg PC/cholesterol (8:1 weight ratio; closed symbols, bottom abscissa). The donor lipid particles were labeled with [1α,2α(N)-^{3}H]cholesterol. Cholesterol absorption from micellar dispersions (open symbols): 0.26 mL of the micellar dispersions (0.7 mg of total lipid/mL, 0.94 μCi of [^{3}H]cholesterol/mL) was mixed with increasing volumes of a brush border membrane suspension (22.3 mg of protein/mL), and to each mixture was added Hepes–Tris buffer to give the same total volume of 1.2 mL. The final concentrations in the incubation medium were [donor lipids] = 0.15 mg/mL containing 0.2 μCi of [^{3}H]cholesterol/mL, and the final brush border membrane protein concentration varied between 0.22 and 17.3 mg/mL (top line). Cholesterol absorption from small unilamellar vesicles (closed symbols): 0.12 mL of lipid dispersion (10 mg of total lipid/mL, 5 μCi of [^{3}H]cholesterol/mL) was mixed with increasing volumes of a brush border membrane suspension (65 mg of protein/mL), and Hepes–Tris buffer was added to a total volume of 1.2 mL. The final concentrations of donor lipids were 1 mg/mL containing 0.5 μCi of [^{3}H]cholesterol/mL, and the final brush border membrane protein concentration varied between 1.2 and 59 mg of protein/mL.

the column. The quantity of radiolabeled lipid transferred from donor to acceptor vesicles was determined by counting the radioactivity in two 0.5-mL aliquots of the eluate in a Beckman LS 7500 liquid scintillation counter. It should be noted that vesicle fusion does not interfere with the assay of cholesterol exchange between two populations of small unilamellar vesicles described above. Fusion between one donor and one acceptor vesicle leads to a reduction of the surface charge density by a factor of 2 while fusion between the same kind of vesicles has no effect on lipid composition. Control experiments showed that vesicles containing half of the egg phosphatidic acid of the donor vesicles are by and large still retained on the DEAE-Sepharose CL-6B column and hence make no contribution to the cholesterol transfer.

DEAE-Sepharose CL-6B Chromatography. DEAE-Sepharose CL-6B (0.6 mL) was packed into a Pasteur pipet which was closed at its tip with a glass wool plug. The column was rinsed with 1 mL of 0.01 M Hepes–Tris buffer, pH 7.3. Acceptor vesicles (0.1 mL containing 3 mg of lipid/mL) were applied to the column in order to saturate nonspecific binding sites. The column was rinsed with 1 mL of Hepes–Tris buffer, pH 7.3, and after this pretreatment, 0.1 mL of the incubation medium was applied, and the acceptor vesicles were eluted with 1 mL of Hepes–Tris buffer.

Other Methods. The ESR spectroscopic and the analytical methods used were described before (Mütsch et al., 1986). The protein concentrations were determined with bicinchoninic acid from Pierce using bovine serum albumin as a standard. ESR signal intensities were measured by double integration of the ESR spectra carried out on a Bruker BNC-12 computer interfaced with the Varian X-band spectrometer (Model E-104A).

Results and Discussion

Figure 1A shows the kinetics of the cholesterol absorption from small unilamellar vesicles of egg PC/cholesterol (8:1 weight ratio) to brush border membrane. The data Figure 1A were linearized (insert, Figure 1A), and pseudo-first-order rate constants were derived from the linear relations using established procedures (Mütsch et al., 1986; McKay, 1938; McLean & Phillips, 1981; Roseman & Thompson, 1980). The same kind of relations as shown in Figure 1A were obtained for the cholesterol absorption from mixed micelles of egg lyso-PC/egg PC (6:4 weight ratio). The cholesterol transfer from micelles is physiologically more relevant since the absorption of cholesterol and other dietary lipids in the small intestine is supposed to take place from mixed bile salt micelles. The results are summarized in Table I. The pseudo-first-order rate constants for cholesterol uptake from micelles are about twice as large as those for cholesterol uptake from small unilamellar vesicles. The k_1 values for cholesterol uptake from small unilamellar vesicles agree within experimental error with

Table I: Pseudo-First-Order Rate Constants k_1 for Cholesterol Transfer

donor[a]	acceptor[b]	acceptor lipid:donor lipid wt ratio	k_1 (h^{-1})[d]	$t_{1/2}$ (h)[c]	order of reaction	remarks
small unilamellar vesicles (egg PC/cholesterol = 8:1 wt ratio)	brush border membrane	50	1.42	0.49	second	
		10	0.286	2.42	second	
small unilamellar vesicles (egg PC/cholesterol = 8:1 wt ratio)	brush border membrane, papain treated	10	0.08	8.7	first	
mixed micelles (egg lyso-PC/egg PC/cholesterol = 60:38:2 wt ratio)	brush border membrane	100	5.82	0.12	second	
		50	2.80	0.25	second	
		10	0.50	1.39	second	
small unilamellar vesicles (egg PC/egg phosphatidic acid/cholesterol = 6.5:1.5:1.0 wt ratio)	small unilamellar vesicles (egg PC/cholesterol = 8:1 wt ratio)	10	0.10	6.3	first	without supernatant
		10	0.55	1.3	first	with supernatant

[a] The donor lipid particles contained a trace amount of [1α,2α(N)-^{3}H] cholesterol. The donor concentration c_D is equal to the total lipid concentration used. [b] The acceptor concentration is equal to the protein concentration of brush border membrane multiplied by a factor of 0.3 (Mütsch et al., 1986). [c] Half times ($t_{1/2}$) were calculated from $t_{1/2} = \ln 2/k_1$. [d] In the case of cholesterol exchange between two populations of small unilamellar vesicles, k_1 is a true first-order rate constant.

the k_1 value measured for the uptake of spin-labeled cholestane under comparable conditions (Mütsch et al., 1986). The rate constants for cholesterol absorption from both micelles and vesicles increase linearly with the weight ratio of acceptor/donor lipid (Figure 1B, Table I). Furthermore, logarithmic plots were made (I) of the initial reaction rate (v_0) as a function of the acceptor concentration (c_A) [keeping the donor concentration (c_D) constant] and (II) of v_0 as a function of c_D (c_A = constant). This was done for cholesterol uptake from micelles as well as from small unilamellar vesicles. The resulting plots were all linear (data not shown). According to the equation:

$$v_0 = k_2 c_A^m c_D^n$$

where k_2 is the second-order rate constant and the sum $m + n$ is identical with the order of the reaction; the slopes of such logarithmic plots are equal to the exponents, yielding values for m and n between 0.8 and 1 ($m + n = 1.6$–2). This result and the data in Figure 1B indicate that cholesterol absorption by brush border membrane from either micelles or small unilamellar vesicles is a second-order reaction. As reasoned below, the kinetic data are consistent with a mechanism involving collision-induced transfer of cholesterol [see also the discussion in the work of McLean and Phillips (1981)]. Although cholesterol absorption from both micelles and small unilamellar vesicles is a second-order reaction, there are significant differences in the uptake depending on the nature of the donor particle. In addition to differences in rate constants (cf. Table I), there are differences in the reaction mechanism. With micelles as the donor particle, there is net transfer of cholesterol; i.e., at equilibrium, all but ~5% of the cholesterol is incorporated in the lipid bilayer of brush border membrane (Mütsch et al., 1983, 1986). In contrast, with small unilamellar vesicles as the donor, there is true mass exchange: at equilibrium, cholesterol is evenly distributed between the donor and the acceptor pool of lipids. The expected equilibrium values calculated on the basis of the lipid composition are indicated by dotted lines (Figure 1A) and agree well with the experimental values.

Cholesterol absorption by brush border membrane from both mixed micelles and small unilamellar vesicles reveals saturation kinetics. The data for cholesterol transfer from either donor particle can be plotted according to the Michaelis–Menten equation or one of the linear transforms of this equation. The transfer of cholesterol from small unilamellar vesicles to brush border membrane vesicles was measured with increasing concentrations of donor lipid (egg PC/cholesterol = 8:1 weight ratio), keeping the brush border membrane concentration constant at 15 mg of protein/mL, corresponding to 12.9 mg of total membrane lipid/mL. Curves similar to those shown in Figure 1A were obtained for each donor concentration. If the data are plotted in a double-reciprocal way according to the Lineweaver–Burk equation, $1/v = 1/v_{max} + (K_M/v_{max})(1/c_D)$, good straight lines result; v is the reaction rate (velocity), v_{max} is the maximum value of the reaction rate, K_M is the Michaelis constant, and c_D is the donor concentration. For instance, if the data for cholesterol absorption from small unilamellar vesicles are plotted according to Lineweaver–Burk, the experimental data are best fitted by a linear regression analysis ($r^2 = 0.999$). The intercept of the resulting straight line on the y axis is $1/v_{max}$ [v_{max} = 31 pmol (mg of protein)$^{-1}$ s^{-1}], and the slope is K_M/v_{max}, yielding a Michaelis constant K_M = 3.8 mM.

Proteolytic treatment of brush border membrane with papain released ~25% of the total membrane protein. After papain treatment, the rate constants for cholesterol absorption by brush border membrane are significantly reduced (Table I). The rate constants are comparable to or smaller than the first-order rate constant measured for transfer (exchange) of cholesterol between two populations of unilamellar vesicles (cf. Figure 2, Table I). Furthermore, the cholesterol transfer from small unilamellar vesicles to papain-treated brush border membranes is independent of the weight ratio acceptor lipid/donor lipid and therefore a first-order reaction. As discussed below (cf. discussion of Figure 2), the kinetic data are consistent with cholesterol desorption from the donor bilayer, diffusion of monomeric cholesterol through the aqueous phase, and finally cholesterol incorporation into the acceptor bilayer. As a result of the papain treatment of brush border membrane, the cholesterol uptake by this membrane changes from a second-order reaction to a first-order one. At the same time, the reaction mechanism changes from collision-induced cholesterol uptake to a mechanism involving the diffusion of monomeric cholesterol through the aquoeus phase.

The protein(s) released into the supernatant by papain treatment of brush border membrane exhibit(s) cholesterol exchange activity. The time course of cholesterol exchange between two different populations of small unilamellar vesicles in the absence and presence of supernatant is shown in Figure 2. The data points represent the mean of three to four experiments. Pseudo-first-order rate constants can be derived from linearized plots (insert, Figure 2) and are included in Table I. These pseudo-first-order rate constants measured in the absence and presence of supernatant are independent of the acceptor concentration. This is different from the absorption of cholesterol by brush border membranes (cf. Figure 1B). In the latter case, k_1 was shown to increase linearly with the acceptor:donor weight ratio (Figure 1B) while the k_1 values

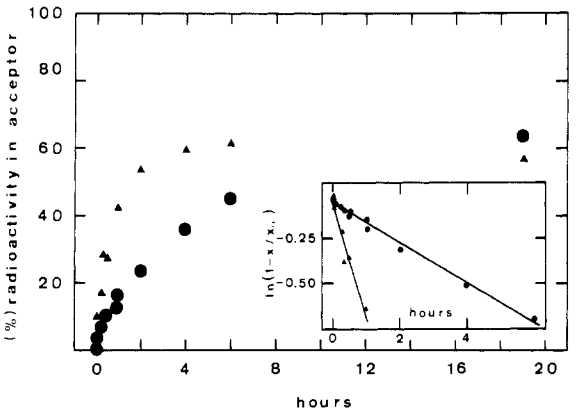

FIGURE 2: Exchange of cholesterol between two populations of small unilamellar vesicles in the presence (▲) and absence (●) of supernatant. The donor vesicles consisting of egg PC/egg phosphatidic acid/cholesterol (6.5:1.5:1.0 weight ratio) containing a trace amount of [1α,2α(N)-^{3}H]cholesterol and the acceptor vesicles consisting of egg PC/cholesterol (8:1 weight ratio) were prepared as described under Materials and Methods. Donor vesicles (0.055 mL at 1 mg/mL, 2.4 μCi of [^{3}H]cholesterol/mL) and acceptor vesicles (0.057 mL at 9.6 mg/mL) were mixed, and to this mixture was added either 0.44 mL of Hepes–Tris buffer in the control experiment or 0.44 mL of supernate–protein in the same buffer. Alternatively, the protein was added in an approximately isoosmolar NaCl buffer (0.01 M sodium phosphate, pH 7.3, 0.14 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3). The final concentrations in the incubation mixture were [donor lipid] = 0.1 mg/mL containing 0.24 μCi of [^{3}H]cholesterol/mL, [acceptor lipid] = 1 mg/mL, and [protein] = 0.53 mg/mL. After timed intervals, the two populations of vesicles were separated on a DEAE-Sepharose CL-6B column (2 cm × 0.6 cm, from Pharmacia) which retained the negatively charged donor vesicles while the neutral acceptor vesicles were eluted from the column. The cholesterol transfer was followed by measuring the radioactivity (%) appearing in the acceptor vesicles. Insert: Linearization of the curves according to $\ln(1 - x/x_\infty) = -k_1[(c_A + c_D)/c_A]t$ (Mütsch et al., 1986). The solid lines are least-squares fits.

for cholesterol exchange between two populations of small unilamellar vesicles in the presence of supernatant were independent of the acceptor:donor weight ratio between 10 and 300 (data not shown). The cholesterol transfer (exchange) between two populations of small unilamellar vesicles is therefore a first-order reaction in the absence and presence of supernate–protein. The order of the reaction is the same as that of cholesterol absorption by papain-treated brush border membrane. As shown in Table I, the effect of protein in the supernatant is to accelerate the cholesterol exchange (by a factor of 5 at 1 mg of protein/mL). The rate constant of cholesterol exchange measured in the absence of supernatant is in good agreement with published values (McLean & Phillips, 1981; Bloj & Zilversmit, 1977; Backer & Dawidowicz, 1979). These authors showed that the cholesterol exchange between two populations of unilamellar vesicles involves cholesterol desorption from the donor bilayer, diffusion of monomeric cholesterol through the aqueous phase, and finally cholesterol incorporation into the acceptor bilayer (McLean & Phillips, 1981). Since this kind of cholesterol exchange is a first-order reaction, the desorption step must be rate limiting. The effect of supernatant is then to facilitate the desorption process probably by collision-induced binding of cholesterol to a protein present in the supernatant (see discussion below).

Evidence that the exchange activity observed in the supernatant is due to protein(s) is provided by the following series of experiments: (I) the activity is nondialyzable and can be sedimented by centrifugation at 120000*g* for 15 h. (II) The activity is destroyed after briefly boiling the supernatant. Plotting the activity vs temperature gives a sigmoidal heat denaturation curve (data not presented) which shows that the cholesterol exchange activity is reduced to 50% at 57 °C. (III) The activity can be precipitated with $(NH_4)_2SO_4$ (2.7 M, 50% saturation). Upon exhaustive dialysis to remove $(NH_4)_2SO_4$, ~95% of the precipitate is redissolved; however, the activity regained is 40 ± 15%. (IV) No cholesterol exchange activity is observed in buffer containing urea at concentrations ≥5 M. Removal of urea by exhaustive dialysis restores 40 ± 10% of the original activity. (V) After proteinase K treatment of the supernatant, the activity is reduced to about 20%. (VI) The cholesterol exchange activity present in the supernatant exhibits a well-defined pH maximum at pH ≃ 7. (VII) The protein(s) present in the supernatant bind(s) cholesterol. This can be demonstrated by injecting an ethanolic solution of a spin-labeled cholesterol analogue (3-doxyl-5α-cholestane) into the supernatant (Figure 3). The spin-label concentration in ethanol is chosen such that a Heissenberg spin exchange spectrum is observed (Figure 3A). In the presence of supernatant, an anisotropic ESR spectrum develops characteristic of fast rotation of the spin-label about the molecular long axis (Figure 3B). The value of the maximum hyperfine splitting derived from the ESR spectrum is $A_\perp = 20 \pm 1$ G $\simeq (A_{zz} + A_{xx})/2$ which is the motional average of the A_{zz} and A_{xx} tensor components. The ESR line shape of the spectrum in the presence of supernatant (Figure 3B) resembles that of spin-labeled bilayers or micelles. In order to rule out the possibility that the ESR spectrum observed in Figure 3B is due to lipid contaminants present in the supernatant, a lipid extract of the supernatant was made with chloroform/methanol (2:1 by volume). TLC analysis of the lipids extracted revealed the presence of a small amount of lyso-PC, but not of diacylphospholipids. Evidence is presented in Figure 3C–E that the ESR spectrum in Figure 3B is due to spin-label–protein interaction and not due to spin-label being incorporated into lyso-PC micelles. The temperature dependence of the cholestane spin-label in the presence of supernatant is quite different from the temperature dependence of the spin-label incorporated into egg lyso-PC micelles (Figure 3C–E). The value of the maximum hyperfine splitting of the label in the presence of supernate–protein at 0 °C is $A_\perp \simeq 29$ G (Figure 3C). In contrast, the maximum hyperfine splitting observed when the label is present in lyso-PC micelles shows no temperature dependence, with $A_\perp = 19 \pm 1$ G at 21 and 0 °C (Figure D,E). The ESR spectrum of the cholestane probe in the presence of supernatant at 0 °C (Figure 3C) indicates that the motional averaging of the probe is significantly reduced compared to the situation when the label is present in lyso-PC micelles. The maximal hyperfine splitting $A_\perp \simeq 29$ G approaches the value of the A_{zz} tensor component under these conditions. This is interpreted to indicate that at 0 °C the spin-label becomes almost immobilized due to interaction with protein(s). This interpretation is corroborated by the following experiment. The two spin-labeled systems behaved quite differently in the presence of the reducing agent sodium ascorbate. At 21 °C and in the presence of excess sodium ascorbate (25 mM), the pseudo-first-order rate constant for the reduction of the cholestane spin-label present in egg lyso-PC micelles is about 8 times larger than that measured for the reduction of the label present in the supernatant (Table II). Furthermore, the reduction of the label in the presence of supernatant showed little temperature dependence whereas the reduction of the spin-labeled lyso-PC micelles was temperature-dependent (cf. Table II). These experiments show that the spin group in the 3-doxyl-5α-cholestane–supernate–protein complex is less accessible to the water-soluble reducing agent sodium ascorbate than when the same label is incor-

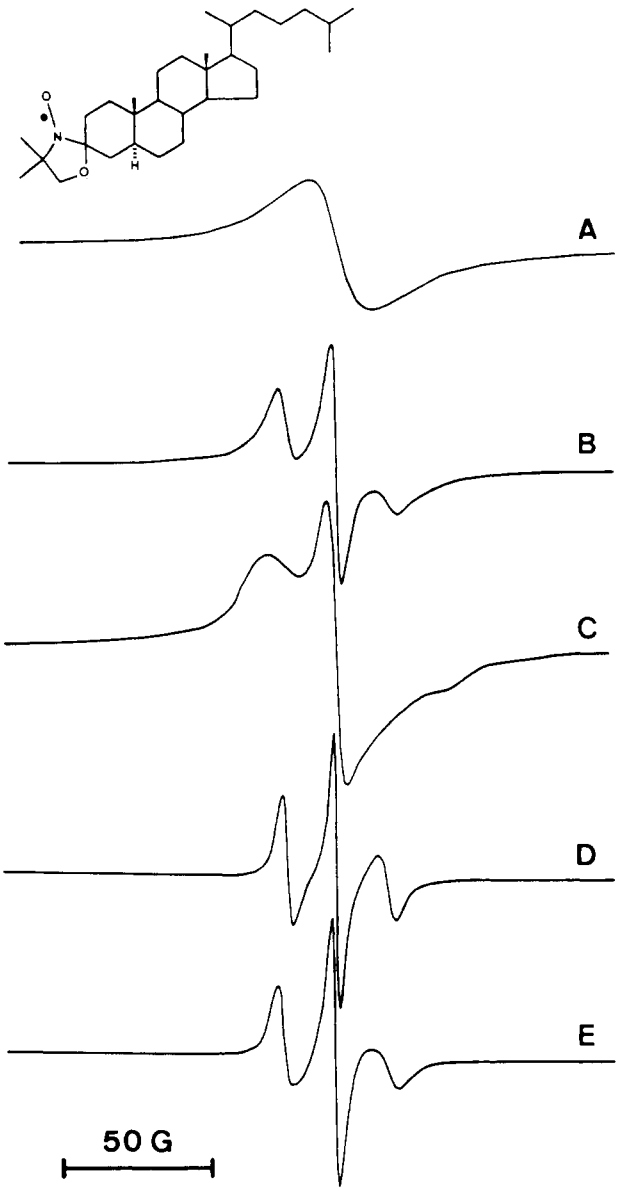

FIGURE 3: ESR spectra of 3-doxyl-5α-cholestane after incubation with supernatant and after incorporation into egg lyso-PC micelles. 16 μL of a 2.5 mM solution of the spin-label in ethanol was added to 64 μL of NaCl buffer (0.01 M sodium phosphate, pH 7.3, 0.14 M NaCl, 2.5 mM EDTA, and 0.02% NaN_3) so that the final spin-label concentration was 0.5 mM and that of ethanol was 20% (by volume). The ESR spectrum shown (A) was time independent. 16 μL of the same solution of spin-label in ethanol was added to 64 μL of NaCl buffer containing the supernate–proteins; the experimental conditions were as in (A). The mixture was incubated at 21 °C, and an anisotropic ESR spectrum was developed with time. The spectrum shown (B) was recorded after incubation for 1 h, at which time equilibrium was attained for all practical purposes. The ESR spectra A and B were recorded at 21 °C. A spectrum of the same sample as described under (B) was recorded at 0 °C (C). The cholestane spin-label was mixed with egg lyso-PC in $CHCl_3/CH_3OH$ (2:1 by volume), the organic solvent was evaporated on the rotary evaporator, and the dry lipid was dispersed in NaCl buffer containing 20% ethanol (by volume). The final concentration of the lysophospholipid was 25 mM, that of the cholestane spin-label 0.5 mM. ESR spectra of this dispersion were recorded at 21 °C (D) and at 0 °C (E).

porated into lyso-PC micelles. Our result with spin-labeled cholestane is similar to a spin-label study of soluble PC exchange protein from bovine liver (Devaux et al., 1977; Wirtz et al., 1980). These authors showed that 1-palmitoyl-2-(16-doxylstearoyl)-*sn*-phosphatidylcholine is strongly immobilized when the spin-label interacts with PC exchange protein. Furthermore, the spin group in the lipid–protein complex is

Table II: Pseudo-First-Order Rate Constants for the Reduction of Cholestane Spin-Label by Excess Sodium Ascorbate

system[a]	temp (°C)	k_1 (min^{-1})	$t_{1/2}$ (min)
3-doxyl-5α-cholestane incubated	21	0.15	4.6
with supernatant	0	0.13	5.3
3-doxyl-5α-cholestane incubated	21	1.2	0.58
with egg lyso-PC micelles	0	0.49	1.4

[a] The spin-label was either incubated with the supernatant of papain-treated brush border membrane or incorporated into egg lyso-PC micelles. For experimental details, see legend to Figure 3.

not accessible to ascorbate. This result was interpreted to indicate that the spin group on the fatty acyl chain of PC is buried in a protein pocket.

The fact that the cholesterol binding protein is liberated by proteolytic treatment of brush border membranes indicates that it is an intrinsic protein. It is not present in the basolateral membrane of the enterocyte, at least not to any significant extent. The protein(s) responsible for cholesterol absorption cannot originate in the endoplasmic reticulum. On the basis of experiments with marker proteins (cf. Materials and Methods), any significant contamination of our brush border membrane preparation by endoplasmic reticulum can be ruled out. Furthermore, our brush border membrane preparation was carefully separated by repeated centrifugation from cytosolic material until no lipid exchange activity was detectable in the dispersion medium. Hence, the protein described here cannot be of cytosolic origin. On the basis of the evidence presented, we propose that the portion of the protein responsible for the binding of cholesterol is extrinsic and exposed to the aqueous phase of the intestinal lumen. This protein has receptor-like properties for cholesterol and is probably associated with the brush border membrane proper via an anchor. A possible function of this protein consistent with the data presented here would be to catalyze the transfer of cholesterol from bile salt mixed micelles to the membrane surface and thus facilitate the uptake of cholesterol. Clearly, more work is required in order to elucidate its function. One approach we have taken is to purify the protein(s) responsible for the cholesterol exchange activity observed in the supernatant of papain-treated brush border membrane. This purification has been accomplished by a combination of gel filtration on Sephadex G-75 and affinity chromatography (data not shown). Raising antibodies against this protein should enable us to identify the intact protein in the brush border membrane. Furthermore, the antibody will be used to isolate and purify the protein(s) responsible for cholesterol absorption. This will be the subject of a future publication.

Conclusions. We can conclude that the cholesterol uptake by intact brush border membrane from either micelles or vesicles is a second-order reaction. The data are consistent with a mechanism involving collision-induced transfer of cholesterol. In the case of small unilamellar vesicles as the donor, fusion can be ruled out as a major mechanism since the observed distribution of radiolabeled cholesterol at equilibrium is close to that predicted for true mass exchange. With mixed micelles as the donor, lipid transfer may be coupled with membrane fusion, leading to the observed net transfer of cholesterol. The uptake of cholesterol by the brush border membrane from both donor particles appears to be saturable and catalyzed by an intrinsic protein of the brush border membrane. After proteolytic digestion of brush border membrane, cholesterol absorption is markedly slowed down. The rate constants measured under these conditions are representative of a passive diffusion mechanism. Protein(s) liberated from the brush border membrane by proteolysis bind(s)

cholesterol and exhibit(s) cholesterol exchange activity between two populations of small unilamellar vesicles. At present, it is unknown how the exchange activity present in the supernatant is related to the facilitated cholesterol absorption exhibited by brush border membrane vesicles.

The main result of our study is that cholesterol absorption by the brush border membrane of enterocytes is a facilitated process. This finding is at variance with current views on cholesterol absorption presented in review articles and text books [e.g., see Johnston and Borgström (1964), Patton (1981), Thomson and Dietschi (1981), Carey et al. (1983), Glickman (1983), Pind and Kuksis (1986), and Shiau (1987)]. The prevailing viewpoint is that cholesterol absorption from mixed micelles is an energy-independent, passive process involving the diffusion of cholesterol down a concentration gradient from the intestinal lumen via the brush border membrane to the cytosol of enterocytes.

We have shown that cholesterol absorption is protein-mediated in a brush border membrane system derived from rabbit small intestine. If generally true, this observation would have important implications. It holds out a prospect of regulating or possibly controlling cholesterol absorption in the gut. Good use could be made of the protein responsible for cholesterol absorption in developing specific inhibitors that partly or totally block cholesterol uptake. It is therefore highly desirable to demonstrate that cholesterol absorption in brush border membrane is protein-mediated under in vivo conditions and that this is a general phenomenon and not restricted to rabbit small intestine. Work along these lines is currently in progress.

ACKNOWLEDGMENTS

We thank Jeannette Stäuble for technical assistance. We thank Dr. B. Mütsch for performing some of the kinetic measurements. We are greatly indebted to Professor G. Schatz for valuable discussions.

Registry No. Cholesterol, 57-88-5.

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