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UPTAKE AND EFFLUX BY EVERTED INTESTINAL SACS OF MICELLAR CHOLESTEROL IN BILE SALTS AND IN NON-IONIC DETERGENT

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SUMMARY

1. Uptake and efflux of labelled micellar cholesterol by everted sacs of rat jejunum was compared for two detergents, (a) pure sodium taurocholate–sodium taurodeoxycholate (4:1, on molar basis) and (b) a high-molecular-weight non-ionic detergent, Pluronic F68. Oleic acid and monoolein were also incorporated in the micellar solutions.

2. Uptake of cholesterol from bile salt micelles was 2–4 times faster than from non-ionic micelles, for incubation periods varying from 10 min to 2 h.

3. Uptake from bile salt micelles increased with temperature (apparent activation energy about 6 kcal/mole cholesterol) but uptake from non-ionic micelles did not. Metabolic inhibitors had little effect.

4. When sacs were loaded with isotopic cholesterol from bile salt micellar media, subsequent efflux into buffer, or into unlabelled cholesterol in non-ionic micelles or in bile salt micelles was slower than was efflux into a given medium after loading with isotopic cholesterol from non-ionic micelles. On the other hand, efflux into bile salt micellar medium was faster for a given loading medium than efflux into non-ionic micelles.

5. It is suggested that cholesterol uptake may involve two stages only one of which is temperature sensitive and is promoted by bile salts.

INTRODUCTION

Little or no cholesterol is absorbed in the absence of bile salts from the intestinal lumen[1–4]. Cholesterol is virtually insoluble in water[5]. Thus bile salts might be obligatory primarily as micellar solubilizers[6], although an additional more specific role has also received some support[7].

If bile salts were important for cholesterol absorption only as micellar solubilizers other detergents should be efficient substitutes provided they were non-toxic. Preliminary experiments on absorption into the lymph in bile-fistula rats did not support this. Only a trace of cholesterol appeared in the lymph when steadily infused into the duodenal lumen as a micellar solution – with fatty acid and monoolein – in a high-molecular-weight non-ionic detergent (Pluronic F68). Cholesterol was readily absorbed when the same lipid mixture was given in bile salt micellar solution. Fatty acid was absorbed equally well from both detergents. It seemed of interest, therefore, to make more detailed comparisons, *in vitro*, by exposing everted sacs of small intestine

to the two types of micellar solution. Uptakes of isotopic cholesterol were compared, varying the duration of exposure, concentration of cholesterol and temperature and in the presence or absence of metabolic inhibitors. Efflux of labelled cholesterol into each type of micellar solution was measured after loading in one or the other type. The results would be consistent with a two-stage uptake of cholesterol, neither stage dependent on metabolic integrity but one of them temperature sensitive and promoted by bile salts.

MATERIALS AND METHODS

Tissue

Male rats of an inbred Wistar strain and weighing approx. 200 g were used. All animals had been fasted 18–120 h prior to use. The proximal half of the small intestine was removed under ether anaesthesia and rinsed in saline at room temperature. The small intestine was then carefully everted on a stainless steel rod and sacs of approx. 300–500 mg wet weight prepared according to the method of WILSON AND WISEMAN[8]. Sufficient warm physiological saline was injected into the serosal space to produce slight distension of the sacs. The sacs from each rat were randomly distributed between test and control groups.

Materials

The non-ionic detergent Pluronic F68 was a gift from Wyandotte Chemical Corporation and was stated to have an average molecular weight of 8000. It was used as supplied. Sodium taurocholate was Puriss grade as purchased from Koch-Light Laboratories Ltd. Sodium taurodeoxycholate was prepared by the HOFMANN[9] modification of the method of Norman. A 2-mg sample of each bile salt ran as one spot on thin-layer chromatography when developed in the system ethyl acetate–methanol–glacial acetic acid (70:20:10, by vol.). [4-^{14}C]Cholesterol was purchased from the Radiochemical Centre, Amersham and was re-purified by thin-layer chromatography before use. Unlabelled cholesterol (British Drug Houses) was used without further purification. The trimethylsilyl ether derivative gave a single peak by gas–liquid chromatography on 3% JXR. Oleic acid purchased from Nutritional Biochem. Corporation, Ohio was shown to be 98.5% pure by gas–liquid chromatography. Glycerol 1-monooleate was shown by thin-layer chromatography to contain a small amount of diglyceride and free fatty acid. When examined by gas–liquid chromatography in this laboratory the percentage fatty acid composition was $C_{12:0}$, 1; $C_{16:0}$, 5; $C_{18:1}$, 85; $C_{18:2}$, 5. NaF and 2,4-dinitrophenol were of lab-reagent quality from British Drug Houses Ltd. Iodoacetamide was purchased from Sigma Chemical Co. and used as supplied. All other chemicals and solvents were of analytical grade and were used as supplied except for ethanol which was redistilled.

Solutions

Incubation solutions were made up in a phosphate buffer (pH 6.4) of the following composition: HPO_4^{2-}, 7.5 mM; $H_2PO_4^-$, 15 mM; Cl^-, 137 mM; Ca^{2+}, 1 mM; Na^+, 157 mM; glucose, 1 mM. This buffer solution was made up fresh daily and oxygenated with O_2–CO_2 (95:5, by vol.) prior to use. The bile salts were used in a total concentration of 10 mM, the mixture consisting of sodium taurocholate–sodium

taurodeoxycholate (4:1, molar ratio). Pluronic F68 was used at a concentration of 50 mg/ml in the incubation solution.

Stock solutions of 80 mM monoolein and 80 mM oleic acid were made up in chloroform. Each of these lipids was used at a concentration of 3 mM in all incubation solutions. [4-^{14}C]Cholesterol *plus* unlabelled cholesterol was made up in a 5 mM stock solution of known specific activity and used at the appropriate concentration for each experiment. Micellar incubation solutions were made up by adding the appropriate weight of either bile salts or Pluronic F68 mixed in 1/3 of the final volume of warm buffer, to the evaporated lipids. Further warm buffer was added to provide the required volume of solution which was then insonated briefly in a Branson Sonifier.

Uptake

5 ml of the appropriate incubation solution was taken in a 10-ml round-bottomed stoppered flask and shaken (125 excursions/min) in a water bath. Except for observations on temperature effects, all incubations were carried out at 35°. After allowing for temperature equilibrium of the incubation solution, one freshly prepared sac was added to each flask and incubated for the appropriate time. For uptake intervals of 10 min or less, sacs were preincubated in phosphate buffer (pH 6.4) at the required temperature for 5 min before transfer to the labelled cholesterol medium. During observations with metabolic inhibitors, the phosphate buffer which was used for pre-incubation also contained the test inhibitor at the required concentration.

Efflux

Everted sacs were incubated in the appropriate labelled cholesterol medium for 30 min after which each sac was transferred into exactly 5 ml of incubation medium containing the required detergent *plus* an identical but initially unlabelled lipid mixture. At the end of 2 min each sac was touched lightly on the flask neck before being transferred to a similar medium and the procedure repeated at 5, 10, 15, 30, 60 and 120 min. Flasks were shaken continuously during efflux experiments.

Extraction and counting

Following incubation sacs were rinsed in chilled 0.15 M NaCl, emptied of serosal fluid and weighed. Mucosal tissue was scraped off, homogenised by insonation in saline and duplicate aliquots from a measured volume of homogenate were then extracted by solvent partition in ethanol-diethyl ether–light petroleum (1:1:1, by vol.) and the lower phase washed twice with preequilibrated upper phase[10]. The solvent was then evaporated off under N_2 and 10 ml scintillant (2 g 2,5-diphenyl oxazole and 0.025 g 1,4-bis-[5-phenyloxazoyl-2]-benzene in 500 ml toluene) added to the residue. Samples were then counted in a Nuclear Chicago liquid scintillation counter. Quench correction was by the channels ratio method[11].

For efflux experiments 4 ml of the initially unlabelled medium in each vessel were extracted as above.

Calculations

From the known initial specific activity of the [4-^{14}C]cholesterol, uptake was expressed as nmoles of cholesterol per g wet weight of tissue. The specific activity

of cholesterol in the incubation medium fell by 20–30% in the first 30 min, with much smaller changes subsequently. This was partly due to shed unlabelled cells and crypt debris as shown by centrifugation. The changes were similar for both media. In efflux experiments the uptake of labelled cholesterol during preloading was calculated from the amount of isotope in the sac at the end of efflux *plus* the total isotope collected in the media during efflux. Efflux has been expressed as per cent loss of isotope. Conversion into mass efflux is precluded by changing overall specific activity of mucosal cholesterol and uncertainty regarding the size of the exchangeable pool. The total mass of mucosal cholesterol in the sacs (about 700 μg)was fairly large relative to that in the medium (about 250 μg).

RESULTS

There was progressive uptake of labelled cholesterol from both types of micellar solution for periods up to 2 h, the rate of accumulation becoming slower after the first 10 min. At all times, uptake was greater from bile salt micellar solution (Fig. 1). The difference increased with time but even in the first 10 min, when back-flux of labelled molecules should have been minimal, uptake was 2–4 times faster from bile salt micelles.

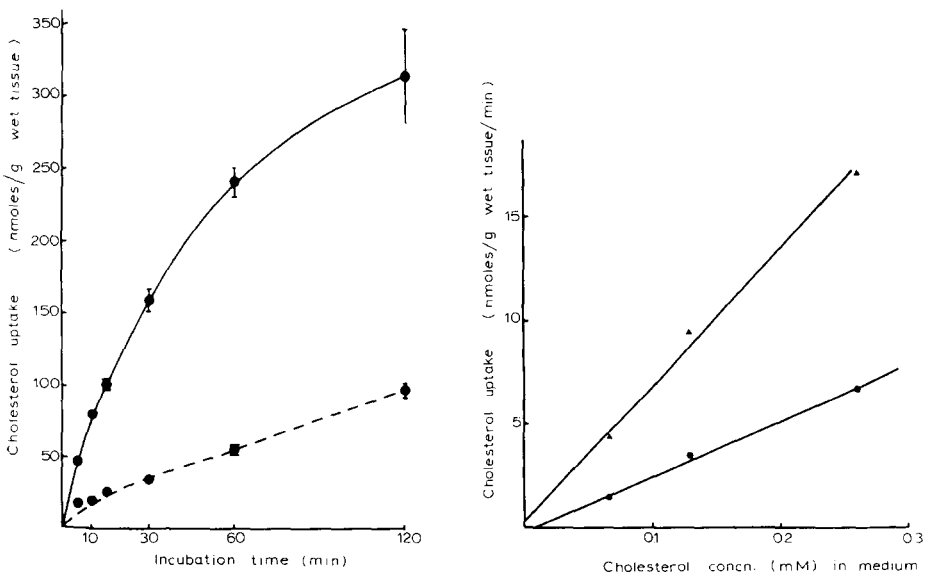

Fig. 1. The uptake of labelled cholesterol from micellar solutions of two detergents. The lipid mixture was 0.125 mM [4-^{14}C]cholesterol, 3 mM oleic acid and 3 mM monoolein in each medium. ●—●, uptake (nmoles/g wet weight of tissue) using 10 mM bile salts, sodium taurocholate–sodium taurodeoxycholate in a 4:1 molar ratio; ●---●, uptake (nmoles/g wet weight of tissue) using 50 mg/ml Pluronic F68. Values represent mean ± S.E. (for 6–35 sacs).

Fig. 2. The effect of cholesterol concentration in the incubation medium on the rate of uptake of labelled cholesterol (nmoles/g wet weight of tissue per min) as measured from 5-min incubations. Values are means for at least 4 sacs. The lipid mixture was 3 mM oleic acid and 3 mM monoolein for each concentration of cholesterol used; ▲—▲, rate of uptake from 10 mM bile salt mixed micelles (sodium taurocholate–sodium taurodeoxycholate in a 4:1 molar ratio); ●—●, rate of uptake from 50 mg/ml Pluronic F68.

Uptake increased linearly with cholesterol concentration up to 0.26 mM (Fig. 2). This concentration was probably too low to saturate the uptake mechanism but the use of higher detergent concentrations to enhance micellar cholesterol seemed undesirable. Uptake from bile salt micelles was high relative to uptake from non-ionic micelles over the range of concentration.

Effect of temperature

As an indication of possible differences in mechanism underlying the differences in rates of uptake from the two types of micelles, the temperature coefficients of uptake were compared. After 5 min pre-incubation in phosphate buffer at the required temperature sacs were incubated for 10 min in labelled micellar cholesterol solutions at temperatures from 4 to 35°. The incubation period was short so that initial linear uptake rates could be measured. Temperatures below 16° were not used for non-ionic micellar solutions since these became cloudy on further cooling.

Uptake from bile salt micelles was clearly temperature dependent whereas uptake from non-ionic micelles was not (Fig. 3A). At 5° uptake from bile salt micelles was the same as the temperature-independent uptake from non-ionic micelles between 16 and 35°. An Arrhenius plot (Fig. 3B) gave an apparent activation energy of about 6 kcal/mole cholesterol for uptake from bile salts.

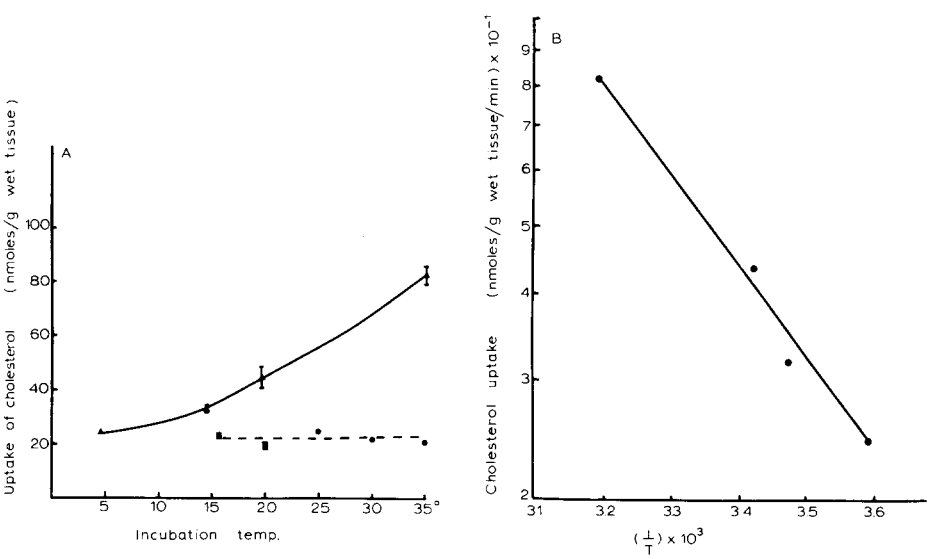

Fig. 3. a. The effect of temperature on [4-^{14}C]cholesterol uptake by everted sacs incubated for 10 min in 10 mM bile salts (sodium taurocholate–sodium taurodeoxycholate in a 4:1 molar ratio) (▲—▲); and 50 mg/ml of incubation medium Pluronic F68 (●---●). The lipid composition of both micellar solutions was 0.125 mM labelled cholesterol, 3 mM oleic acid and 3 mM monoolein. Values are means (nmoles/g wet weight of tissue) ± S.E. (The variance was small for Pluronic F68 at all temperatures and the standard error of the mean is therefore shown only for two points.) At least 4 sacs were used at each temperature. b. Arrhenius plot showing rate of uptake of labelled cholesterol (nmoles/g wet weight tissue per min) *vs.* the reciprocal of absolute temperature. Sacs were incubated in 10 mM bile salts (sodium taurocholate–sodium taurodeoxycholate in a 4:1 molar ratio), 3 mM monoolein, 3 mM oleic acid and 0.125 mM [4-^{14}C]cholesterol for 10 min. 8 sacs from 4 rats were used at 35°; at other temperatures, values represent the mean for 4 sacs from 2 rats.

TABLE I

THE EFFECT OF SOME METABOLIC INHIBITORS ON UPTAKE OF LABELLED CHOLESTEROL

Except for the 6 mM sodium taurodeoxycholate medium, the lipid composition of all incubation solutions was 3 mM monoolein, 3 mM oleic acid, and 0.125 mM [4-^{14}C]cholesterol. The lipid composition of the 6 mM sodium taurodeoxycholate medium consisted of 5.4 mM monoolein and 0.125 mM [4-^{14}C]cholesterol. 10 mM bile salt solutions were made up of sodium taurocholate–sodium taurodeoxycholate in a molar ratio of 4:1. Pluronic F 68 was used at a concentration of 50 mg/ml. 8 pairs of sacs from 4 rats were used for each experiment and values represent means ± S.E.

Inhibitor	*Medium*	*Incubation time (min)*	*Cholesterol uptake (nmoles/g wet tissue)*		*Change in uptake (% of control)*	*P*
			Controls	*Test*		
5 mM NaF	10 mM bile salt micelles	60	259.7 ± 6.4	210.9 ± 4.8	−19	<0.001
5 mM NaF	Pluronic F68 micelles	60	58.2 ± 2.1	78.3 ± 2.7	+34	<0.001
Iodoacetamide (200 μg/ml)	10 mM bile salt micelles	30	165.2 ± 4.7	162.2 ± 7.0	−1.8	Not significant
Iodoacetamide (200 μg/ml)	6 mM sodium taurodeoxycholate micelles	30	107.4 ± 1.8	136.2 ± 2.7	+27	<0.005

Metabolic inhibitors

Although uptake from bile salt micelles was temperature dependent the effect was not large enough to indicate unequivocally that metabolic energy was involved. The results of further experiments, using metabolic inhibitors, are summarized in Table I.

200 μg/ml iodoacetamide produced no significant alteration in uptake from mixed taurocholate–taurodeoxycholate micellar solution. An increase in uptake with iodoacetamide has been reported for cholesterol in monoolein–taurodeoxycholate micelles[12]. This was confirmed (Table I). Interpretation is complicated by the effects of differences in the bile salt mixture and in other lipids in the mixed micelles. With 5.4 mM monoolein–6 mM taurodeoxycholate, cholesterol uptake in the absence of iodoacetamide was less than from the bile salt–lipid mixture used in the present experiments. It was also significantly less (8 sacs, $P < 0.01$) than from 3 mM monoolein–3 mM oleic acid in 6 mM taurodeoxycholate. 5 mM NaF produced a statistically significant but small inhibition of uptake after 60 min incubation in mixed bile salt micellar solution of cholesterol. It was interesting, though puzzling, to find some enhancement by F^- of the normally small uptake from non-ionic micelles. In concentrations ($1 \cdot 10^{-4}$ M) known to uncouple oxidative phosphorylation, 2,4-dinitrophenol had no significant effect.

Efflux experiments

The results of experiments with metabolic inhibitors still left open the possibility that the enhancement of uptake by bile salts relative to non-ionic micelles might

reflect differences in binding of labelled cholesterol by the absorptive cells. Efflux experiments were carried out in which sacs were first loaded with isotopic cholesterol in one of the two types of micellar solution and then transferred for efflux into unlabelled cholesterol solution either in the same detergent or in the alternative one (Fig. 4B).

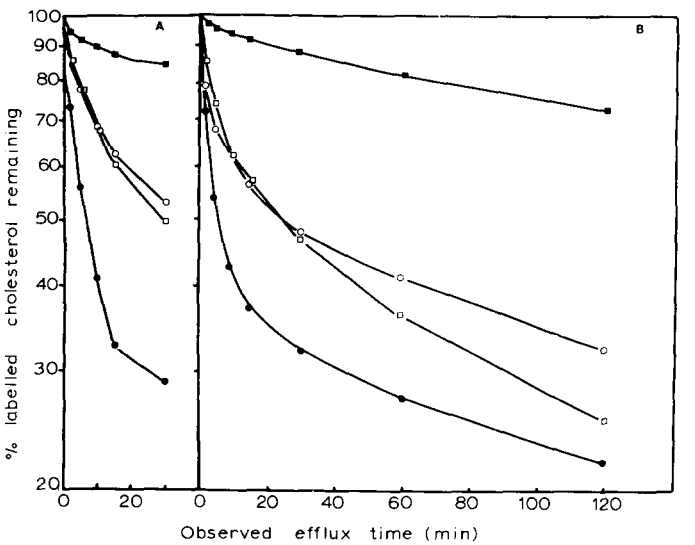

Fig. 4. Efflux of labelled cholesterol from sacs preloaded for 30 min in 0.125 mM [4-^{14}C]cholesterol, 3 mM monoolein, 3 mM oleic acid solubilised in either of the two detergents. A. 30-min efflux into a cholesterol-free medium containing 3 mM oleic acid, 3 mM monoolein and either of the two detergents. B. 120-min efflux into 3 mM monoolein, 3 mM oleic acid, non-labelled 0.125 mM cholesterol and either of the two detergents. In both uptake and efflux media, either 10 mM bile salts or Pluronic F68 (50 mg/ml) was used to solubilise the lipids; □—□, sacs preloaded in bile salt micelles, and effluxed into bile salt micelles; ○—○, sacs preloaded in Pluronic F68 and effluxed into Pluronic F68; ■—■, sacs preloaded in bile salt medium and effluxed into Pluronic F 68 micelles; ●—●, sacs preloaded in Pluronic F68 micelles and effluxed into bile salt micelles.

Efflux did not appear to be strictly exponential although it could be approximately resolved into an initial rapid phase, $t_{\frac{1}{2}}$ usually about 10 min, and a subsequent slower efflux, $t_{\frac{1}{2}}$ about 5 h. While the data do not justify a kinetic analysis it is interesting to note that very little isotopic efflux occurred in the first 20 min when sacs loaded from micellar solution in bile salts were exposed to unlabelled cholesterol in non-ionic micelles. On the other hand a major part of the isotopic cholesterol effluxed when sacs loaded in non-ionic micellar cholesterol were exposed to unlabelled cholesterol in bile salts. Early rapid efflux was intermediate for sacs in which the same type of micelles were used for loading as for efflux and the proportion of isotope lost was much the same for both types. This suggests that the type of detergent may influence the mode of adsorption of cholesterol to the cell membrane or its penetration and thus its accessibility for subsequent efflux.

To eliminate the effect of exchange on efflux of labelled cholesterol, the experiments were repeated with efflux media containing no cholesterol but otherwise identical with those used before. During a 30-min period efflux into cholesterol-free media was virtually the same as into those containing cholesterol, 0.125 mM (*cf.*

Figs. 4A and 4B). In contrast to uptake, preliminary experiments revealed no effect of temperature on efflux into bile salt micellar media.

DISCUSSION

These experiments *in vitro* showed that uptake of labelled cholesterol from a lipid mixture in micellar solution in bile salts differed quantitatively from uptake when the micellar solubilizer was a non-ionic detergent. Uptake from non-ionic micelles was only 1/4–1/2 that from bile salt micelles. There was an even greater discrepancy, *in vivo*, in a few preliminary experiments on lymphatic absorption in unanaesthetized rats.

Quantitative differences in uptake from micellar solutions might be related to differences in partition between micelles and cell membrane, *in vitro*, since the system is a closed one with little or no translocation of lipid from absorbing cell to tissue fluid. This was emphazied by FELDMAN AND BORGSTROM[13] who found for example that cholesterol ethers were taken up *in vitro* in larger amounts than cholesterol but were transported to the lymph *in vivo* more slowly than cholesterol. In their experiments the uptake of different sterols was compared using the same micellar solubilizer. In the present experiments two types of micellar solubilizers were compared using the same sterol. To explain these results by differences in partition, non-ionic micelles would have to show a greater affinity for cholesterol than bile salt micelles, relative to the cell membrane. However, this would be difficult to reconcile with the results of efflux experiments. This was seen more clearly with efflux into cholesterol-free media, in which exchanges of labelled for unlabelled cholesterol could be excluded. Efflux into bile salt micellar media was faster than into non-ionic micellar media, whereas the reverse should be true if the non-ionic micelles had a higher affinity for cholesterol relative to the cell membrane.

Both uptake and efflux were faster with bile salt micellar media. This would be compatible with rate-limiting diffusion to and from the surface of the cell membrane, faster for the smaller bile salt micelles. A small apparent activation energy, about 6 kcal/mole, would not exclude diffusion but it would still be necessary to explain why uptake from bile salt micellar media was temperature sensitive but not uptake from non-ionic micelles nor efflux into either medium. Perhaps uptake is a two-stage process[14]. It might be that only the second stage is temperature sensitive and that this is mediated by bile salts but not by non-ionic micelles. Such a hypothesis would be consistent with (i) slower efflux from sacs *in vitro* after uptake from bile salt micellar media and (ii) failure of transfer of cholesterol from non-ionic micelles to lymph *in vivo*. If there is a second stage in uptake it would seem to be distinct from subsequent esterification of cholesterol, which was minimal *in vitro*. It will be interesting to compare uptake of cholesterol from the two types of micellar solution by brush border preparations. It will also be necessary to test the effect of other detergents since Tween 80 has been reported to promote cholesterol absorption[15].

The possibility must also be taken into account, as with all *in vitro* experiments, that the results are an artifact of the model and attributable to different rates of deterioration from the state *in vivo*. Controls of this possibility are seldom mentioned. In the present experiments there was morphological evidence of deterioration. This was by no means as severe as that found by LEVINE *et al.*[16]. It consisted of subepithelial

edema followed later by evidence of autolysis and irregular loss of cells from the villi. Early changes were seen at 30 min with non-ionic detergent and were more obvious with bile salts. Such changes probably accounted in part for slower uptake with prolonged incubation. They could hardly explain the large differences within the first 10 min incubation on which quantitative comparisons have mainly been based. It should also be mentioned that evidence has been given[17] for an increase of epithelial permeability to small molecules on exposure of everted sacs to conjugated bile salts. Much of the work was done with concentrations of 10 mM and higher of taurodeoxycholate[17]. It is always difficult to exclude the possibility of contamination by unconjugated dihydroxy bile acids, which are known to be toxic *in vitro*[18]. The conjugated bile salts used in the present experiments were synthesized in this laboratory and were tested chromatographically. The concentration of dihydroxy conjugates in the mixture was deliberately kept low.

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