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Cholesterol-transfer protein located in the intestinal brush-border membrane. Partial purification and characterization

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Cholesterol absorption by small intestinal brush border membrane vesicles from taurocholate mixed micelles is a second-order reaction. From a comparison of reaction rates and order before and after proteinase K treatment of brush-border membrane vesicles, it is concluded that cholesterol absorption is protein-mediated. It is shown that the desorption of cholesterol from taurocholate mixed micelles is by a factor of about 10^4 faster than that from egg phosphatidylcholine bilayers. When brush border membrane vesicles are stored at room temperature, intrinsic proteinases are activated and proteins are liberated from the brush border membrane. These proteins collected in the supernatant catalyze cholesterol and phosphatidylcholine exchange between two populations of small unilamellar phospholipid vesicles. One of the active proteins present in the supernatant is purified by a two-step procedure involving gel filtration on Sephadex G-75 SF and affinity chromatography on a Nucleosil-phosphatidylcholine column. The protein thus obtained is pure by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. It has an apparent molecular weight of slightly less than 14000 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and a value of 11500 determined by gel filtration on Sephadex G-75 SF.

Introduction

Previously, we reported that the absorption of cholesterol by small intestinal brush border membrane from either mixed micelles or small unilamellar phosphatidylcholine vesicles is protein-mediated [1]. With micelles as the donor particles, there is net transfer of cholesterol from micelles to the brush border membrane while with small unilamellar phospholipid vesicles there is true mass exchange: cholesterol at equilibrium appears to be evenly distributed between the lipid pools of the donor and acceptor particles. We were also able to show that the protein-mediated cholesterol absorption is abolished by proteolytic treatment of brush border membrane [1].

After papain digestion of the brush border membrane, cholesterol absorption was significantly reduced. The residual cholesterol absorption observed was a 'passive' process: the mechanism involves cholesterol desorption from the donor particle, diffusion of monomeric cholesterol through the aqueous phase and incorporation of cholesterol into the bilayer of the brush border membrane [1]. After papain digestion of the brush border membrane, cholesterol absorption is therefore mechanistically different from the protein-mediated process. It is a true first-order reaction while the protein-mediated absorption is a second-order reaction.

We previously reported that the protein responsible for cholesterol absorption is liberated from brush border membrane by proteolysis [1,2]. It is very likely that we are dealing with an integral membrane protein. It is also very likely that after proteolytic treatment, a water-soluble portion of the integral membrane protein containing the lipid binding site is released. It was stressed [2] that proteolysis is due to intrinsic proteinases and the rate of proteolysis can be enhanced by the addition of external

Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; CHAPS, 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulfonate; IAM, immobilized artificial membrane; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

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proteinases, such as papain. The protein(s) thus liberated from the membrane structure exhibit(s) lipid exchange activity in a model system. The water-soluble protein(s) released into the supernatant by proteolysis, catalyze(s) the exchange of cholesterol as well as phosphatidylcholine between two populations of small unilamellar phospholipid vesicles [1,2].

Here, we report on cholesterol absorption by brush border membrane vesicles from mixed micelles. We confirmed that: (i) there is net transfer of cholesterol from mixed micelles to brush border membrane; (ii) cholesterol absorption is a second-order reaction and protein-mediated; (iii) cholesterol absorption is much more efficient from taurocholate mixed micelles than from lysophosphatidylcholine/phosphatidylcholine mixed micelles. The composition of the taurocholate mixed micelles resembles more closely that of mixed micelles present in the small intestine. Proteolysis of brush border membrane abolishes the efficient absorption of cholesterol. In the second part of the paper, the purification of a protein is described that binds cholesterol and is probably involved in cholesterol absorption in the brush border membrane. Some physicochemical properties of this protein are discussed.

Materials and Methods

Materials

Egg phosphatidylcholine, egg lysophosphatidylcholine, and egg phosphatidic acid were purchased from Lipid Products (South Nutfield, Surrey, U.K.), cholesterol (puriss.) from E. Merck (Darmstadt, F.R.G.), radiolabeled [1α,2α(n)-^{3}H]cholesterol ([^{3}H]cholesterol, specific activity 44 Ci/mmol) from Amersham International (Amersham, Buckinghamshire, U.K.), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, >98%) from Fluka Chemie AG (Buchs, Switzerland), 1,12-dodecanedicarboxylic acid from Aldrich-Chemie (Steinheim, F.R.G.), taurocholate and α-chymotrypsin from bovine pancreas from Sigma Chemical Company (St. Louis, MO), monooleoylglycerol and oleic acid from Applied Science (State College, PA), Sephadex G-75 SF (superfine), Sephacryl S-200 HR and Blue dextran 2000 from Pharmacia (Dübendorf, Switzerland), Bio-Gel P 10 from Bio-Rad (Glattbrugg, Switzerland), [*carboxyl*-^{14}C]cholic acid and [U-^{14}C]sucrose (specific activity 4 mCi/mmol) from NEN (Du Pont de Nemours International S.A., Regensdorf, Switzerland), porcine thyroglobulin, bovine serum albumin, ovalbumin and cytochrome *c* from Serva Feinbiochemica GmbH & Co. (Heidelberg, F.R.G.). 1-Myristoyllysophosphatidylcholine was custom-synthesized by Avanti Polar Lipids, Inc. (Birmingham, AL). Nucleosil-phosphatidylcholine was prepared as described previously [3,4]. Sodium taurocholate was recrystallized twice from ethanol/diethyl ether (17:40, v/v). All lipids used were pure by TLC standard. All other chemicals used were of analytical grade. The water for preparing aqueous solutions and lipid dispersions was double-distilled in a quartz apparatus.

Methods

Preparation of brush border membrane vesicles

Rabbits of different breed were killed in a rabbit slaughter house and small intestines were excised, cut into pieces of about 30 cm length, and each piece was thoroughly rinsed with physiological saline. The pieces were frozen and stored at −80°C prior to the preparation of brush border membrane vesicles. The preparation of these vesicles was carried out routinely following the procedure outlined in a previous publication [1]. Unless stated otherwise, the resulting brush border membrane vesicles were suspended in buffer A (10 mM Hepes adjusted with Tris to pH 7.3, 0.3 M D-mannitol, 5 mM EDTA, 0.02% NaN_3). Vesicles that were not used immediately after preparation were frozen with liquid nitrogen and stored at −35°C. The purity of the brush border membrane preparation was checked as described [1]. This brush border membrane preparation is not contaminated with basolateral membrane, other cell organelle membranes, or cytosolic compounds.

Preparation of small unilamellar phospholipid vesicles and mixed micelles

Small unilamellar vesicles of egg phosphatidylcholine/cholesterol (1:0.25, w/w) and egg phosphatidylcholine/egg phosphatidic acid/cholesterol (1:0.2:0.15, w/w) containing a trace of [^{3}H]cholesterol were made by sonicating phospholipid dispersions in buffer A as described [2]. Mixed micelles of egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol (60:38:2, w/w) containing a trace amount of [^{3}H]cholesterol and of taurocholate/oleic acid/monooleoylglycerol/cholesterol (88:6:3.6:2.6, w/w) containing a trace of [^{3}H]cholesterol were made as follows: the lipids dissolved in $CHCl_3/CH_3OH$ were mixed in the appropriate wt. ratio and the organic solvent was removed by rotary evaporation at room temperature. The resulting lipid film was dried under vacuum at a pressure of 0.04/hPa for at least 1 h. The dried lipid was dispersed in the appropriate volume of buffer A to yield the desired micellar concentration.

Preparation of supernate-proteins

It was reported that proteins are liberated from brush border membrane vesicles during storage at temperatures >2°C [1,2]. The proteins released by this treatment are referred to as supernate-proteins. Water-soluble supernate-proteins were produced by suspending

brush border membrane vesicles in buffer A and storing the suspension at 2 ± 2°C for 2 h. At the end of the incubation, 15 volumes of buffer B (10 mM sodium phosphate (pH 7.3), 0.14 M NaCl, 2.5 mM EDTA and 0.02% NaN_3) were added and the proteins released into the supernatant were separated from brush border membrane vesicles by centrifugation at 120000 × *g* for 30 min. Supernate-proteins were fractionated by gel filtration on Sephadex G-75 SF or Sephacryl S-200 HR as described [3].

Cholesterol absorption by brush border membrane vesicles from mixed micelles

Brush border membrane vesicles (0.2 to 15 mg protein/ml) as the acceptor were incubated at room temperature with mixed micelles of egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol (60 : 38 : 2, w/w; 0.15 mg/ml) as the donor particles. The latter particles contained a trace of radioactive [^{3}H]cholesterol so that the kinetics of cholesterol absorption by brush border membrane could be followed. The final concentrations of the components of the mixed micelles were [egg lysophosphatidylcholine] = 0.17 mM, [egg phosphatidylcholine] = 0.075 mM and [cholesterol] = 7.75 μM. After timed intervals, 0.1 ml of the incubation medium were diluted with 0.05 ml buffer A and donor and acceptor particles were separated by centrifugation at 100000 × *g* for 10 min in a Beckman airfuge (Beckman Instruments International SA, Zürich). The radioactivity of the micelles remaining in the supernatant was determined in a Beckman LS 7500 liquid scintillation counter.

Cholesterol absorption by brush border membrane vesicles from mixed micelles consisting of taurocholate/oleic acid/monooleoylglycerol/cholesterol (88 : 6 : 3.6 : 2.6, w/w) and a trace amount of [^{3}H]cholesterol was too fast to be measurable as described above. The kinetics of cholesterol absorption from this kind of mixed micelles was measured using a home-made apparatus previously described [4]. Cholesterol absorption was initiated by mixing a droplet of 4 μl brush border membrane suspension (34 mg protein/ml) in buffer A with a droplet of 4 μl of a dispersion of taurocholate mixed micelles (6 mg lipid/ml). Both droplets were placed on the bottom of a clear polystyrene test tube (diameter 9 mm, length 67 mm) and mixing of the two droplets was accomplished by vibration. The frequency was about 60 Hz and both frequency as well as amplitude of the vibration were electronically controlled. The final concentrations of components of the taurocholate mixed micelles were: [taurocholate] = 4.8 mM, [oleic acid] = 0.6 mM, [monooleoylglycerol] = 0.3 mM and [cholesterol] = 0.2 mM. The reaction was stopped by automatic injection of 2 ml of ice-cold buffer A into the test tube. The contents of the test tube was poured onto a cellulose nitrate filter (mean pore size 0.65 μm) and the test tube was washed twice with 2 ml ice-cold buffer A.

The cellulose nitrate filters retained only 80 ± 10% of brush border membrane vesicles and about 6 ± 2% of the taurocholate micelles as determined in control experiments. Values for cholesterol absorption by brush border membrane were therefore corrected by taking into account, that retention of brush border membrane vesicles was incomplete, and that approx. 6% of taurocholate micelles were absorbed to the filter.

Determination of the cholesterol exchange activity in the supernatant of brush border membrane vesicles

Supernate-proteins were produced as described above. It was reported [1,2] that these proteins catalyze cholesterol and phosphatidylcholine exchange between two populations of small unilamellar vesicles. The donor vesicles consisted of egg phosphatidylcholine/egg phosphatidic acid/cholesterol (1 : 0.2 : 0.15, w/w) and a trace amount of [^{3}H]cholesterol while the acceptor vesicles were phosphatidylcholine/cholesterol vesicles (1 : 0.25, w/w) [1]. The exchange of radiolabelled cholesterol between donor and acceptor vesicles was measured as described [1,2].

Purification of supernate-proteins

(i) Gel filtration on Sephadex G-75 SF. Supernate-proteins produced as described above were concentrated in an Amicon ultrafiltration equipment using Amicon PM 10 or YM 10 filters with a cut-off molecular weight of about 10^4 (Grace AG, Amicon Schweiz, Wallisellen, Switzerland). About 10 ml of the concentrated protein-solution (approx. 25 mg/ml) in buffer B were applied to a Sephadex G-75 SF column (29.7 × 4.4 cm) which was equilibrated and eluted with buffer B at a flow rate of approx. 29 ml/h. About 6-ml fractions were collected in an automatic fraction collector and each fraction was analyzed by UV absorption at 254 nm, for protein content and phosphatidylcholine as well as cholesterol exchange activity [1,2]. The protein recovery was 80 ± 5%, the yield of the phosphatidylcholine activity was 80 ± 15% (three experiments). The column was calibrated using the following proteins as markers: thyroglobulin (apparent molecular weight $M_r \approx$ 660 000); bovine serum albumin ($M_r \approx$ 67 000); ovalbumin ($M_r \approx$ 45 000); α-chymotrypsin ($M_r \approx$ 25 000); cytochrome *c* ($M_r \approx$ 12 400). The distribution function $K_d = (V_e - V_o)/(V_t - V_o)$ was plotted semi-logarithmically as a function of the apparent molecular weight M_r of the marker proteins yielding a good straight-line relationship. V_e is the elution volume of the protein, V_o and V_t are the column void volume and total volume, respectively. V_o and V_t were determined by chromatographing Blue dextran 2000 and [^{14}C]sucrose. Supernate-proteins were also fractionated on smaller

Sephadex G-75 SF columns and on Sephacryl S-200 HR for analytical purposes [3].

(ii) Immobilized artificial membrane (IAM) chromatography. Column chromatography based on phosphatidylcholine-bonded silica was introduced by one of us [5,6]. 1-Myristoyl-2-(13-carboxyltridecanoyl)-*sn*-phosphatidylcholine was bonded to silica particles in a four step process as described by Pidgeon and his co-workers [6]. As silica particles Nucleosil-300 ($7NH_2$) was used which is a brand name for silica derivatized with propylamine. The surface density of the propylamine groups was similar to the density of hydrocarbon chains present on the surface of chromatographic support material used for reverse phase chromatography. The resulting chromatographic material was denoted as Nucleosil-phosphatidylcholine [6]. It consists of silica particles of 7 μm diameter with 30 nm pores to which dimyristoylphosphatidylcholine is covalently linked via an amide bond as was demonstrated by infrared spectroscopy [6]. Nucleosil-phosphatidylcholine is one type of immobilized artificial membrane. It was packed into a HPLC column (dimensions 10 cm × 0.46 mm) and rinsed with acetone. Prior to use, acetone was replaced by distilled water and the column was equilibrated with buffer B containing 0.125% CHAPS. The fractions of peak 3 obtained by gel filtration of supernate-proteins on Sephadex G-75 SF were pooled and concentrated using the Amicon filtration equipment and YM 10 Amicon filters. To the concentrated protein solution containing approx. 4.8 mg/ml, CHAPS was added to a final concentration of 0.125%. The solution was centrifuged at about 100 000 × *g* for 20 min in a Beckman TL 100 ultracentrifuge with a TLA 100.3 rotor, and 1.9 ml of the supernatant were applied to the Nucleosil-phosphatidylcholine column using a LKB 2152 controller equipped with two LKB 2150 pumps (from Pharmacia LKB). All solvents and solutions were filtered through a Millipore filter (mean pore size 0.45 μm) and degassed prior to their application to the Nucleosil-phosphatidylcholine column. The column was eluted with buffer B at a flow rate of 0.7 ml/min and 0.7 ml fractions were collected in an automated fraction collector and analyzed for protein. Since the presence of CHAPS interfered with the determination of lipid exchange activity, 0.2 ml of each fraction were dialyzed exhaustively against buffer B using Visking dialysis tubing (Union Carbide, cut-off molecular weight 6000–8000). After dialysis, the lipid exchange activity was determined as described above.

(iii) Reverse phase chromatography. Reverse phase chromatography was performed on a butyl-Nucleosil column of dimension (6 × 0.4 cm). The butyl-Nucleosil particles of 5 μm diameter containing 30 nm pores were obtained from Macherey-Nagel AG (Oensingen, Switzerland). The column was equilibrated with 0.1% trifluoroacetic acid. Proteins applied to the column were eluted with a linear gradient of *n*-propanol in 0.1% trifluoroacetic acid (0–50% in about 30 min).

Analytical procedures

Lipid phosphorus was determined according to Chen et al. [7], protein according to Lowry et al. [8] or alternatively with bicinchoninic acid [9] using bovine serum albumin as the standard. The reaction kit for the latter method was obtained from Pierce Europe (Oud Beijerland, The Netherlands). The lipid content of brush border membrane vesicles was determined gravimetrically or by phosphorus analysis after lipid extraction. To this end, brush border membrane lipids were extracted with chloroform-methanol. The extraction procedure and TLC analysis of lipids were carried out as detailed before [10]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out using the Mini-Protean II Dual Slab Cell from Bio-Rad according to the Bio-Rad instruction manual. Bio-Rad low molecular weight markers were used as standards. Proteins were visualized by Coomassie blue or silver staining.

Results

Kinetics of cholesterol uptake from mixed micelles

Fig. 1 shows the kinetics of cholesterol absorption by brush border membrane from mixed micelles consisting of egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol (60 : 38 : 2, w/w). Fig. 1A gives the amount of radioactive cholesterol remaining in the donor micelles as a function of the time of incubation of donor and acceptor particles. The data of Fig. 1A were linearized (Fig. 1B) assuming that the radioactivity of the donor particles decreases exponentially [11–14]. Pseudo-first-order rate constants were derived from the linear relations shown in Fig. 1B and the values for the pseudo-first-order rate constants thus obtained are listed in Table I. The pseudo-first-order rate constants increased linearly with the weight ratio of acceptor/donor lipid as shown in Fig. 1C (cf. Table I) suggesting that cholesterol absorption from mixed micelles is a second-order reaction. This was confirmed by plotting the data according to the general rate law expressed in Eqn. 1:

$$-(d[D]/dt)_{initial} = k[A]_0^a[D]_0^d \quad (1)$$

where $(d[D]/dt)_{initial}$ is the initial rate of cholesterol absorption, $[A]_0$ and $[D]_0$ are the initial acceptor and donor concentrations, respectively, a and d are the order of the reaction with respect to the acceptor and donor concentration, respectively, and k is the rate constant. Plotting log of the initial rate of cholesterol absorption versus log of the initial acceptor concentration keeping the donor concentration constant yielded a straight line of slope $a = 1.1$ (data not shown). Cholesterol absorption by brush border membrane is

therefore a first-order reaction in the brush border membrane (acceptor) concentration and a second-order reaction overall.

The kinetics of cholesterol absorption from taurocholate mixed micelles are shown in Fig. 2. The taurocholate mixed micelles behaved quite differently from

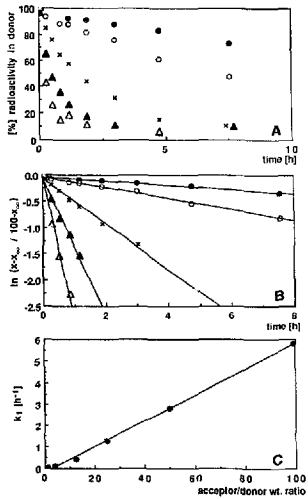

Fig. 1. (A) Time course of cholesterol absorption from mixed micelles (donor particles) to brush-border membrane vesicles (acceptor particles). The donor particles were mixed micelles consisting of egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol (60:38:2, wt. ratio) labeled with a trace of [1α,2α(n)-^{3}H]cholesterol. The acceptor particles were brush-border membrane vesicles. Dispersions of donor and acceptor particles in buffer A were mixed so that the final concentration of donor lipids was 0.15 mg lipid/ml containing 0.2 μCi [^{3}H]cholesterol/ml and the final brush border membrane concentrations were 0.22 (0.19) (●) 0.73 (0.63) (○), 2.2 (1.9) (×), 4.4 (3.8) (▲) and 8.7 (7.5) (△) mg protein/ml. The numbers in parentheses are the brush-border lipid concentration which was calculated as 0.86 × protein concentration. After timed intervals brush-border membrane vesicles were separated from the donor micelles by centrifugation at 100000 × g for 10 min. The micelles remained in the supernatant and the amount of radioactive cholesterol remaining in the micelles was determined in a Beckman LS 7500 liquid scintillation counter. (B) The curves depicted in (A) were linearized according to $\ln[(x - x_\infty)/(x_0 - x_\infty)] = -kt$ where $x_0 = 100\%$ at $t = 0$ and x and x_∞ represent the fractional transfer of radioactivity at time t and at equilibrium ($t \to \infty$), respectively. (C) Pseudo-first-order rate constants k_1 (h^{-1}) as a function of the weight ratio of brush-border membrane lipid to total lipid present in mixed micelles. k_1 values were determined for the following lipid wt. ratios: 1.25, 4.2, 12.5, 25, 50, 99. The solid line is a least-squares fit to the experimental data ($r^2 = 0.999$).

TABLE I

Pseudo-first-order rate constants k_1 and half times $t_{1/2}$ for cholesterol absorption by brush border membrane

As donor particles, two types of mixed micelles were used, either micelles consisting of egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol (60:38:2, w/w), or taurocholate mixed micelles consisting of taurocholate/oleic acid/monooleoylglycerol/cholesterol (88:6:3.6:2.6, w/w), both containing a trace of [^{3}H]cholesterol. The pseudo-first-order rate constants (k_1) were derived from the linearization of the curves shown in Figs. 1A and 2 as discussed in the text. The decrease in cholesterol content of the taurocholate mixed micelles shown in Fig. 2 is not a simple exponential function. Instead, the initial rapid decrease of radioactivity within the first 10 s (Fig. 2) can be fitted by the sum of two exponentials. Therefore, two rate constants were derived for cholesterol absorption from mixed taurocholate micelles.

Donor	Acceptor	Acceptor lipid/donor lipid (wt. ratio)	k_1 (h^{-1})	$t_{1/2}$
Egg lyso-phosphatidyl-choline mixed micelles	brush border membrane vesicles	13	0.45	1.5 h
		25	1.3	0.53 h
		50	2.8	0.25 h
		100	5.9	0.12 h
Tauro-cholate mixed micelles	brush border membrane vesicles	3.3	$1.0 \cdot 10^4$	0.24 s
			$2.8 \cdot 10^2$	9.0 s
Tauro-cholate mixed micelles	brush border membrane vesicles stored at room temperature for two hours	3.3	$2.1 \cdot 10^3$	1.2 s
			2.0	0.34 h
Tauro-cholate mixed micelles	brush border membrane vesicles after proteinase K treatment	3.3	0.63	1.1 h

egg lysophosphatidylcholine/egg phosphatidylcholine mixed micelles. Comparing the time courses of cholesterol absorption (Fig. 1A and Fig. 2), it is obvious that cholesterol absorption is much faster from the taurocholate mixed micelles. The halftime $t_{1/2}$ of cholesterol absorption from taurocholate mixed micelles is in the order of 1 s. Under comparable conditions, the halftime $t_{1/2}$ of cholesterol absorption from egg lysophosphatidylcholine/egg phosphatidylcholine mixed micelles is in the order of several hours, i.e., the absorption of cholesterol from taurocholate mixed micelles is about 10^4 times faster. Furthermore, as mentioned above each curve shown in Fig. 1A represents a single exponential; in contrast, the decrease in radioactivity of the taurocholate micelles is not a simple exponential function. The initial rapid decrease in radioactivity (Fig. 2) occurring during the first 10 s can be fitted by the sum of two exponentials. The two k values thus derived

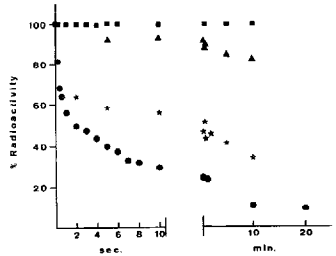

Fig. 2. Time course of cholesterol absorption from taurocholate mixed micelles as the donor to brush-border membrane vesicles as the acceptor. The taurocholate mixed micelles consisted of taurocholate/oleic acid/monooleoylglycerol/cholesterol = 88 : 6 : 3.6 : 2.6 (by weight) containing a trace of [1α,2α(n)-^{3}H]cholesterol. Donor and acceptor particles both suspended in buffer A were mixed so that the final micellar concentration was 3 mg total lipid/ml and the lipid concentration of brush-border membrane was 10 mg lipid/ml. The cholesterol absorption was stopped and donor and acceptor particles were separated by filtration as described in Materials and Methods. The amount of radiolabeled cholesterol remaining in the micelles was determined by scintillation counting in a Beckman LS 7500 liquid scintillation counter. Untreated brush border membrane (●); brush-border membrane incubated with taurocholate mixed micelles containing a trace of [*carboxyl*-^{14}C]cholate (■); brush border membrane after proteinase K treatment (▲); brush-border membrane stored at room temperature for 2 h before cholesterol absorption was determined (★).

were $k_1 = 1.0 \cdot 10^4$ h^{-1} and $k_1' = 2.8 \cdot 10^2$ h^{-1} corresponding to half-lives of 0.24 s and 9.0 s, respectively (Table I).

In a double-labeling experiment taurocholate mixed micelles were used containing trace amounts of [^{3}H]cholesterol and sodium [^{14}C]cholate. As shown in Fig. 2, the absorption by brush border membrane of cholate from these micelles was negligible. Also included in Fig. 2 are the effects of storing brush border membrane vesicles at room temperature and the digestion of these vesicles by proteinase K. After storing brush border membrane at room temperature for 2 h, cholesterol absorption was slowed down (Fig. 2). Again, the initial cholesterol uptake within the first 10 s could be fitted by the sum of two exponentials yielding k values of $k_1 = 2.1 \cdot 10^3$ h^{-1} ($t_{1/2} = 1.2$ s) and $k_1' = 2.0$ h^{-1} ($t_{1/2} = 0.34$ h) (Table I). After proteinase K treatment, the brush border membrane apparently lost its ability to take up cholesterol effectively from taurocholate mixed micelles (Fig. 2). Even after proteinase K treatment, about 5% of the radioactive cholesterol present in taurocholate mixed micelles appeared to be taken up rapidly by brush border membrane. The mechanism of this uptake is unknown. However, apart from this minor contribution, cholesterol absorption after proteolysis was independent of acceptor concentration and hence a first-order reaction (data not shown) with a rate constant $k_1 = 0.63$ h^{-1} ($t_{1/2} = 1.1$ h) (Table I).

All cholesterol uptake measurements were performed at room temperature. Cholesterol uptake at 37°C, though physiologically relevant, was too fast to be measurable with the methods used here.

Purification of a protein that catalyzes cholesterol exchange and phosphatidylcholine exchange between two populations of small unilamellar vesicles

Supernate-proteins were produced as described in Materials and Methods. It can be shown that these proteins are active and catalyze the exchange of both cholesterol and phosphatidylcholine between two populations of small unilamellar vesicles. These experiments were described and discussed in some detail previously [1,2]. It suffices to say that cholesterol exchange was accelerated about 5-fold in the presence of supernate-proteins (1 mg/ml) [1] and phosphatidylcholine exchange by a factor of about 200 [2].

The active protein(s) released from brush border membrane by proteolysis, and responsible for cholesterol and phosphatidylcholine exchange between two populations of small unilamellar vesicles were purified by two steps:

(i) gel filtration on Sephadex G-75 SF and

(ii) by immobilized artificial membrane (IAM) chromatography on a Nucleosil-phosphatidylcholine-HPLC column. The gel filtration pattern of supernate-proteins on Sephadex G-75 SF is shown in Fig. 3A. Most of the protein (80–85%) was eluted as an asymmetric peak at the column void volume V_o. Only minor protein peaks appeared at elution volumes $V_e > V_o$. The eluate was also analyzed for phosphatidylcholine and cholesterol exchange activity. The phosphatidylcholine exchange activity was eluted in three well resolved peaks (designated peaks 1 to 3, Fig. 3A). The elution volume of all three peaks was well reproducible within the error of the measurement. Using the calibration curve (cf. inset, Fig. 3A), elution volumes were converted to apparent molecular weights and the values thus obtained for peaks 1, 2 and 3 were $M_r \geq 70\,000$, $M_r = 22\,000 \pm 2\,000$ and $M_r = 11\,500 \pm 1\,250$, respectively. The relative phosphatidylcholine exchange activities in peaks 1,2 and 3 were 29 ± 6%, 29 ± 4% and 42 ± 8%, respectively (n = five experiments). The relative intensities of the three peaks varied from experiment to experiment as indicated by the large standard deviations of the mean. Protein present in peak 3 was pooled, concentrated, and rechromatographed on Sephadex G-75 SF. Peak 3 was eluted as a single peak at an elution volume V_e consistent within experimental error with the first chromatogram shown in Fig. 3A. The same was true when peak 2 was rechromatographed. In order to check the homogeneity of peak 3, 0,1 ml of the protein solution of

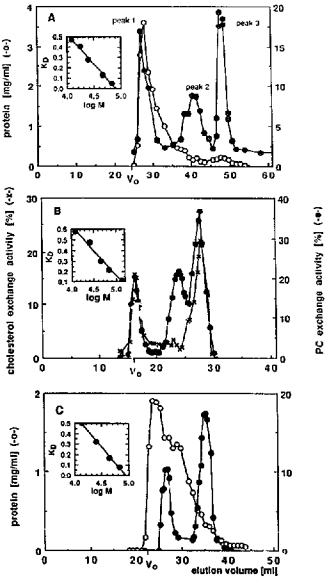

Fig. 3. (A) Gel filtration on Sephadex G-75 SF of supernate-proteins liberated from brush-border membrane by proteolysis (see Materials and Methods). About 10 ml of solution of supernate-proteins in buffer B (approx. 25 mg protein/ml) were applied to the Sephadex G-75 SF column (29.7×4.4 cm), and the protein was eluted with buffer B at a flow rate of approx. 29 ml/h. Fractions of 6 ml were collected in an automatic fraction collector and analyzed for protein (○——○) and phosphatidylcholine exchange activity (●——●). V_o = 24.3 ml, V_t = 70.0 ml. (B) Gel filtration of supernate-proteins on Sephadex G-75 SF. 0.8 ml of the supernate-proteins in buffer B (approx. 37 mg protein/ml) were applied to the Sephadex G-75 SF column (50×1 cm) and the protein was eluted with buffer B at a flow rate of 4 ml/h. Fractions of 0.3 ml were collected in an automatic fraction collector and analyzed for cholesterol exchange (×——×) and phosphatidylcholine exchange activity (●——●). V_o = 16.0 ml, V_t = 40.5 ml. (C) Gel filtration of supernate-proteins on Sephacryl S-200 HR. 0.8 ml of protein solution (21 mg/ml) were applied to the Sephacryl S-200 HR column (53×1.1 cm) and the protein was eluted with buffer B at a flow rate of 6,5 ml/h. Fractions of 0.44 ml were collected in an automatic fraction collector and analyzed for protein (○——○) and phosphatidylcholine exchange activity (●——●). V_o = 22.5 ml, V_t = 48.7 ml. Calibration curves of the columns are presented as insets. The logarithm of the apparent molecular weight of various marker proteins in units of thousands is plotted as a function of $K_d - (V_e - V_o)/(V_t - V_o)$ where V_e is the elution volume of the protein, V_o and V_t are the void and total volume of the column, respectively.

peak 3 (5.91 mg protein/ml) was chromatographed on a Bio-gel P 10 column (18.5 × 0.84 cm) which was equilibrated and run with 10 mM sodium phosphate buffer (pH 7.3) containing 0.14 M NaCl, 2.5 mM EDTA and 0.02% NaN_3. The protein elution pattern consisted of a main slightly asymmetric peak with a shoulder at the tailing edge. This peak exhibited also phosphatidylcholine exchange activity but the peak position of the phosphatidylcholine exchange activity was displaced (to a larger elution volume) relative to that of the protein peak (data not shown). The displacement of the two peaks indicates that protein of peak 3 is inhomogeneous with respect to size (see below). The peak position in the phosphatidylcholine exchange activity pattern was close to that of ribonuclease A corresponding to a molecular weight of 13 700.

The cholesterol exchange activity was eluted from the Sephadex G-75 SF column in two well resolved peaks, the first one in the column void volume V_o and the second one at V_e consistent with peak 3 (Fig. 3B). The elution pattern was similar to that of the phosphatidylcholine exchange activity except that there was little cholesterol exchange activity between peak 1 and 3.

For comparison, supernate-proteins were also chromatographed on Sephacryl S-200 HR (Fig. 3C). From a comparison of the gel filtration patterns obtained on Sephadex G-75 SF and Sephacryl S-200 HR (cf. Figs. 3A and 3C), it is clear that the protein eluted as peak 1 from Sephadex G-75 SF is highly inhomogeneous with respect to size. Peak 1 protein was eluted from Sephacryl S-200 HR as a broad peak consisting of three partly resolved peaks. The elution volumes of these three peaks ranged from the column void volume V_o to V_e = 30 ml corresponding to a molecular weight range from 65 000 to greater than 190 000 (Fig. 3C). The phosphatidylcholine exchange activity was associated with the centre peak at V_e = 27 ml (Fig. 3C) corresponding to an apparent molecular weight of 100 000 ± 10 000 (n = 3). Phosphatidylcholine exchange activity was eluted in a second peak well-resolved from the 100 000 protein. This second peak is asymmetric (Fig. 3C) with a shoulder at the tailing edge, its elution volume V_e = 36 ml corresponding to an apparent M_r = 16 000. A comparison with the gel filtration pattern on Sephadex G-75 SF (cf. Figs. 3A and 3C) reveals that this peak is a composite one containing both the 22 000 and the 12 000 protein. Apparently, in contrast to gel filtration on Sephadex G-75 SF, these two proteins are not resolved on Sephacryl S-200 HR.

Protein present in peak 3 exhibiting both cholesterol and phosphatidylcholine exchange activity was further purified by IAM-chromatography. The choice of peak 3 was based on the observation that the active protein present in this peak was least contaminated with other proteins. Plotting specific phosphatidylcholine exchange activities instead of absolute ones as in Fig. 3 would

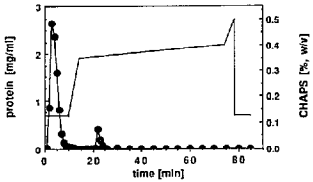

Fig. 4. Purification of proteins contained in peak 3 by immobilized artificial membrane chromatography on a Nucleosil-phosphatidylcholine HPLC column. The pressure used was 60–70 bar, flow rate 0.7 ml/min, protein loading approx. 7 mg and protein recovery approx. 90%. The HPLC column (10 cm × 4.6 mm) was equilibrated with buffer B containing 0.125% CHAPS. 1.9 ml of protein solution (approx. 3.6 mg/ml) in buffer B containing 0.125% CHAPS were applied to the column. About 90–95% of the protein were eluted in the column pass through peak with buffer B containing 0.125% CHAPS. The protein retained on the column was eluted with a linear CHAPS gradient from 0.125 to 0.5%.

yield a different intensity pattern: peak 3 would have maximum intensity and the intensities of both peaks 2 and 3 would be much greater than that of peak 1 (data not shown). This was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis: peak 3 gave about 7 protein bands, peak 2 about 20, and peak 1 more than 50 bands using silver staining (data not shown).

To the concentrated protein solution of peak 3 in buffer B, CHAPS was added in the same buffer to a final concentration of 0.125%. 1.9 ml of the protein solution (3.6 mg/ml) were applied to the Nucleosil-phosphatidylcholine column and the column was eluted with buffer B containing 0.125% CHAPS. 90–95% of the applied protein were eluted from the column in this way (Fig. 4). The remaining protein (7.5 ± 1.0%) that was bound to the column material was eluted with a linear CHAPS gradient (0.125 to 0.5% (w/v)) as shown in this figure; the protein was eluted at a CHAPS concentration of approx. 0.3%. Upon further raising the detergent concentration to 0.5%, no more protein was eluted from the column (Fig. 4). The protein thus obtained was exhaustively dialyzed against buffer B to remove the detergent, and the lipid exchange activities were determined in the detergent free protein solution: 31 ± 7% of the original phosphatidylcholine and cholesterol exchange activity were recovered from the Nucleosil-phosphatidylcholine column. The purification steps and their effects on the purity and recovery of the active protein(s) are summarized in Table II. The purification of peak 3 by IAM-chromatography on Nucleosil-phosphatidylcholine led to an increase in the specific lipid exchange activity by a factor of approx. 80–90. More importantly, each purification step produced a similar increase in the specific cholesterol and phosphatidylcholine exchange activity at least within the error of the measurement. This finding suggests that probably a single protein is responsible for both exchange activities.

Characterization of the active protein purified by IAM-chromatography of peak 3

The purity of the active protein purified by IAM-chromatography on Nucleosil-phosphatidylcholine was checked by reverse phase chromatography on butyl-Nucleosil (particle size 5 μm, 30 nm pores, column dimension 6 × 0.4 cm) equilibrated with 0.1% trifluoroacetic acid. The protein was eluted with a gradient of

TABLE II

Purification of lipid exchange protein from rabbit small intestine

Purification step	Protein (mg)	Protein recovery (%)	Exchange activity (mU) [a]		Recovery of exchange activity (%)		Specific exchange activity (mU/mg protein) [a]		Purification factor [a]	
			phosphatidylcholine	cholesterol	phosphatidylcholine	cholesterol	phosphatidylcholine	cholesterol	phosphatidylcholine	cholesterol
(1) Starting material: Brush border membrane vesicles	1490	–								
(2) Production of supernate-proteins	300	100	1040	71	100	100	3.4	0.23	1	1
(3) Peak 3 produced by gel filtration on Sephadex G-75 SF	7.4	2.4	450	29	43	41	61	4.1	18	18
(4) Protein purified by IAM-chromatography	0.55	0.18	160	10	16	14	300	19	88	80

[a] The exchange activity, specific exchange activity, and the purification factor are referred to phosphatidylcholine and cholesterol exchange between two populations of small unilamellar vesicles. 1 unit of activity U = 1 μmol lipid transferred per min.

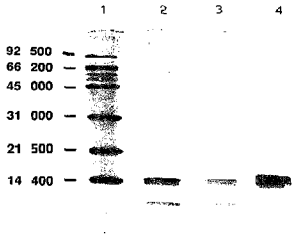

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis was carried out with 15% gels as described in Materials and Methods. The protein bands were stained with Coomassie blue. Molecular weight standards (lane 1), protein of peak 3 (lane 2), pass through peak from the IAM-column (lane 3), protein retarded and eluted from the IAM-column with a CHAPS gradient (lane 4).

n-propanol (0–50%) in 0.1% trifluoroacetic acid yielding a single, slightly asymmetric peak with a small shoulder at the tailing edge. Some minor peaks were eluted in front of the main protein peak amounting to less than 1% of the total protein eluted.

The purified protein gave a single band when examined by electrophoresis on 15% polyacrylamide gels in the presence of sodium dodecyl sulfate using Coomassie blue staining (Fig. 5). Silver staining of the electrophoresis pattern revealed a second minor band with a slightly higher mobility. The position of the single or main band obtained with the purified protein on sodium dodecyl sulfate polyacrylamide gel electrophoresis corresponds to an apparent molecular weight slightly smaller than that of lysozyme ($M_r = 14400$). For comparison, the electrophoresis patterns of peak 3 and of the first protein peak eluted with 0.125% CHAPS from the Nucleosil-phosphatidylcholine column (cf. Fig. 4) are included in Fig. 5. These electrophoresis patterns confirm that most of the protein applied to the IAM column is not bound to the column support but eluted right away with the column buffer.

The apparent molecular weight of the purified protein was also determined by gel filtration on a Sephadex G-75 SF column which was calibrated with various marker proteins (see inset of Fig. 3). The apparent molecular weight was 11500 ± 1250. This is in reasonably good agreement with the apparent molecular weight derived from polyacrylamide gel electrophoresis.

Isoelectric focussing of the protein purified by IAM-chromatography revealed two main bands with slightly different charge characteristics, one protein having an isoelectric point of 9.1, and the other of 9.4. There was a very weak band corresponding to an isoelectric point of 8.5.

Discussion

The first part of our work deals with cholesterol absorption by brush border membrane from mixed micelles as the donor. Two kinds of mixed micellar systems were used: (i) mixed micelles consisting of egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol (60 : 38 : 2, w/w), and (ii) mixed micelles consisting of taurocholate/oleic acid/monooleoylglycerol/cholesterol (88 : 6 : 3,6 : 2,6, w/w). The physicochemical properties of mixtures of egg lysophosphatidylcholine/egg phosphatidylcholine were the subject of a previous study [15]. The composition of the mixed taurocholate micelles was the same as that used by other groups [16,17]. The taurocholate mixed micelles are physiologically more relevant than the egg lysophosphatidylcholine/egg phosphatidylcholine micelles. Another reason for choosing taurocholate as a bile salt was the observation that taurocholate is a mild detergent as to its membrane-solubilizing power. Taurocholate had little effect on the integrity of the brush border membrane at least up to concentrations of about 5 mM used in this work. This is consistent with a report on the membrane-solubilizing properties of taurocholate [17].

Electron microscopy of freeze-fractured samples confirms that the two kinds of donor particles used here consist mainly of small micelles. Electron micrographs show that egg lysophosphatidylcholine/egg phosphatidylcholine/cholesterol dispersions consist mainly of micelles of a diameter of up to about 15 nm (cf. Ref. 15). Besides micelles, some larger spherical particles are present with diameters greater than approx. 25 nm representing small unilamellar vesicles (data not shown). In contrast, electron micrographs of freeze-fractured preparations of taurocholate mixed micelles revealed smooth fracture-surfaces with no particulate matter. Apparently, the taurocholate mixed micelles are too small to be visible by freeze-fracture electron microscopy. The molecular weight of the taurocholate mixed micelles was determined by gel filtration as 23000 [17].

Cholesterol absorption by small intestinal brush border membrane from both mixed micellar systems is a second order reaction. In both cases, there is net transfer of cholesterol, i.e., at equilibrium practically all cholesterol is present in the brush border membrane. The cholesterol absorption from both micellar systems is protein-mediated. This conclusion is based on proteolytic treatment of brush border membrane. For example, proteinase K treatment produces profound changes: (i) after proteinase K treatment cholesterol absorption is mechanistically different from the protein-mediated process. After proteinase K treatment, cholesterol absorption becomes a true first-order reaction. (ii) The rate of cholesterol absorption by brush border membrane was dramatically reduced. After proteinase K

treatment, k_1 values were measured which were smaller by a factor of about $5 \cdot 10^3$ (cf. Table I). These k_1 values were identical within experimental error with the k_1 values for cholesterol absorption by small unilamellar egg phosphatidylcholine vesicles from taurocholate mixed micelles (Table I). This process can be shown to be also a true first-order reaction, i.e., to be independent of the egg phosphatidylcholine or donor concentration. These results indicate that the residual cholesterol absorption measured with brush border membrane after proteinase K treatment is a passive process: it involves cholesterol desorption from the taurocholate mixed micelle, diffusion of monomeric cholesterol through the aqueous phase and incorporation of cholesterol into the bilayer of brush border membrane. Since we are dealing with a true first-order reaction, the desorption step of cholesterol from the taurocholate mixed micelle must be rate-limiting.

Brush border membrane stored at room temperature seems to loose its ability to take up cholesterol efficiently. The rate of cholesterol absorption by brush border membrane stored at room temperature for 2 h was reduced by a factor of about 10 (Fig. 2 and Table I). The loss of activity of the brush border membrane is accompanied by the concomitant release of fragments of integral membrane proteins which can be collected in the supernatant and which are referred to as supernate-proteins. This finding is a manifestation of the instability of our brush border membrane vesicles at temperatures above freezing. As was previously discussed [2], intrinsic proteinases are responsible for the observed membrane instability. It is not known how these intrinsic proteinases are controlled in the intact brush border membrane and by which procedures and manipulations of membrane vesicles they are activated. Once activated, the intrinsic proteinases apparently attack and cleave integral membrane proteins leading to the observed reduction in activity of various functional proteins.

Although the cholesterol absorption by brush border membrane from both micellar systems is the same regarding the reaction order and mechanism, there are, however, significant differences regarding the reaction rates. Cholesterol absorption from taurocholate mixed micelles is about 10^4-times faster than from egg lysophosphatidylcholine/egg phosphatidylcholine mixed micelles. In control experiments, cholesterol transfer was measured from taurocholate mixed micelles to small unilamellar vesicles of egg phosphatidylcholine. The first-order rate constant of this reaction was $k_1 = 0.63$ h^{-1} ($t_{1/2} = 1.1$ h). These values should be compared with corresponding values for cholesterol transfer from egg lysophosphatidylcholine/egg phosphatidylcholine mixed micelles to small unilamellar egg phosphatidylcholine vesicles: $k_1 = 0.08$ h^{-1} ($t_{1/2} = 8.7$ h) and for cholesterol transfer between two populations of small unilamellar phospholipid vesicles reported by McLean and Phillips [13]: $k_1 = 0.070$ h^{-1} ($t_{1/2} = 10$ h) or by Thurnhofer and Hauser [1]: $k_1 = 0.10$ h^{-1} ($t_{1/2} = 6.3$ h). Comparing these data, it is clear that cholesterol transfer from taurocholate mixed micelles is faster than from any other donor particle. This is in agreement with a recent finding that the transfer of phosphatidylcholine is much faster from taurocholate micelles than from small unilamellar vesicles as donors [18,19]. It seems that bile salts increase the rate of transfer of cholesterol as well as phosphatidylcholine in exchange reactions that are not protein-mediated. We emphasize that cholesterol absorption from taurocholate mixed micelles is not accompanied by taurocholate absorption. As shown convincingly in Fig. 2, during the fast uptake of cholesterol practically no cholate is incorporated in the brush border membrane. The same is true for the first-order reaction of cholesterol transfer from taurocholate mixed micelles to small unilamellar egg phosphatidylcholine vesicles. The slow uptake of cholesterol by egg phosphatidylcholine bilayers is not paralleled by uptake of cholate (data not shown). The following conclusions can be drawn from these control experiments: All cholesterol transfer reactions between micelles as the donor and small unilamellar egg phosphatidylcholine vesicles as the acceptor, as well as between two populations of small unilamellar lipid vesicles, are first-order reactions and as such the cholesterol desorption must be rate-limiting. The control experiments discussed above show that the desorption of cholesterol is approx. 10-times faster from taurocholate mixed micelles than from other donor particles be it mixed micelles of egg lysophosphatidylcholine/egg phosphatidylcholine/ cholesterol or phospholipid bilayers [1,13]. The control experiments also rule out the possibility that cholesterol is absorbed as a cholesterol-taurocholate complex. The physiological role of bile salts in lipid digestion and absorption is to efficiently emulsify the products of lipid digestion and to assist in the absorption process of these lipids. In which way, bile salts and their conjugates assist lipid absorption has not been specified. From the data presented here it is tempting to speculate on this point. It is conceivable that the fast off-rate of cholesterol observed with bile salt mixed micelles is of physiological significance. Subjecting this hypothesis to experimental test will be the subject of a future study.

Supernate-proteins liberated from brush border membrane vesicles by proteolysis are active in catalyzing both cholesterol and phosphatidylcholine exchange between two populations of small unilamellar lipid vesicles [1,2]. As detailed previously [1,2], proteolysis is due to the activity of intrinsic proteinases. The basic rate of proteolysis can be significantly enhanced by the addition of extrinsic proteinases such as papain [1]. It was shown before that the inhibition of phosphatidylcholine absorption by brush border membrane as a result of papain digestion is accompanied by the con-

comitant appearance of proteins in the supernatant. The supernate-proteins are active in phosphatidylcholine exchange [1]. Based on this observation, we propose that both activities are associated with the same integral membrane protein of the brush border membrane.

The gel filtration experiments summarized in Fig. 3 shed light on the question of the size of the integral membrane protein responsible for cholesterol absorption and cholesterol and phosphatidylcholine exchange between two populations of small unilamellar vesicles. The gel filtration patterns of Figs. 3A and 3C taken together indicate that proteolysis produces several water-soluble fragments of the integral membrane protein, the smallest one (peak 3) with $M_r \approx 11\,500$, an intermediate one (peak 2) with $M_r = 22\,000$ and the largest one (peak 1) with $M_r \geq 70\,000$. Gel filtration on Sephadex G-75 SF clearly shows that there is no equilibrium between these fragments, for example the protein with an apparent molecular weight $M_r = 22\,000$ is not a dimer of the smallest fragment. All these fragments are water-soluble, the largest one exhibiting lipid exchange activity has a molecular weight $M_r = 100\,000$ as determined by gel filtration on Sephacryl S-200 HR. Therefore, the molecular weight of the total integral membrane protein is on the order of 100 000 or larger depending on the mass of the peptide anchoring the protein to the lipid bilayer of the brush border membrane. It is unlikely that the 100 000 protein and the smaller proteins obtained by proteolysis are of cytosolic origin. Extensive proteolysis particularly by papain could make the brush border membrane leaky and as a result, cytosolic proteins entrapped within brush border membrane vesicles would be released into the aqueous medium. The following experiments rule out this possibility. After controlled papain digestion of brush border membrane vesicles described in Ref. 2, the brush border membrane was still intact as judged from the observed glucose overshoot in the presence of a Na^+ gradient ($[Na^+]_{external}$ was ten times larger than $[Na^+]_{internal}$). The observation of glucose transport against a concentration gradient is taken as evidence for the intact barrier properties of brush border membrane after papain digestion. Furthermore, it can be shown that external proteins added to brush border membrane are not digested by active intrinsic proteinases of this membrane. Hence, the smaller fragments identified by gel filtration (Fig. 3) must be the result of intrinsic proteinases cleaving integral membrane proteins.

All three fragments separated on Sephadex G-75 SF catalyze phosphatidylcholine exchange between two populations of small unilamellar phospholipid vesicles. It is not clear why protein of peak 2 (apparent molecular weight $M_r = 22\,000$) has little or no cholesterol exchange activity. Clearly more work is required to clarify this question. Table II shows that the purification of supernate-proteins [5] by gel filtration and IAM-chromatography leads to an active protein that is pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purification factor is about 80–90, but more importantly each purification step leads to about the same increase in the specific phosphatidylcholine and cholesterol exchange activity. The observation that the two specific activities increase in parallel is interpreted to indicate that both activities are associated with one and the same protein.

The essential step of the purification of protein of peak 3 (Fig. 3A) is IAM-chromatography on Nucleosil-phosphatidylcholine (Fig. 4). The mechanism of this chromatography probably involves both affinity and distribution chromatography. It was shown before that supernate-protein(s) bind(s) both spin-labeled phosphatidylcholine [2] and 3-doxyl-5α-cholestane [1]. Hence, it is reasonable to assume that the active protein of peak 3 binds to Nucleosil-phosphatidylcholine. This is indeed the case as is evident from Fig. 4. It is quite likely that the protein not only binds to the surface of the IAM-column material, but also unfolds at the water/Nucleosil-phosphatidylcholine interface. Hydrophobic domains of the protein that become exposed in the course of unfolding would then distribute preferentially into the hydrophobic matrix of the Nucleosil-phosphatidyl-choline resin. Unfolding and association of hydrophobic protein domains with the hydrocarbon phase of the column are probably irreversible processes. Such irreversible processes could be responsible for the low yield of about 30% of the phosphatidylcholine exchange activity recovered from the Nucleosil-phosphatidylcholine column. The purification of protein of peak 3 was achieved effectively in a single chromatographic step though it seems at the expense of a great loss in lipid exchange activity.

Recovery of total protein was almost quantitative averaging at $90 \pm 5\%$. The recovery of the activity was, however, only approx. 30%. The actual value of protein recovery depends crucially on a number of factors, among others the total protein mass applied to the column and the hydrophobic-hydrophilic balance are important.

We note that Nucleosil-phosphatidylcholine, synthesized in our lab, performs chromatographically similar to the commercially available IAM-phosphatidylcholine at least with regard to column selectivity. However, with regard to protein recovery from the column, Nucleosil-phosphatidylcholine performs virtually identical to the commercially available IAM-phosphatidylcholine columns (Pidgeon, C., submitted for publication). During the purification of cytochrome *P*-450, 60–90% of the cytochrome *P*-450 injected onto IAM-phosphatidylcholine columns is recovered, in almost pure form, from a single injection. Eight forms of cytochrome *P*-450 have been purified with similar results. Since only 30% of the cholesterol transfer protein was recovered from

the Nucleosil-phosphatidylcholine column, the protein recovery depends on the type and amount of injected protein.

An approximate estimate of the apparent molecular weight of the purified protein was obtained by two independent methods: polyacrylamide gel electrophoresis in sodium dodecyl sulfate and gel filtration on calibrated Sephadex G-75 SF yielded a value between 12 000 and 14 000. The purified protein is a basic protein with a *pI* between 9.1 and 9.4.

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