Role of the Intestinal Brush Border in the Absorption of Cholesterol in Rats

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1. Short-term incubation of the everted intestinal sacs of rats in media containing cholesterol oleate or cholesterol plus oleic acid resulted in rapid hydrolysis, but no synthesis, of the sterol ester. 2. On separation of the brush border from the rest of the mucosal cell, almost all of the hydrolytic activity and appreciable amounts of the synthetic activity of the whole cell were found to be present in the brush-border fraction. 3. The isolated brush-border fraction contained considerable amounts of cholesterol, which was always present in the unesterified state; the rest of the cell contained about an equal amount of unesterified cholesterol, but, in addition, small but definite amounts of the esterified sterol were also found in this fraction. 4. On feeding rats with [4-¹⁴C]cholesterol, which was diluted with 3mg. of cholesterol, it was found that the brush border very rapidly took up the fed sterol without changing its net content of cholesterol. No traces of radioactive cholesterol ester could ever be detected in the isolated brush border after feeding with ¹⁴C-labelled esterified or unesterified cholesterol. 5. The appearance of the labelled sterol was quite rapid in the rest of the cell also, where small proportions were found in the esterified state. 6. Therefore the sequence of events in the absorption of cholesterol appears to be: the dietary cholesterol esters are hydrolysed by the cholesterol ester hydrolase of pancreas or of the mucosal brush border or both, after which the brush border rapidly absorbs the de-esterified sterol and transfers it into the mucosal cell, by a mechanism of displacement, where it is slowly re-esterified for transport through the lymph.

Several studies on the mechanism of absorption of cholesterol have established that the sterol can enter the mucosal cells only as unesterified cholesterol (Friedman, Byers & St George, 1956; Treadwell, Swell & Vahouny, 1962) and that the absorbed sterol appears in the lymph largely as its ester (Swell, Trout, Hopper, Field & Treadwell, 1959; Treadwell, Swell, Vahouny & Field, 1959). However, little is known about the fate of the absorbed sterol inside the mucosal cell before it eventually appears in the lymph. Nevertheless, the hydrolysis of cholesterol esters and the subsequent esterification of most of the free cholesterol seem to be essential steps in the overall process of absorption of cholesterol esters, and it has been demonstrated that both pancreas and intestinal mucosa of rats contain both cholesterol ester hydrolase and synthetase (Murthy, Mahadevan & Ganguly, 1961a; Murthy, Mahadevan, Seshadri Sastry & Ganguly, 1961b; Murthy & Ganguly, 1962). It is also known that the mucosal enzymes are not of pancreatic origin and are much less active than the pancreatic

enzymes (Murthy *et al.* 1961b; Murthy & Ganguly, 1962; Lossow, Migliorini, Brot & Chaikoff, 1964).

The role of the brush border in the absorption of cholesterol is not clearly understood. By isolating the brush border of rat intestine, we have shown that it contains almost all of the hydrolytic activity of the mucosal cells and that, during the absorption of cholesterol, it rapidly takes up the de-esterified sterol and transfers it into the mucosal cell, where it is re-esterified.

We had previously shown that the hydrolytic and synthetic activities of rat pancreas towards cholesterol esters are due to two separate enzymes (Murthy & Ganguly, 1962). By separating the brush border from the rest of the mucosal cell, we have demonstrated a differential distribution of the two enzymes of rat-intestinal mucosa.

MATERIALS AND METHODS

[4-¹⁴C]Cholesterol was obtained from The Radiochemical Centre, Amersham, Bucks. [4-¹⁴C]Cholesterol stearate and oleate were prepared enzymically with acetone-dried goat

pancreas as the source of enzyme and under the conditions described by Murthy & Ganguly (1962), who also give the sources of the other materials used.

Normal male albino rats of this Institute strain, weighing about 150 g., were used in all experiments.

The procedure of Miller & Crane (1961) was adopted for the isolation of the brush border of rat-intestinal mucosa. Miller & Crane (1961) used the term 'supernatant' to mean the mucosal cell stripped of the brush border, and we have used the same terminology here. These workers had shown that 75–86% of the total invertase (Miller & Crane, 1961) and 68–75% of the total alkaline phosphatase (Holt & Miller, 1962) of the intestinal mucosa of hamster are localized in the brush-border fraction. When we assayed the invertase according to Miller & Crane (1961) and alkaline phosphatase according to Heppel (1955), in the supernatant and brush-border fractions obtained from rat-intestinal mucosa, we found that about 75% of the total invertase activity of the intestinal mucosa could be recovered in the brush border, the rest being present in the supernatant; we found that, on first sedimentation after the separation of the brush border from the mucosal cell, about 55–60% of the total activity of the alkaline phosphatase was recovered in the brush-border fraction. But during the course of purification, which was carried out by successive washing of the brush-border fraction with ice-cold 5 mM-EDTA, most of the alkaline phosphatase was released from the sediment into the washing medium, which did not occur with the invertase. When examined under the phase-contrast microscope, our preparations resembled the description of the isolated brush border given by Miller & Crane (1961).

In feeding experiments the rats were first starved for 24 hr., after which they were given 0.4 μ C of [4- 14 C]cholesterol diluted with 3 mg. of cholesterol and dissolved in 0.2 ml. of groundnut oil. Labelled cholesterol stearate was administered in a similar manner after diluting it with 3 mg. of cholesterol stearate. At suitable time-intervals the animals were killed by heart puncture under light ether anaesthesia and the whole intestine was removed into beakers maintained at 0°. The intestinal contents were immediately washed out with ice-cold 0.9% NaCl, and the mucosa was scraped off with the blunt end of a scalpel. The brush border was then separated from the rest of the mucosa according to the procedure of Miller & Crane (1961).

The cholesterol ester-hydrolysing and -synthesizing activities of the mucosal brush border and of the supernatant fractions, as well as of the 0.25 M-sucrose homogenates of the whole mucosa, were assayed by the method of Murthy & Ganguly (1962).

In experiments in which everted sacs were used, the whole intestine of each individual rat was cut into five segments, each approx. 18 cm., which were weighed and then everted as described by Wilson & Wiseman (1954). The five segments from each rat were incubated in separate flasks containing 30 ml. each of the same reaction mixture. Unless otherwise stated, the reaction mixture for the cholesterol ester-synthesizing activity consisted of 30 ml. of 0.1 M-veronal buffer, pH 6.1, 30 μ moles of cholesterol, 60 μ moles of oleic acid and 120 μ moles of sodium taurocholate; for the cholesterol ester-hydrolysing activity it was 30 μ moles of cholesterol oleate and 120 μ moles of sodium taurocholate in 30 ml. of 0.1 M-veronal buffer,

pH 8.6. The substrates (cholesterol plus oleic acid or cholesterol oleate) were first dissolved in ethanol and then dispersed in the respective buffers to give a final concentration of 10% of ethanol in the media. The serosal medium for the synthetic activity was 0.1 M-veronal buffer, pH 6.1, and for the hydrolytic activity it was 0.1 M-veronal buffer, pH 8.6. The incubations were carried out with constant shaking in a metabolic shaker, which was maintained in a water bath at 37°. At the desired time-intervals 2 ml. portions of the incubation medium were withdrawn from each flask and pooled for subsequent analysis.

Extraction and chromatographic separation of cholesterol and its esters, and their radioactivity and chemical measurements, were carried out as described by Murthy, David & Ganguly (1963). Proteins were estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

RESULTS

We had demonstrated in our earlier work that the intestinal mucosa of rats contains both the hydrolytic and synthetic activities of cholesterol esterases (Murthy *et al.* 1961b; Murthy & Ganguly, 1962). Fig. 1 shows that incubation of the everted sacs with cholesterol oleate in the medium resulted in rapid hydrolysis of this ester, the rate of hydrolysis being linear up to 30 min., after which time the activity had distinctly slowed down, but continued up to 20 hr. This hydrolytic activity, when

Fig. 1. Cholesterol ester hydrolysis and synthesis by the everted intestinal sacs. The experimental conditions are described in the Materials and Methods section. No perceptible esterification of the medium cholesterol took place at the earlier time-intervals, as can be seen from the points (○) on the base line. ●, Hydrolysis of cholesterol oleate; ○, synthesis of cholesterol oleate.

expressed in terms of μmoles of cholesterol liberated/g. of intestine/hr., was 1800, 630, 460 and 315 at $\frac{1}{2}$, 2, 3 and 20 hr. respectively. In striking contrast with the very rapid hydrolysis of the ester present in the medium there was no esterification of the free sterol of the medium even after 3 hr. incubation; however, on prolonging the incubation to 20 hr. some esterification took place.

Although the everted sacs could not effect any esterification of the cholesterol of the medium at the earlier time-intervals of incubation, it is shown in Table 1 that rapid synthesis of the sterol ester can be demonstrated if the mucosal cells are broken by homogenization. The sterol-esterifying activity, as manifested by the everted sacs only after prolonged incubation (Fig. 1), is therefore probably due to the disruption of the mucosal cells caused by such an incubation, which might result in the release of the hitherto inaccessible esterifying enzyme. The rapid hydrolysis of the medium ester by the everted sacs, on the other hand, made it obvious that the hydrolytic enzyme is in intimate

contact with the substrate. One possibility is that the hydrolytic enzyme is situated on the brush border. That this is indeed so is shown in Table 2, where it is demonstrated that the brush border, when separated from the rest of the mucosal cell, almost quantitatively accounts for the entire cholesterol ester-hydrolytic activity of the whole mucosa. Table 2 also shows that, in addition to the presence of about 92% of the hydrolytic activity, nearly 33% of the synthetic activity of the whole mucosal cell was recovered in this fraction.

These results disagreed sharply with the data of Gallo & Treadwell (1963), who claimed that the brush border of rat intestine is devoid of either of the two activities. The conditions of assay employed by us and by these workers were quite different, which led us to repeat the experiments under conditions similar to those described by them. Table 2 shows that under their conditions the two enzyme activities were about equally distributed between the supernatant and brush-border fractions. The discrepancy between the results obtained under the two different sets of conditions is probably due to the different periods of incubation. The shorter period of 2–3 hr. (Murthy & Ganguly, 1962) is likely to give a more correct picture of the enzyme activities than the period of 18–24 hr. used by Gallo & Treadwell (1963).

It might be argued that in our experiments with the everted sacs the use of fixed pH for the respective reactions could have given such differential activities. But it is shown in Fig. 2 that on gradually changing the pH from 5.1 to 9.6 no synthesis of the sterol ester could be noticed in the medium at any pH, although the pH optimum for the cholesterol ester-synthesizing enzyme of rat intestine has been shown to be at pH 6.1 (Murthy & Ganguly, 1962). On the other hand, similar changes in the pH of the incubation medium led to very sharp changes in the efficiency of hydrolysis of the sterol ester of the medium. Strangely enough the pH optimum

Table 1. *Hydrolysis and synthesis of cholesterol esters by the mucosal homogenates and everted sacs of rat intestine*

The mucosa was removed from the weighed intestine and homogenized in 0.25 M-sucrose. Everted intestinal sacs were prepared and incubated as described in the Materials and Methods section. Enzyme activities are expressed as μmoles of cholesterol liberated or esterified/hr./g. of intestine. The results are averages of four separate experiments in each group. The figures in parentheses represent the range of each value.

Intestinal preparation	Hydrolytic activity	Synthetic activity
Mucosal homogenate	1300 (1240–1450)	2000 (1800–2200)
Everted sacs	1450 (1360–1590)	Nil

Table 2. *Distribution of the hydrolytic and synthetic activities for cholesterol esters in the brush-border and supernatant fractions of rat-intestinal mucosa*

Enzyme activities are expressed as μmoles of cholesterol liberated or esterified/mg. of protein/hr. and are averages of six or more individual experiments. The figures under (a) represent the values obtained with the method of Murthy & Ganguly (1962) and those under (b) are the corresponding values obtained by using the procedure of Gallo & Treadwell (1963). Figures in parentheses are the ranges for each mean value.

	Hydrolytic activity		Synthetic activity	
	(a)	(b)	(a)	(b)
Mucosal fraction				
Brush border	83 (72–95)	37 (28–43)	48 (41–56)	36 (27–39)
Supernatant	9 (0–14)	38 (24–42)	99 (79–110)	43 (33–51)

Fig. 2. Effect of pH on the hydrolysis and synthesis of cholesterol ester by the everted intestinal sacs. The conditions of the experiments are similar to those described in the Materials and Methods section. Veronal buffer (0.1M) was used throughout and all reactions were stopped after incubation for 2hr. Here also there was no perceptible esterification of the sterol in the medium, as can be seen from the points (○) on the base line. ●, Hydrolysis of cholesterol oleate; ○, synthesis of cholesterol oleate.

obtained with the everted sac was identical with that found for the solubilized mucosal hydrolytic enzyme (Murthy & Ganguly, 1962).

The above experiments have thus established that the brush border displays a high hydrolytic activity towards cholesterol ester, and this could lead to the assumption that this particular fraction may take an active part in the overall process of absorption of cholesterol, because this sterol is absorbed only in the unesterified form (Friedman *et al.* 1956; Treadwell *et al.* 1962). The results presented in Table 3, however, show that, apart from its enzymic potential, the brush border also plays a vital role in the actual entry of cholesterol into the mucosal cells. Thus, not only is the brush border very rich in its endogenous cholesterol content, but it also rapidly takes up the fed sterol. It can be seen from Table 3 that, compared with the rest of the cell, the brush border takes up the fed sterol earlier, and that the specific activity of the sterol in this fraction is not only higher at all periods but also reaches its peak earlier. What is most striking is the total absence of cholesterol ester from the brush-border fraction even after feeding with cholesterol, the sterol in it always being in the unesterified state. The supernatant fraction also is rich in its endogenous cholesterol content, but, in contrast with the total absence of the esterified sterol from the brush border, this fraction always contained

small amounts of the sterol esters. Here also the fed labelled sterol appeared rapidly, although a little later than in the brush border. The specific activities of both the esterified and unesterified sterol in the supernatant reached their peak at about the same time and declined at about the same rate.

Further, that prior hydrolysis of the fed cholesterol ester is essential for the absorption of the sterol into the mucosal cells is demonstrated in Table 3, where it will be seen that, after feeding with [4-¹⁴C]cholesterol stearate, at no time-interval were we able to detect even traces of the esterified labelled sterol in the brush-border fraction, whereas it always contained significant amounts of the unesterified labelled sterol. The supernatant fraction, however, gave a picture similar to that obtained after feeding with the unesterified labelled sterol. In these experiments the uptake of the fed esterified sterol was slower than that of the unesterified sterol; this is probably because the fed ester has to await hydrolysis before absorption.

DISCUSSION

The existence of the brush border on the columnar cells of the villi of the small intestine has been known for a long time. Yet its precise role in absorption from the intestine has not been properly understood. The classical work of Gomori (1941) had demonstrated histochemically the presence of highly active phosphatase in the brush border, but this work did not define the possible role, at the enzyme level, the brush border might play in absorption in general. More recent biophysical techniques, e.g. electron microscopy, have revealed the ultrastructure of the brush border (Dalton, 1950, 1951; Granger & Baker, 1950; Weiss, 1955a,b; Gol'din, 1956; Zetterqvist, 1956; Palay & Karlin, 1959; Millington & Finean, 1962, 1963; Sjöstrand, 1963). But these demonstrations have led to the general assumption that the main, if not the only, function of the brush border is to act as a sieve and expose enormously large surface areas, thereby facilitating very rapid absorption. However, little attempt was made, till very recently, to study the enzymes of the brush border; clearly, in the absence of such vital information the precise biochemical functions of the brush border in the overall processes of absorption can be little understood.

Serious attempts at the separation of the brush border from the intestinal mucosal cells seem to have been made only recently, as testified by the successful isolation of the brush border of hamster intestine by Miller & Crane (1961). These workers also demonstrated almost quantitative localization of invertase and maltase of the mucosal cells in

Table 3. *Distribution, during absorption, of [4-¹⁴C]cholesterol in the brush-border and supernatant fractions of rat-intestinal mucosa*

Values are expressed as counts/min./mg. of cholesterol. Figures in parentheses represent the net amounts of chemically determined unesterified or esterified cholesterol. Results are averages of four separate experiments in each group in those cases where cholesterol was fed, and of two such experiments in those cases where its ester was given. In the fourth column all values are nil.

Form of sterol fed	Time (min.)	Brush border		Supernatant	
		Unesterified	Esterified	Unesterified	Esterified
Unesterified	15	1076 (1.66)	Nil	553 (3.28)	539 (0.36)
	30	1300 (2.24)		706 (2.27)	590 (0.33)
	90	1498 (2.12)		1196 (2.88)	655 (0.36)
	120	1685 (1.90)		1295 (2.94)	897 (0.11)
	150	2459 (2.08)		1422 (1.60)	1200 (0.33)
	180	2288 (2.08)		1635 (3.54)	1918 (0.24)
	240	2196 (2.01)		1521 (3.18)	1227 (0.18)
	360	1397 (2.24)		401 (2.88)	1000 (0.24)
	720	596 (2.24)		283 (2.60)	550 (0.30)
	1440	186 (2.40)		205 (2.90)	270 (0.13)
	2880	77 (1.40)		74 (1.70)	139 (0.3)
Esterified (stearate)	5	90 (2.74)		15 (3.00)	Nil (0.25)
	10	180 (1.90)		36 (3.00)	Nil (0.26)
	60	320 (2.01)		136 (3.00)	Nil (0.24)
	120	613 (1.61)		368 (2.24)	632 (0.24)
	180	1125 (3.10)		992 (3.12)	745 (0.19)

this fraction. Holt & Miller (1962) demonstrated the predominance of yet two more enzymes, namely leucine amino peptidase and alkaline phosphatase in the isolated brush border of hamster mucosa. Gallo & Treadwell (1963) found about 96% of the total invertase activity of the rat-mucosal cells in the isolated brush-border fraction, although Dahlqvist & Thomson (1964) do not seem to believe that the total invertase activity of the rat-mucosal cells resides in this fraction only. Our results fully confirm the claims of Miller & Crane (1961); the values reported by Gallo & Treadwell (1963) appear to be on the high side. We therefore believe that invertase can be used as a marker for this fraction, and have followed it in this work. Although a very active alkaline phosphatase is present in the brush border (Holt & Miller, 1962),

this enzyme cannot be as good a marker as invertase, because, as indicated before, during the successive stages of washing of the brush border it tends to leave the particulate sediment and appear in the supernatant.

The present work seems to be the first attempt at defining a role that the brush border might play in the absorption of cholesterol. Thus in the experiments with the everted intestinal sacs we found very rapid hydrolysis of the cholesterol ester; this suggested that the hydrolytic enzyme of the everted sacs must be in very intimate contact with the substrate. In fact, we have shown that, when separated from the rest of the mucosal cell, 92% of the total cholesterol ester-hydrolytic activity of the whole cell can be recovered in the brush-border fraction. In contrast with the very rapid hydrolysis

of the medium ester, the everted sacs showed no synthesis of cholesterol ester even up to 3 hr. of incubation. Some esterification, however, did take place when incubations were carried over a very long period. This was probably due to the disruption of the cells caused by the prolonged incubation, which was accompanied by constant shaking. Such an interpretation would appear to be valid, because, although the intact cells of the everted sacs showed no esterification, homogenization of the mucosal cells revealed the presence of a highly active esterifying enzyme in the cell. But a part of the esterifying activity of the whole cell was recovered in the brush border. Therefore it appears that in the intact intestine the hydrolytic activity of the mucosa must be situated on the outer surface of the brush border and must be oriented in such a way that it constantly meets its substrate. The esterifying enzyme of the brush border, on the other hand, is probably oriented towards the inner side of the cell. Such a spatial arrangement of this enzyme would explain the inability of the everted sacs to esterify the medium sterol. These results closely resemble our earlier observations on the localization of the hydrolytic enzyme for retinyl esters on the mucosal membrane, because in these experiments also we demonstrated a similar rapid hydrolysis of the medium retinyl esters by the everted intestinal sacs of rats (Mahadevan, Seshadri Sastry & Ganguly, 1963).

We had previously shown that, after feeding with [4-¹⁴C]cholesterol, the ester fraction of the labelled sterol of rat-intestinal mucosa is found distributed among the various fractions of the mucosal cell, with the microsomal fraction showing the highest activity, followed by the cell-sap fraction (Murthy *et al.* 1963). In the present experiments we have never found even traces of the ester of the labelled sterol in the isolated brush-border fraction (Table 3). It would thus be quite logical to conclude that the brush border isolated by us is free of contamination from the rest of the mucosal cell or its organelles. Therefore the activity of the synthetic enzyme as found in the isolated brush border must belong to this fraction. Such a highly active enzyme does not seem to play any role in esterifying the sterol during its absorption and it is pertinent to query whether the mere demonstration of the presence of an enzyme in any tissue must necessarily lead to a claim for its metabolic role in that particular tissue.

In our previous work, where water-soluble enzymes prepared from acetone-dried rat pancreas were used, we effected a clear separation of the hydrolytic and synthetic cholesterol esterases, and we concluded that the two activities are due to separate enzymes (Murthy & Ganguly, 1962). We have now demonstrated a markedly different dis-

tribution of the two activities in the different areas of the cell of rat-intestinal mucosa, and this was achieved by the minimum dislocation of the normal organization of the mucosal cell. Shyamala, Lossov & Chaikoff (1965) have reported a similar differential localization of the two activities in the homogenates of bovine adrenal glands.

We had earlier suggested that cholesterol is probably absorbed by a mechanism of displacement of the endogenous cholesterol of the lipoproteins that constitute the membranous materials of the mucosal cells (Ganguly, Krishnamurthy & Mahadevan, 1959). Subsequent work with labelled sterol substantiated such a hypothesis, because we demonstrated rapid and preferential appearance of the fed labelled sterol in the microsomal fraction of the mucosal-cell homogenates (Murthy *et al.* 1963). In all this earlier work we used the whole mucosal cell and did not attempt to isolate the brush border. Separation of the brush border from the rest of the cells has now enabled us to demonstrate not only that the brush border is very rich in endogenous cholesterol, but also that it rapidly takes up the fed cholesterol without effecting any quantitative changes in its cholesterol content. Therefore the theory of the absorption of cholesterol by a mechanism of displacement can be extended to the brush border also. Further, at the earlier time-intervals after the feeding of labelled sterol, not only was the specific activity of the labelled sterol in the brush border markedly higher than that in the rest of the cell, but also its peak appeared earlier in the brush border than in the rest of the cell. Even more significant is the total absence of any cholesterol ester in the brush-border fraction despite the presence of abundant amounts of the substrate and a very active esterifying enzyme in the isolated brush border. Moreover, the total absence of esterified sterol from the brush-border fraction, even after feeding of the sterol ester, confirms the observations of the earlier workers that the cholesterol esters have to be totally de-esterified before the sterol can be absorbed (Friedman *et al.* 1956; Treadwell *et al.* 1962); this de-esterification seems to be a significant rate-limiting step, because, at all time-intervals after feeding of comparable amounts of the labelled sterol in the esterified or unesterified state, markedly higher radioactivity could consistently be located in all the fractions of the mucosal cell of those rats that received the unesterified sterol. The brush border thus appears to play at least two major roles in the absorption of cholesterol: it can complete the hydrolysis of the fed sterol esters, which under normal circumstances is probably initiated by the more powerful pancreatic enzyme; this is then followed by the rapid uptake of the de-esterified sterol by the brush border.

Another important event in the process of absorption of cholesterol is its re-esterification, because the absorbed sterol eventually appears in the lymph mostly as its ester (Swell *et al.* 1959; Treadwell *et al.* 1959). It has already been discussed that no sterol ester can be detected in the isolated brush border after feeding with esterified or unesterified cholesterol. On feeding the labelled unesterified sterol the specific activity of the cholesterol ester in the supernatant steadily increased, reaching a peak at 180 min., after which it declined, and at all time-intervals the net amount of cholesterol ester in this mucosal fraction remained fairly constant (Table 3). These results show that re-esterification takes place inside the mucosal cell and that the rate of re-esterification is parallel to the rate of removal of the ester from the mucosal cell. On the basis of the results of the present work it is therefore possible to visualize the following sequence of events in the absorption of cholesterol: the dietary esters of cholesterol are hydrolysed by the cholesterol ester hydrolase of either the pancreas or the mucosal brush border, or by both, after which the de-esterified sterol is rapidly taken up by the brush border and transferred into the mucosal cell, where it is slowly re-esterified for transport through lymph.

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