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STUDIES ON THE MECHANISM OF CHOLESTEROL UPTAKE AND ON THE EFFECTS OF BILE SALTS ON THIS UPTAKE BY BRUSH-BORDER MEMBRANES ISOLATED FROM RABBIT SMALL INTESTINE

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The effect of bile salts and other surfactants on the rate of incorporation of cholesterol into isolated brush-border membranes was tested. At constant cholesterol concentration, a stimulatory effect of taurocholate was noticed which increased as the bile salt concentration was raised to 20 mM. Taurodeoxycholate was as effective as taurocholate at concentrations of up to 5 mM and inhibited at higher concentrations. Glycocholate was only moderately stimulatory whereas cholate was nearly as effective as taurocholate at concentrations above 5 mM. Other surfactants such as sodium lauryl sulfate and Triton X-100 were very inhibitory at all concentrations tried whereas cetyltrimethyl ammonium chloride was stimulatory only at a very low range of concentrations. These micellizing agents all caused some disruption of the membranes and the greater effectiveness of taurocholate in stimulating sterol uptake was partly relatable to the weaker membrane solubilizing action of this bile salt. Preincubation of membranes with 20 mM taurocholate followed by washing and exposure to cholesterol-containing lipid suspensions lacking bile salt, did not enhance the incorporation of the sterol. In the absence of bile salt the incorporation of cholesterol was unaffected by stirring of the incubation mixtures. Increasing the cholesterol concentration in the mixed micelle while keeping the concentration of bile salt constant caused an increase in rate of sterol incorporation. This increased rate was seen whether the cholesterol suspension was turbid, i.e., contained non-micellized cholesterol, or whether it was optically-clear and contained only monomers and micelles. When the concentration of taurocholate and cholesterol were increased simultaneously such that the concentration ratio of these two components was kept constant, there resulted a corresponding increase in rate of cholesterol uptake. The initial rates of cholesterol incorporation from suspensions containing micellar and monomer forms of cholesterol were much larger than from solutions containing only monomers of the same concentration. The rates of incorporation of cholesterol and phosphatidylethanolamine from mixed micelles containing these lipids in equimolar concentrations were very different. The results as a whole suggest at least for those experimental conditions specified in this study, that uptake of cholesterol by isolated brush-border membranes involves both the monomer and micellar phases of the bulk lipid and that the interaction of the micelles with membrane does not likely involve a fusion process.

Introduction

On the basis of evidence obtained from various in vitro experiments it was suggested that the absorption of lipids by brush-border membranes involves monomers rather than micelles of the bulk phase [1–4]. The mechanism of absorption however, may vary depending on the lipid composition of the suspension such that micelles could be implicated in uptake also [5]. Addition of bile

salt [1–4] and other surfactants [6] to the lipid bulk phase facilitates absorption, an effect which has been explained on the basis of a reduction in the effective resistance of the unstirred water layer [1–4].

Recent studies in our laboratory have indicated that isolated brush-border membrane preparations can readily incorporate phosphoglyceride and neutral lipids via a process markedly stimulated by Ca^{2+} [7]. It was also shown [8] that in the absence of Ca^{2+}, but not in its presence the rate of cholesterol uptake was sufficiently slow to allow its measurement. This rate was greatly influenced by the lipid composition of the mixed micelles containing the cholesterol. Whereas the presence of phosphatidylcholines with long acyl chains decreased the rate of cholesterol uptake, analogous lipids containing ethanolamine enhanced this rate. The effect of a lipid appeared to depend on its ability to increase or decrease cholesterol retention in the micellar phase.

The present study reveals the influence of other factors such as bile salts and certain detergents on cholesterol uptake. It is also concerned with the mechanism of uptake of this sterol.

Materials and Methods

[^{32}P]Phosphatidylethanolamine was prepared from *Escherichia coli* cells grown in medium containing [^{32}P]orthophosphate [9]. [7(n)-^{3}H]Cholesterol was purchased from New England Nuclear Corp. and diluted to required specific activity. Coliform phosphatidylethanolamine, cholesterol, oleic acid and monoolein were purchased from Sigma Chemicals. These lipids were found to be chromatographically pure except monoolein which contained a trace amount of free fatty acid.

Rabbit brush-border membranes were isolated from strips of small intestine according to the method of Selhub and Rosenberg [10] and suspended in 10 mM Tris buffer (pH 7.4). The purity of the membranes was ascertained by marker enzymes and by electron microscopy as described previously [8].

[^{3}H]Cholesterol-containing micelles were usually prepared by dissolving this labelled sterol in chloroform together with oleic acid, monoglyceride and other lipid components using concentrations specified. The solvent was evaporated by prolonged exposure to a jet of nitrogen and the residue was suspended in 10 mM Tris buffer (pH 7.4), containing taurocholate, other bile salts or detergents at concentrations specified in the text. The lipids were dispersed by sonication at 250–300 watts with the large probe of an Ultrasonics sonifier. Sonication was continued until the suspensions clarified no further. The sols were then centrifuged at 10^7 $g \cdot$ min in a Beckman ultracentrifuge equipped with a 65 or 70:1 Ti rotor. The amount of lipid remaining in the supernatant was usually estimated from the radioactivity recovered or by determining the lipid phosphorus [11] in cases when phosphoglyceride has also been added to the micellar mixture.

The incubation mixtures usually contained 0.1 ml of brush-border membrane suspension, corresponding to 0.2–0.5 mg protein as determined by the method of Lowry et al. [13], 0.4 ml of clear micellar suspension and 0.5 ml of 10 mM Tris buffer (pH 7.4). Except for time studies, incubations were for 15–30 min as indicated. Following this period, the suspensions were sedimented by centrifuging at 38 8000 $\times$ g for 30 min and washed once with 10 mM Tris buffer (pH 7.4). When turbid suspensions were used (Fig. 6a) the incubation mixture was centrifuged over 2.5 volumes of 10% Ficoll [7,8]. For time studies, zero-time controls were subtracted from all the values. For other studies, blanks without membranes were prepared in the same manner and subtracted from the experimental values. Unspecific binding due to inulin space was $\leqslant 2\%$ of the incorporated lipid and was considered negligible. The pellets were suspended in 0.2 ml of water and transferred together with a 0.2 ml wash to counting vials containing toluene and PCS (Amersham) 1:1 v/v. The samples were counted in a Beckman LS 133 spectrometer using a channels ratio method.

For one set of experiments micelles were prepared by first dissolving enough [^{3}H]cholesterol, monoolein, oleic acid and taurocholate together in ethanol/chloroform (1:1, v/v) to prepare aqueous suspensions of the following respective, final concentrations; 0.26 mM, 0.3 mM, 0.6 mM and 20 mM. The solutions were then dried under a stream of nitrogen and further dried in vacuo at room temperature overnight. The residue was then

sonicated in 10 mM Tris buffer (pH 7.4) to give an apparently clear sol. The suspension was further centrifuged at 10^7 $g \cdot$ min and 10 ml of the supernatant was saturated with nitrogen and placed in a Spectrapore 1000 dialysis tube (Spectrum Medical Industries Inc. Los Angeles: pore diameter < 15 Å, M_r cutt off 1000) and dialysed against 20 ml of 10 mM Tris buffer (pH 7.4) saturated with nitrogen. The dialysis was continued with agitation for three days in an air-tight tube containing nitrogen. At this time, the radioactivity outside the dialysis tubing increased no further indicating that the diffusion process had reached equilibrium. Although precise values for the size of monoglyceride-fatty acid-cholesterol-charged taurocholate micelles prepared for this experiment are unknown, one can deduce from published data that pure taurocholate micelles in a buffered medium of low ionic strength, such as ours, would have a diameter of at least 20–30 Å with an aggregate number of 3–4 (M_r approx. 2000). Adding monoglyceride even in small amounts and other lipids to these micelles would tend to increase their size [12] such that they would be retained in the dialysis tubing. For this same experiment, 10 ml of centrifuged lipid suspension were mixed directly with 20 ml of buffer in another tube and shaken under nitrogen for the same period of time. This solution containing both monomers and micelles and the other containing monomers only, of the same concentration, were each incubated with membranes and the rate of incorporation was measured in each case. For this experiment, the dilution due to addition of the membranes to the lipid suspensions was no more than 1.5%.

Results are all means ± S.E. of 4–20 determinations obtained with membranes from 2–4 rabbits except in the case of Fig. 4 where the values represent the average of duplicate determinations with membranes from a single rabbit. Except for time-course studies the incorporations obtained represent rates reported as nmoles per mg protein for the standard incubation time of 30 min. Incorporations were found to be linear with time up to 30 min when lipids were micellized in taurocholate.

Results

A detailed examination of the effect of taurocholate and other surfactants on the rate of incorporation of cholesterol was made. In the presence of 10 mM taurochlolate, this rate was markedly stimulated and was characterized by a linear incorporation up to approx. 30 min under the conditions specified. In the absence of bile salt, a slower rate was seen and the increase was not linear with time (Fig. 1). The stimulatory effect of taurocholate on the rate increased almost linearly as the concentration was raised to 20 mM. Taurodeoxycholate was as effective as taurocholate at concentrations of up to 5 mM but a much decreased incorporation was seen at a concentration of 20 mM (Fig. 2a). Glycocholate was only mildly stimulatory whereas cholate was almost as effective as taurocholate at concentrations above 5 mM (Fig. 2b). Other detergents such as sodium lauryl sulfate and Triton X 100 were very inhibitory whereas cetyl trimethyl ammonium chloride was stimulatory within the narrow concentration range of 0.016–0.064 mM and inhibitory at higher concentrations (Figs. 3a–c).

Results illustrated in Fig. 4 indicate the solubilizing effect of taurocholate and other surfactants on the brush-border membranes as could be ascer-

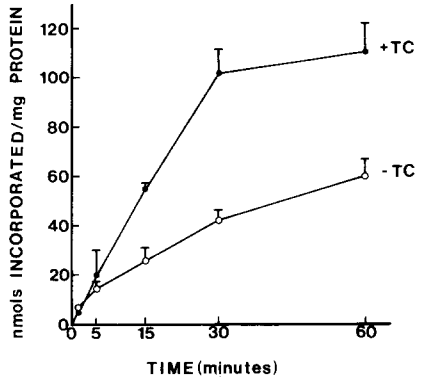

Fig. 1. Effect of taurocholate on the time-course of cholesterol incorporation. The incubation mixture contained in 1.0 ml, brush-border membranes (0.20–0.25 mg protein) 10 mM Tris buffer (pH 7.4) and sonicated, optically-clear suspension of lipids containing 0.26 mM [^{3}H]cholesterol, 0.6 mM oleic acid 0.3 mM monoolein and 10 mM taurocholate. The incubations were at 37°C. ○, without and ●, with taurocholate (TC)

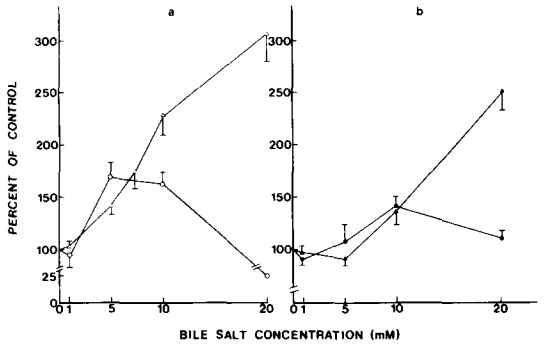

Fig. 2. Effect of bile salts on incorporation of [^{3}H]cholesterol. (a) Taurocholate, △; taurodeoxycholate, ○. (b) Glycocholate, ●; cholate, ▲. The conditions were as indicated for Fig. 1 except the bile salt concentration was varied and the time of incubation was 30 min at 37°C. The zero-detergent control corresponded to an incorporation of 63 ± 12 nmol/mg protein.

tained by the recovery of protein in the washed membrane pellets following incubation. Except in the case of taurocholate, the size of the pellets was very visibly diminished at higher surfactant concentrations. Taurocholate appeared to have the mildest membrane-solubilizing effect of the bile salts tested and this could at least partly explain its apparently greater efficiency in stimulating cholesterol uptake.

The effect of preincubating membranes with and without taurocholate followed by incubation in the absence of this bile salt is seen in Fig. 5.

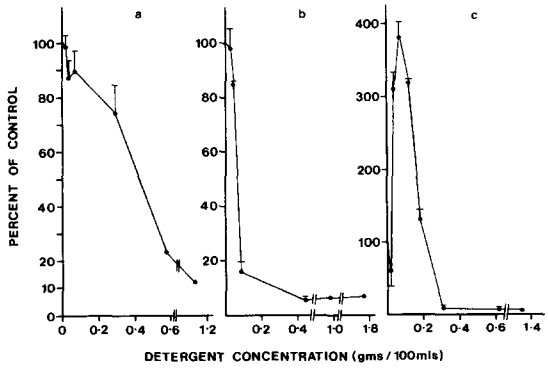

Fig. 3. Effect of detergents on incorporation of [^{3}H]cholesterol. (a) Sodium lauryl sulfate. (b) Triton X-100. (c) Cetyltrimethylammonium chloride. The conditions were as stated for Fig. 2.

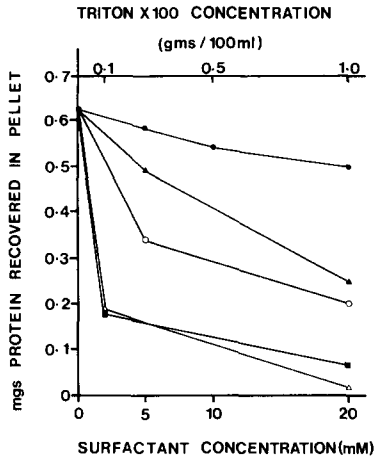

Fig. 4. Solubilizing effect of surfactants on brush-border membranes. Membranes were incubated with or without detergents in the presence of lipids under conditions stated for Figs. 1 and 2. In the zero-detergent control, 0.62 mg of protein were recovered after 30 min out of 0.69 mg added: taurocholate, ●; Triton X-100, ■; cetyltrimethylammonium chloride, △; taurodeoxycholate, ○; glycocholate, ▲.

Preincubation with taurocholate did not accelerate the incorporation of cholesterol. Infact the initial rates of incorporation were slightly higher for membranes incubated with buffer alone.

The effect of increasing chlolesterol concentra-

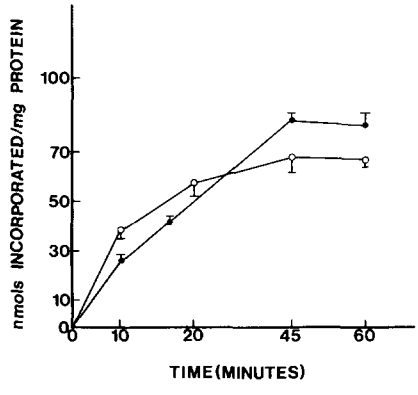

Fig. 5. Effect of preincubating membranes with bile salt. Membranes (0.15–0.23 mg protein) were incubated for 30 min in 10 mM Tris buffer (pH 7.4) containing (●) or lacking (○) 20 mM taurocholate. After 15 min, the membranes were isolated by centrifugation, washed and then resuspended in the usual buffer (0.5 ml) and incubated with lipid suspension lacking bile salt such that the final concentrations of cholesterol, oleic acid and monoolein were 0.26 mM, 0.6 mM and 0.3 mM, respectively.

tion in 20 mM taurocholate on rate of incorporation, reported as nmol/mg protein in 30 min was tested with several types of suspensions. Firstly, chloroformic cholesterol solutions were dried with a stream of nitrogen such as to form in each case a thin film of residue at the bottom of a beaker. This material was then suspended by sonication in various volumes of 20 mM taurocholate to give required concentrations of the sterol and centrifuged 10 min at $3500 \times g$ to remove titanium particles. Opalescence, which could not be eliminated by further sonication and equilibrium at room temperature for several hours, was detected in suspensions with concentrations $\geqslant 0.1$ mM. Incubations with such suspensions gave a linear relationship between rate and concentrations up to approx. 0.3 mM cholesterol (Fig. 6a). Secondly, when these same suspensions were completely clarified by centrifugation at 10^7 $g \cdot$ min, about 70–80% of the suspended material was lost in the pellet. Somewhat higher rates of incorporation were obtained than before centrifugation (Fig. 6b). This must mean that some of the larger aggregates (cholesterol crystals) responsible for opalescence were reactive.

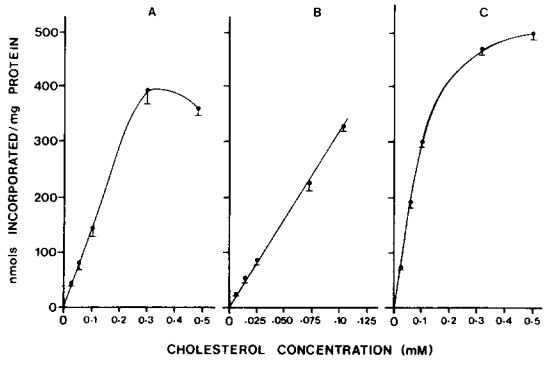

Fig. 6. The effect of [^{3}H]cholesterol concentration on incorporation. (A) Suspensions of cholesterol in 20 mM taurocholate were prepared by sonication without centrifugation and incubated. This solutions were faintly opalescent to turbid as the sterol concentration was increased from 0.1 mM to 0.48 mM. (B) The suspensions described in A, were clarified before incubation, by centrifugation at 10^7 $g \cdot$min. Approximately 20% of the sterol was recovered in the supernatant. (C) Suspensions were prepared by dissolving the sterol and taurocholate together in ethanol. Following solvent evaporation, the residue was sonicated in 10 mM Tris buffer (pH 7.4) to yield the required final concentrations of sterol and taurocholate. Incubation conditions were as stated for Fig. 2.

In a third type of experiment the bile salt and cholesterol were dissolved in ethanol/chloroform (1 : 1, v/v) and after evaporation of the solvent in vacuo, the residue was dispersed by sonication in 10 mM Tris buffer to yield a suspension containing 0.52 mM cholesterol and 20 mM taurocholate. This solution, which appeared to be completely clear, was centrifuged 10 min at $3500 \times g$ to sediment titanium particles. It was then diluted, to yield required concentrations, with 10 mM Tris buffer containing 20 mM taurocholate. The rates of incorporation are given in Fig. 6c, and these are the same as for Fig. 6b and about twice those found for the case of turbid solutions (Fig. 6a) when the linear part of the curves are considered. A plateau is seen at a taurocholate/cholesterol ratio $< 130 : 1$ where there is no further micellization of sterol possible [12]; however, this plateau may simply indicate that the uptake process is saturable.

When taurocholate and cholesterol concentrations were increased together such that their concentration ratio was kept constant, the rate of incorporation of cholesterol increased linearly (Fig. 7). The results were essentially the same when the mixed micelles were prepared by suspending the

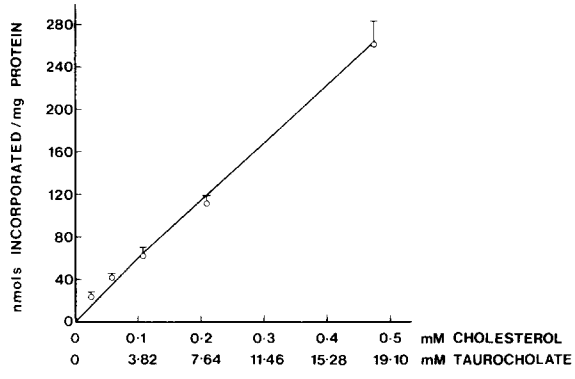

Fig. 7. The effect of varying [^{3}H]cholesterol and taurocholate concentration concurrently. [^{3}H]Cholesterol, oleic acid and monoolein in the proportions stated in Fig. 1 were dissolved in chloroform and following evaporation of this solvent the residue was sonicated in 20 mM taurocholate to yield a slightly opalescent suspension which was clarified by centrifugation at 10^7 $g \cdot$min. The solution was diluted with Tris buffer to yield solutions of required [^{3}H]cholesterol and bile salt concentration which were used as described for Fig. 2.

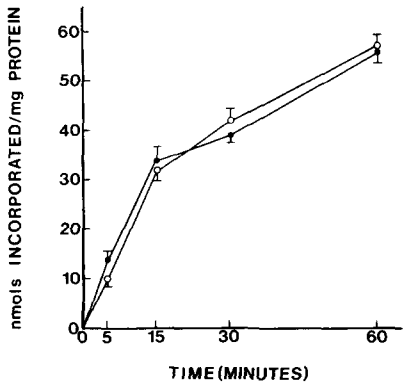

Fig. 8. The effect of stirring on incorporation of [^{3}H]cholesterol. A lipid suspension was prepared as stated in Fig. 1 but without taurocholate and centrifuged at 10^7 $g \cdot$min. The supernatant was then incubated with membranes (0.23 mg of protein) and incubated for various times without stirring (●); or with stirring at 480 rpm (○).

lipid mixture in 20 mM taurocholate and diluting this solution to required concentrations or when fixed amounts of lipids and taurocholate were mixed by dissolving these substances together, in chloroform/ethanol, followed by evaporation of solvent and sonication in different volumes of buffer to obtain required concentrations.

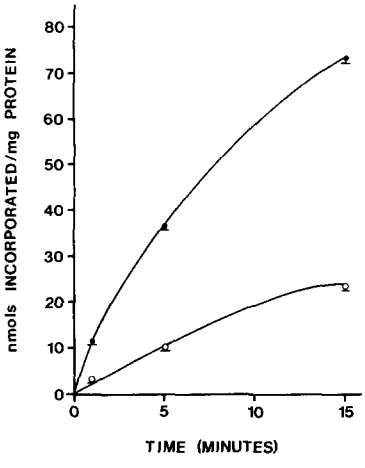

Fig. 9. The rate of [^{3}H]cholesterol incorporation from monomer solutions (○) and from suspensions containing monomers and micelles (●). 0.9 ml of each of these solutions was mixed with 14 μl of membrane suspension (0.15–0.20 mg protein) and incubated for various times at 37°C.

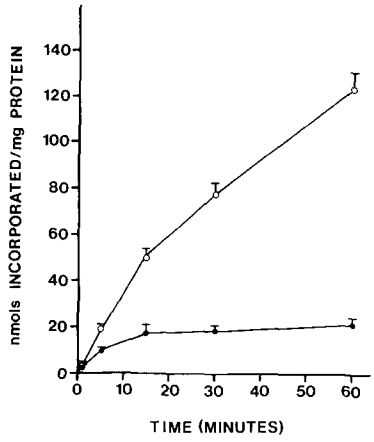

Fig. 10. The rates of incorporation of [^{3}H]cholesterol (○) and [^{32}P]phosphatidylethanolamine (●) from mixed micelles. A clarified lipid suspension containing 0.5 mM oleic acid, 0.3 mM monoolein, 0.26 mM [^{3}H]cholesterol and 0.26 mM [^{32}P]phosphatidylethanolamine and 7.2 mM taurocholate were incubated with brush-border membranes (0.15–0.20 mg protein) for various times at 37°C.

To test whether an unstirred water layer was an important diffusion barrier for incorporation of cholesterol, cholesterol-containing, bile salt-free micelles were incubated with membranes in a medium that was either unstirred or stirred at 480 r.p.m. Fig. 8 illustrates very similar rates of incorporation under both conditions. It seems therefore that with isolated membranes there is no sizeable diffusion barrier in the form of an unstirred water layer.

Results illustrated in Fig. 9 indicate that the initial rates of cholesterol incorporation from solutions containing micellar and monomer forms of cholesterol are much larger than from solutions containing the monomer form of cholesterol only, both types of solutions having equal monomer concentrations.

Results illustrated in Fig. 10 indicate that the rates of incorporation of cholesterol and phosphatidylethanolamine from mixed micelles containing these lipids in equimolar concentrations were very different.

Discussion

It is known that the unstirred water layer bathing the surface of the brush-border membranes in

situ is an important diffusion barrier for hydrophobic lipids such as long chain fatty acids and cholesterol [1,2,14]. Bile salts are believed to facilitate the absorption of these lipids by decreasing the effective resistance of the unstirred water layer [1,2,14]. Recent evidence has indicated however that the main diffusion barrier may be the glycocalyx extending from the surface of the enterocyte [15] rather than the unstirred water layer. Although it is uncertain how the glycocalyx offers resistance to diffusion of highly apolar molecules, it may be that this barrier is partly due to unstirred water trapped in the spaces between mucoprotein strands [16].

That the monomer phase is involved in the absorption of lipids is based partly on the observation that increasing the bile salt concentration while keeping the lipid concentration constant in mixed micelles, causes a decrease in absorption rate [1,2,17]. This decrease is explained on the basis that bile salts retain lipid in the micellar phase and decrease the monomer concentration. However, irrespective of whether monomers or micelles are involved, solubilization of the membrane by higher concentrations of bile salt is a definite possibility as previous [4] and our present results indicate and this could at least partly explain the observed decrease in absorption rate as bile salt to lipid ratios are increased. At low bile salt to lipid ratio, a less extensive solubilization would be expected because the micelles are more nearly saturated with lipid.

The involvement of the monomer phase is also deduced from the fact that increasing the micellar concentration while keeping the bile salt/lipid ratio constant produces only a small initial increase in absorption rate which eventually levels off when the maximum monomer concentration, in equilibrium with the micelles, is attained [1,2]. However, a similar effect on rate of absorption could be seen even if micelles are directly involved in the uptake process since the inter-mucoprotein space of the glycocalyx might be expected to harbor a definite volume of micelles which could no longer increase with bulk phase concentration.

Our present results with isolated brush-border membranes do definitely indicate that not only the monomer phase is involved with uptake of lipids but the micellar phase as well. Indeed, when the taurocholate to cholesterol ratio was increased in mixed micelle suspensions (Fig. 2a), there was an increased rate of sterol incorporation. Consequently monomers could not be the only reacting species concerned in uptake.

The effect of taurocholate did not seem to involve the membrane directly. However, our results only indicate that preincubation with 20 mM taurocholate produced no long lasting changes in membrane permeability to cholesterol, of a type which could persist after subsequent washing of the membranes in buffer. Despite this uncertainty, it can be suggested as a most likely explanation that the effect of bile salt is to increase the accessibility of the bulk phase lipid due to micellization which decreases the aggregate size. In absence of micellizating agents, cholesterol exists in water as a monomer of very low concentration, $\leqslant 10^{-8}$ M, or as relatively large crystals of cholesterol and cholesterol monohydrate [18], which are probably less reactive.

That micelles are directly implicated in the uptake process is infact deducible from the linear increase in incorporation rate with increase in micelle concentration that was noticed when the bile salt/lipid ratio was kept constant (Fig. 7). Finally, another proof of direct micellar involvement in the uptake consists of the fact that the initial rate of absorption of cholesterol from solutions containing only monomers is much lower than from suspensions of equal monomer concentration but also containing micelles.

Although it could be shown that micelles interact with brush-border membranes directly, the absorption process did not, under the conditions specified, involve a fusion with membrane because cholesterol and phosphatidylethanolamine, added in equal concentrations, to constitute mixed micelles, were absorbed at very different rates. It is not known, however, whether or not fusion might occur with other types of lipid mixtures or when Ca^{2+} is present in the medium.

This cation is known to promote fusion of membrane [18] and an earlier report [7] indicated that it stimulated cholesterol uptake by brush-border membranes. In studies, described in the literature, involving uptake of lipids by in vitro preparations of small intestine, Ca^{2+} was invariably omitted from the bulk phase assumably be-

cause it may cause coalescence of the micellized lipids. In our present study it was omitted principally because it accelerated cholesterol uptake to a point that rates could no longer be measured under all conditions tested. Unpublished results obtained in our laboratory indicated that in the presence of this divalent cation a stimulatory effect of bile salts on the incorporation of cholesterol was also seen but the results obtained in this case represented equilibrium values. This means that the bile salt can act by increasing the proportion of reactive mesomorphs in the bulk phase as was mentioned previously and thus alter the partition coefficient in favor of the membrane. The enhancement could also be due to a direct effect of bile salt on the membrane.

Deprived of their microvilli contour and probably much of their glycocalyx [15], the isolated brush-border membrane would be expected to be much more accessible to bulk phase lipids of monomer and micellar form. In fact our present study failed to demonstrate a diffusion barrier of the type attributable to an unstirred water layer (cf. Fig. 8). The presence in situ of an unstirred water layer would not influence the ability of the enterocyte membrane to react directly with lipid micelles; however, the presence of a glycocalyx might well have such an influence. By analogy, one could consider the uptake of lipids by coliform organisms. This uptake does involve the vesicle phase of the lipids and is far greater in strains lacking polysaccharide strands protruding from the outer membrane [19]. Although the glycocalyx could limit access of micelles to the membrane there is nevertheless evidence indicating that a direct interaction of the micellar phase may occur with brush-border membranes in situ depending on the lipid composition [5], of the micelles. Our results do infact support this conclusion by revealing that nothing inherent to the basic structure of the brush-border membrane can prevent a direct interaction with cholesterol of the micellar phase.

Acknowledgement

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