Distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase and alkaline phosphatase activities in isolated ileal epithelial cells of fed, fasted, cholestyramine-fed, and 4-aminopyrazolo[3,4-*d*]pyrimidine-treated rats

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Abstract 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, E.C. 1.1.1.34), the major rate-limiting enzyme of the sterol biosynthetic pathway, was studied in ileal epithelial cells isolated in a villus-to-crypt gradient according to Weiser (Weiser, M. M. 1973. *J. Biol. Chem.* **248:** 2536–2541). Alkaline phosphatase (E.C. 3.1.3.1) served as a marker for the mature villus cells. Protease effects on activity determinations were negligible. The intracellular location of HMG-CoA reductase could not be precisely determined. The activity of ileal reductase was predominantly associated with the less differentiated lower villus and crypt cells, while the reverse gradient occurred with alkaline phosphatase. This distribution of enzymes persisted in both fed and fasted rats injected with control saline–phosphate, although fasting decreased total reductase units in the ileum by 86% in 72 hr. Treatment with cholestyramine and with 4-aminopyrazolo[3,4-*d*]pyrimidine (APP) enhanced reductase activity in ileal cells. The percent stimulation in both cases was higher in the upper villus cells than in the crypt cells, leading to abolition of the gradient in enzyme activity. However, APP treatment caused a 98% loss in total alkaline phoshatase units and a 55% loss in total epithelial cell protein in 72 hr. Thus, there was no increase in total reductase units. These data show that APP affects ileal cell metabolism directly. Furthermore, it appears that the regulation of sterol synthesis in the intestinal mucosa, via HMG-CoA reductase, involves a complex interplay of the effects exerted by the level of alimentation, the enterohepatic circulation of bile, and the levels of plasma lipoproteins.—**Panini, S. R., G. Lehrer, D. H. Rogers, and H. Rudney.** Distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase and alkaline phosphatase activities in isolated ileal epithelial cells of fed, fasted, cholestyramine-fed, and 4-aminopyrazolo[3,4-*d*]-pyrimidine-treated rats. *J. Lipid Res.* 1979. **20:** 879–889.

Supplementary key words villus cells · crypt cells · sterol synthesis

It is known that the small intestine of the rat is capable of rapid synthesis of cholesterol (1). The contribution of the latter to the serum cholesterol levels has also been documented (2). However, while an enormous amount of literature has accumulated on sterol synthesis and its relationship to the major rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (E.C. 1.1.1.34) in the liver, available information in the case of the small intestine is quite meager. Considering that the intestine is the major extrahepatic tissue that contributes to plasma lipoproteins and that it is second only to the liver in its extent of cholesterol synthesis (3–5), interest in intestinal HMG-CoA reductase activity seems justified. Earlier studies suggested that both cholesterol synthesis and HMG-CoA reductase activity were predominantly located in the crypt cells of the intestinal epithelium in the rat (3, 6). However, more recent studies have indicated that the villus cells display activity that is equal to or even greater than that of the crypt cells (7, 8). The latter study (8) utilized a method developed by Weiser (9) to isolate intestinal epithelial cells through incubation of gut loops in EDTA buffers. This method appears to be considerably less injurious to the cells than the earlier scraping technique (3) as determined by cell viability and enzyme activities (7, 9).

Present knowledge of the regulation of sterol synthesis in the ileum implicates three major parameters: the level of alimentation, the bile acid-mediated uptake of lumenal sterols, and the level of plasma lipoproteins (3–5, 10–12). The first parameter has usually been studied via fasting the animals and the second through interference with enterohepatic circulation of the bile. The study of the third

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonate; DTT, dithiothreitol; APP, 4-aminopyrazolo[3,4-*d*]pyrimidine; UV, upper villi; MV, middle villi; LV, lower villi; C, crypt.

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parameter has been made possible by the finding that administration of the adenine analog, 4-aminopyrazolo-[3,4-*d*]pyrimidine (APP), to rats results in a marked reduction in hepatic secretion of lipoproteins and in plasma lipoprotein and cholesterol levels (13, 14). It has recently been reported that this acute reduction in plasma lipoprotein cholesterol levels is associated with enhanced sterol synthesis in several tissues, including the ileum, and with increased HMG-CoA reductase activity in the kidney, lung, and the adrenal (12, 15, 16).

In the present studies, we have isolated the intestinal epithelial cells from rat ileum in a villus-to-crypt gradient using Weiser's method (9). The activity of alkaline phosphatase (E.C. 3.1.3.1) served as a marker for the well-differentiated villus cells. We have examined the interplay of the factors mentioned above on the activity and the distribution of HMG-CoA reductase in these cells. The results demonstrate the primary association of the HMG-CoA reductase activity with the less differentiated lower villus and crypt cells in the normal rat ileum. Cholestyramine feeding and APP treatment alter this association as well as affect the specific activities and total enzyme levels in the ileum. In addition, APP also appears to have a direct effect on the ileal cell metabolism.

MATERIALS AND METHODS

Materials

Labeled substrate, *R,S*-[3-^{14}C]HMG-CoA (50 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Unlabeled HMG-CoA was obtained from P-L Biochemicals, Milwaukee, WI. Other chemicals were obtained from the following sources: DL-[2-^{3}H]-mevalonic acid lactone was from Amersham-Searle, Arlington Heights, IL; *p*-nitrophenylphosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, soy bean trypsin inhibitor (Type I-S), and dithiothreitol were from Sigma Chemical Co., St. Louis, MO; APP was from Aldrich Chemical Co., Milwaukee, WI; bovine serum albumin was from Miles Laboratories, Inc., Kankakee, IL; and cholestyramine resin (Questran) was from Mead Johnson Laboratories, Evansville, IN.

Animals and treatment

Male, Sprague-Dawley rats in the weight range of 200–300 g were maintained under a reversed light–dark cycle (lights on from 3 PM to 3 AM) and were fed Purina laboratory chow ad libitum except as noted below. One group of rats received cholestyramine at a level of 2.5% (w/w) in the diet for 3 days. APP was dissolved in 0.154 M NaCl containing 0.025 M phosphate, pH 2.5, and injected intraperitoneally once a day into another group at a level of 20 mg per kg bodyweight for the indicated number of days. These rats were deprived of food completely but were allowed free access to water during APP treatment. Control rats, both fed and fasted, received the same amount of 0.154 M NaCl–0.025 M phosphate, pH 2.5. All injections were given between 9 and 10 AM, in the middle of the dark phase, and rats were killed 24 hr after the last injection.

Isolation of epithelial cells

Rats were killed by decapitation and 50 cm of the small intestine proximal to the ileo-cecal junction was quickly removed and flushed thoroughly with cold 0.154 M NaCl solution. The intestine was tied at the cecal end with a piece of string and distended with 15–20 ml of a solution (Buffer A) containing KCl, 0.0015 M; NaCl, 0.096 M; sodium citrate, 0.027 M; KH_2PO_4, 0.008 M; and Na_2HPO_4, 0.0056 M, pH 7.2, and was clamped at the other end. The distended intestine was immersed in Buffer A in a beaker and was incubated at 37°C for 20 min in a Blue M constant temperature shaker bath (Blue M Electric Co., Blue Island, IL) with oscillation (60 cycles/min). At the end of 20 min, the intestine was emptied by holding the tied end and opening the clamp at the other end. The intestine was handled only at the ends to achieve a good cell gradient. The intestine was redistended with Buffer B (pH 7.2) containing KCl, 0.0027 M; NaCl, 0.137 M; KH_2PO_4, 0.0015 M; Na_2HPO_4, 0.0043 M; EDTA, 0.0015 M; and DTT, 0.01 M; it was clamped, immersed in a beaker containing Buffer B, and then incubated as before for 4 min.

The intestinal contents were then emptied into a plastic centrifuge tube to yield the first epithelial cell population. The process of filling with Buffer B and collecting the washings was repeated eight more times for different time periods of incubations as described by Weiser (9). Cells in each tube were sedimented by centrifugation at 500 *g* for 5 min and resuspended in 3–4 ml of Buffer B. Inclusion of DTT in Buffer B during both isolation and resuspension helped in breaking up clumps of cells. The mucolytic action of DTT has been documented (17).

The cell suspensions were homogenized by sonication either with a sonicator cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY) using a microtip and a power output of 55 watts for 15 sec, or with a Kontes microultrasonic cell disruptor (Kontes Glass Co., Vineland, NJ) at a power setting of 8 for 60–90 sec. The cell suspensions were immersed in an ice bath during homogenization and the homogenates were

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stored on ice and used within 1–2 hr. The completeness of homogenization was confirmed by microscopic examination. For some experiments, the cells or the homogenates of cell suspensions, in their sequence of dissociation from the intestine, were pooled into four fractions on the basis of their protein content and alkaline phosphatase activity. These were designated as upper villi (UV), middle villi (MV), lower villi (LV), and crypts (C), containing approximately 20 (UV), 30 (MV), 30 (LV), and 20 (C) percent of the total protein, respectively. Viability of the cells was checked by their ability to exclude the dye Trypan blue. Freshly isolated cells in all fractions were 90–95% viable by this criterion.

Enzyme assays

The activity of HMG-CoA reductase was assayed in a final reaction volume of 1 ml containing NADP, 3 μmol; glucose-6-phosphate, 30 μmol; glucose-6-phosphate dehydrogenase, 1 unit; potassium phosphate buffer, pH 7.2, 100 μmol; KCl, 200 μmol; DTT, 10 μmol; and *R,S*-[3-^{14}C]HMG-CoA (1000 dpm/nmol), 200 nmol. All the components of the reaction system except HMG-CoA were incubated at 37°C for 20 min and the reaction was initiated by the addition of HMG-CoA. After 20–30 min at 37°C, the reaction was terminated by the addition of 0.1 ml of 10 N HCl. A solution containing 5 mg of DL-[2-^{3}H]mevalonate (90,000 dpm) in 0.1 ml was added and the tubes were further incubated at 37°C for 30 min to ensure complete lactonization. Each tube was extracted with two 10-ml portions of diethyl ether after the addition of saturating amounts of anhydrous Na_2SO_4. Pooled ether extracts were evaporated to dryness under N_2. The residue was dissolved in a few drops of acetone and its components were separated by thin-layer chromatography on activated silica gel G plates (500 μm thick) in acetone–benzene 1:1. The mevalonolactone band was identified by observing the plates under long-wave ultraviolet light. The band was scraped and counted in a Beckman LS-3145P liquid scintillation counter using Formula 950A scintillation fluid (New England Nuclear, Boston, MA). The radioactivity was corrected for quenching by the external standard ratio method, for extraction efficiency, using the tritium internal standard and for background values, obtained from assays to which no enzyme sample was added. The omission of a NADPH-generating system from the reaction mixture yielded similar background values. Mevalonolactone extraction efficiencies typically ranged from 65 to 70%. The results are expressed as pmol of MVA formed per min per mg of protein. Alkaline phosphatase activity was assayed as described by Weiser (9) except that the concentration of *p*-nitrophenylphosphate was changed to 1 mM. One unit of alkaline phosphatase is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per 15 min under the assay conditions (9). Protein determinations were carried out by the method of Gornall, Bardawill, and David (18) after precipitation with trichloroacetic acid, using bovine serum albumin as the standard.

RESULTS

Kinetic studies on ileal cell homogenates

Ileal epithelial cells, pooled as described in Methods into four cell types, were sonicated and the homogenates were assayed for HMG-CoA reductase activity at different protein concentrations for different time periods. As shown in **Fig. 1*A*,** reductase activity was linear with protein concentration at least up to 1 mg in all cell populations. It was also linear with time up to 30 min in all cell populations (Fig. 1*B*).

Distribution of HMG-CoA reductase and alkaline phosphatase in epithelial cells of ileum

The results shown in Fig. 1 indicated that there is a progressive increase in reductase specific activity from the upper villus cells to the crypt cells. This was confirmed by assaying individual cell homogenates for reductase and alkaline phosphatase activities. Alkaline phosphatase is known to be a marker enzyme for villus cells (9). The results in **Fig. 2** show that there was indeed a progressive increase in the specific activity of reductase as one proceeded from the villus tip to the crypt zone. The concomitant drop in the specific activity of alkaline phosphatase indicated that a separation of villus and crypt cells was being achieved under these conditions. **Table 1** shows that the upper and middle villus cells together accounted for about 50% of the total cell protein but only for 30–35% of the total reductase activity. The lower villus cells and the crypts together accounted for the other 50% of the protein and 65–70% of the total reductase activity. This would indicate that HMG–CoA reductase is primarily a crypt cell enzyme and that the enzyme activity is being progressively lost as the cells move up to the villus tip. In contrast, alkaline phosphatase appeared to be primarily of villus origin since the upper and middle villus cells accounted for about 85% of the total activity while the lower villus and crypt cells contributed only about 15%.

Subcellular localization of HMG-CoA reductase in ileal epithelial cells

Unlike the liver where HMG-CoA reductase is predominantly microsomal, the intestinal enzyme has

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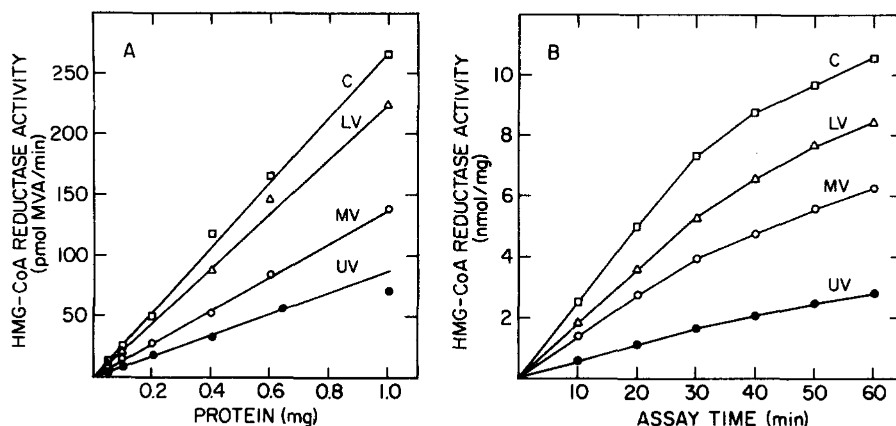

Fig. 1. Kinetic studies on ileal cell homogenates. Isolated ileal epithelial cells were pooled into upper villus (UV), middle villus (MV), lower villus (LV), and crypt (C) cell populations and were assayed for HMG-CoA reductase as described in Methods. The specific activity of *R,S*-[3-^{14}C]HMG-CoA used in these experiments was 3,100 dpm/nmol. *A*, increasing aliquots of whole homogenates were assayed for 30 min. *B*, aliquots of homogenates yielding 0.74 mg (UV), 0.82 mg (MV and LV), and 0.73 mg (C) of protein were assayed for the indicated length of time.

been reported to be present in both mitochondria and microsomes (6). Merchant and Heller (8) found that the enzyme activity was present in all subcellular fractions in their preparations and attributed this to cross-contamination and aggregation of organelles in the presence of mucus. Since we observed that inclusion of high concentrations of DTT in buffers during both isolation and resuspension of cells appeared to eliminate cellular aggregation considerably, we tried subcellular fractionation of ileal cells in such buffers. Briefly, ileal cells isolated in Buffer B were collected by centrifugation and resuspended in Buffer C containing 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA, 0.003 M DTT, and 0.05 M phosphate buffer, pH 7.2. The cells were pooled on the basis of their packed wet weight into four populations (UV, MV, LV, and C) as described before. The cell suspensions were homogenized by sonication and the homogenates were diluted with Buffer C to yield a protein concentration of 5–10 mg/ml. The homogenates were centrifuged at 700 *g* for 10 min to collect "nuclear" fraction which was washed once with Buffer C. The supernatant fraction was centrifuged at 8,500 *g* for 10 min to yield "mitochondria" which were washed once with Buffer C. "Microsomes" and "cytosol" were isolated from the postmitochondrial supernatant by centrifugation at 175,000 *g* for 45 min. The entire fractionation procedure was carried out at 0–4°C in a minimum amount of time and aliquots of all fractions including the homogenates were assayed for protein and for reductase activity. Unlike Merchant and Heller (8), we did not encounter any problems with a "fatty" layer.

As apparent from **Table 2,** HMG-CoA reductase activity was present in all subcellular fractions even though in each population the microsomes accounted for 40–50% of the total enzyme and had the highest specific activity. The recoveries of enzyme activity ranged from 87 to 97% of homogenate activities. The progressive increase in enzyme activity observed in homogenates of the four cell types was also evident in all the subcellular fractions. Since the reductase activity could not be exclusively localized to any one particular subcellular fraction, the whole cell homogenates were used in subsequent experiments.

Effects of soy bean trypsin inhibitor on distribution of HMG-CoA reductase in ileal epithelial cells

In a recent paper Sugano et al. (19) reported that they could measure reductase activity in microsomes, prepared from villus cells by a scraping technique, only in the presence of inhibitors of proteolytic enzymes. The activity in microsomes prepared from crypts did not require the addition of such an inhibitor. This raised the possibility that the gradient observed in the reductase activity from villus to crypt cells may be an artifact of the homogenization procedure. Since the well-differentiated villus cells are comparatively richer in hydrolytic enzymes, sonication may bring them in contact with the reductase, thereby inactivating it. This possibility was tested by studying the effect of the addition of soy bean trypsin inhibitor during both homogenization and enzyme assay of UV, MV, LV, and C cells. Pooled isolated cells in Buffer B were divided into two portions and soy bean trypsin inhibitor dissolved in Buffer B was added to one to yield a final concentration of 133 μg/ml. Both portions were homogenized by sonica-

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tion and the homogenates were assayed for protein, HMG-CoA reductase, and alkaline phosphatase. The incubation medium for reductase assay in the case of trypsin inhibitor-treated homogenates also contained the inhibitor at 133 μg/ml. As the results in **Table 3** show, soy bean trypsin inhibitor had no effect on reductase activity in any of the cell populations. Inhibitor treatment of homogenates also did not affect the values of alkaline phosphatase (data not shown). Moreover, the distribution of HMG-CoA reductase activity in subcellular fractions of ileal cells was also not altered by the presence of soy bean trypsin inhibitor during fractionation (data not shown).

Effect of various treatments on the distribution of enzymes in ileal epithelial cells

Sterol synthesis in the intestine has been reported to be regulated by dietary sterols and/or bile acids (10, 11). Evidence is also available which suggests that plasma cholesterol may play an equally important regulatory role (12). Therefore, it was of interest to study the effect of APP treatment and of cholestyramine feeding on the distribution of HMG-CoA reductase activity in ileal epithelial cells. The activity of alkaline phosphatase from the same populations of cells served as a guide for proper separation of villus and crypt cells under different experimental conditions. In preliminary experiments, the animals injected with APP were allowed free access to food and water. However, in agreement with the observations of Andersen and Dietschy (12), these animals ate very little in the first 24 hr and nothing thereafter. The control animals appeared to eat normally. Therefore, in the experiments reported here the APP-treated animals were entirely deprived of food and two sets of control animals were used with one set being allowed free access to food and the other set being fasted.

The results presented in **Table 4** show the distribution of alkaline phosphatase specific activity in APP-treated animals and saline–phosphate-injected controls. Even though there is a wide variation in the enzyme activities among the various groups, the villus-to-crypt gradient in specific activity was always maintained. This suggested that a proper separation of the different cell populations was being achieved under all treatment conditions. Injection of control saline–phosphate appeared to cause a "stress effect" in the fed controls. There was approximately a 50% drop in the specific activity of alkaline phosphatase in all cell populations in 24 hr. However, these values returned to normal by 72 hr, even though the 72-hr fed controls were injected twice more than the 24-hr controls. It is interesting to note that there was no

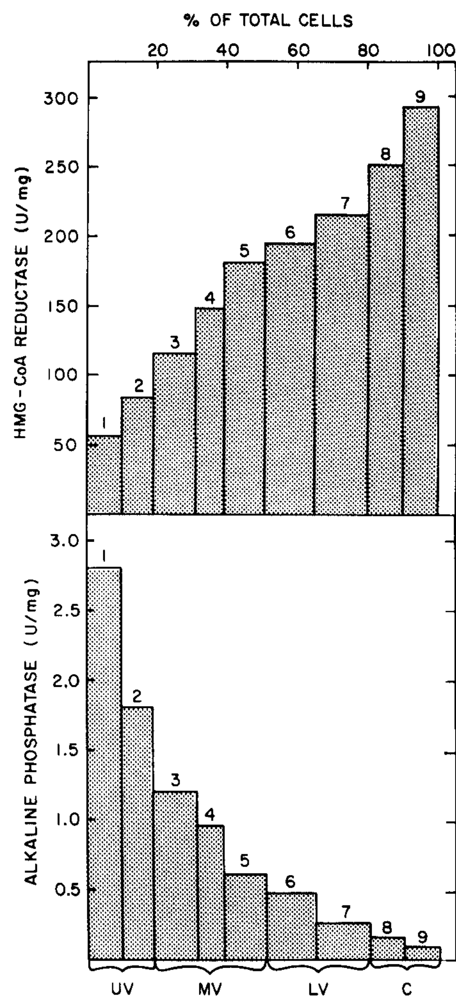

Fig. 2. Specific activities of alkaline phosphatase and HMG-CoA reductase in isolated ileal epithelial cells. The percentage of cells isolated in each successive cell population (the width of a bar) was determined by the proportion of total cell protein isolated in a given population. The numbers at the top of each bar represent the sequence of dissociation. A typical way of pooling the cells into the four populations is indicated at the bottom of the histograms. Enzyme units are definded in the legend to Table 1.

significant change in enzyme activities in injected fasted controls. Treatment with APP, however, caused a striking loss of alkaline phosphatase activity in all cell populations. This loss was progressive with the period of treatment. Addition of APP (1.0 mM) to the assay mixture did not affect the activity of alkaline phosphatase in the homogenates of any of the cell populations (data not shown). Alkaline phosphatase activities of cells from cholestyramine-fed rats were indistinguishable from those from normal rats.

The corresponding changes in the specific activity of HMG-CoA reductase are documented in **Table 5.** Once again, as in the case of alkaline phosphatase, the injection of saline–phosphate alone appeared to

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TABLE 1. Distribution of enzymes in ileal epithelial cells

Cell Type	% Total Protein	HMG-CoA Reductase		Alkaline Phosphatase	
		U/mg[a]	% Total Activity	U/mg[b]	% Total Activity
Upper villus	17.9 ± 2.0	79 ± 10	8.2 ± 1.0	2.14 ± 0.19	48.8 ± 3.8
Middle villus	31.1 ± 2.2	141 ± 9	25.3 ± 1.6	0.89 ± 0.09	35.2 ± 2.1
Lower villus	32.2 ± 2.0	216 ± 23	40.1 ± 4.3	0.31 ± 0.04	12.7 ± 1.5
Crypt	18.6 ± 1.6	248 ± 30	26.5 ± 3.2	0.14 ± 0.02	3.3 ± 0.4

[a] HMG-CoA reductase, 1 U equals one pmol of MVA formed/min.
[b] Alkaline phosphatase, 1 U equals one μmol of *p*-nitrophenol formed/15 min.
Results are the average of six different experiments (rats). All values are mean ± SEM. The cell populations were pooled as described in the text.

cause a change in enzyme activity. However, in contrast to alkaline phosphatase, there appeared to be approximately a 50% increase in reductase specific activity in all cell populations in 24 hr. These values returned to near normal after 72 hr of treatment. There was a significant decrease in the specific activities of reductase in all the groups as early as 24 hr after deprivation of food. This loss increased progressively with increasing time of fasting. In spite of these changes in individual cell types in both fed and fasted controls, the villus-to-crypt gradient in reductase specific activity was maintained as in the case of alkaline phosphatase. Treatment with APP, however, appeared to abolish this gradient. Considering that the effect of APP is superimposed upon that of fasting, the upper villus cells appeared to show the most dramatic response to APP treatment. By 72 hr, the specific activities of reductase in cells from APP-treated rats were 10- to 20-fold higher than those of corresponding fasted controls. The effect of cholestyramine feeding was even more interesting. The gradient in enzyme activity was abolished here, too, and once again the upper villus cells were the most responsive group. These cells now became as active as crypt cells in converting HMG-CoA to mevalonate. Compared to normal rats, cholestyramine feeding caused a greater increase in the specific activity of reductase in all the cell types (3.1- to 9.3-fold) than did APP treatment (1.6- to 4.4-fold).

Changes in epithelial cell protein content and enzyme activities of rat ileum

It was of interest to see if the qualitative changes observed in the specific activities of both reductase and alkaline phosphatase after various treatments would translate into changes in the total activities of the ileum as a whole. As seen from **Table 6,** the epithelial cell protein content of the ileum, as judged from protein recovered in the different cell populations, varied widely with different treatments administered to the rat. Intraperitoneal injection of saline–phosphate at a pH of 2.5 to fed controls appeared to enhance the epithelial cell protein content. The yield of cell protein was 50% higher per unit length of ileum after 72 hr of treatment. In fasted controls, there appeared to be a small (15%) decrease in the yield by 48 hr, which did not change appreciably after 72 hr. In APP-treated rats, there was a large decrease in protein yield by 48 hr. After 72 hr the yield of cell protein in these rats was less than half that in the untreated normal rats. Cholestyramine

TABLE 2. Distribution of HMG-CoA reductase in subcellular fractions of ileal epithelial cells

Fraction	UV[a]		MV		LV		C	
	U/mg	%	U/mg	%	U/mg	%	U/mg	%
Homogenate	96	100	130	100	203	100	262	100
Nuclei	97	17	137	22	272	23	318	17
Mitochondria	167	25	189	22	221	28	257	22
Microsomes	177	49	226	48	343	41	453	44
Cytosol	5	2	13	4	32	5	33	4
Total recovery		93		96		97		87

[a] UV, upper villi; MV, middle villi; LV, lower villi; C, crypts.
Isolated ileal epithelial cells were pooled into different populations and fractionated as described in Methods section. Accomplishment of villus-to-crypt gradient was confirmed by measuring the alkaline phosphatase activity of the homogenates. The percentages were calculated taking the total activity in the homogenate to be 100%.

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TABLE 3. Effect of soy bean trypsin inhibitor on the distribution of HMG-CoA reductase in ileal epithelial cells

Cell Type	HMG-CoA Reductase Activity (U/mg protein)	
	− Trypsin Inhibitor	+ Trypsin Inhibitor
UV	85	80
MV	170	164
LV	219	220
C	252	267

Pooled epithelial cells were homogenized in the presence or absence of soy bean trypsin inhibitor as described in the text. The trypsin inhibitor was also present in the enzyme assay medium at 133 μg/ml for inhibitor-treated homogenates. The homogenates were also assayed for the marker enzyme alkaline phosphatase.

feeding did not alter the yield of epithelial cell protein.

In rats fed normally and injected with saline–phosphate, the combined specific activity of alkaline phosphatase in ileal cells decreased by half after 24 hr but returned to normal after 72 hr. Total alkaline phosphatase units per 50-cm length of ileum also decreased by half after 24 hr but were 60% higher than normal after 72 hr. This "overshoot" response appeared to be entirely due to increased cell protein content. The combined specific activity of HMG-CoA reductase, on the other hand, increased by 40% after 24 hr but returned to normal by 72 hr. Total reductase units, however, remained 40% higher throughout, reflecting the increased cell protein content. It thus appears that in the fed and saline–phosphate-injected controls, the increase in cell protein content seen at 72 hr of treatment was not confined to one cell type but occurred in both villus and crypt cells.

In the case of fasting, combined specific activity of alkaline phosphatase showed a small (19%) decrease by 48 hr but returned to normal by 72 hr in rats injected with saline–phosphate. Total alkaline phosphatase units showed similar changes. The combined specific activity and total units of HMG-CoA reductase showed similar progressive decreases with fasting. By 72 hr about 85% of reductase activity was lost in ileal epithelial cells from fasted rats.

Cholestyramine feeding had no effect on alkaline phosphatase activity of ileal cells whether measured as the specific activity or as total units. The combined specific activity of reductase was enhanced 4.4-fold, accounting for the increase in total enzyme units.

Treatment with APP caused a drastic loss of both the specific activity and total units of alkaline phosphatase. Total HMG-CoA reductase units were essentially unchanged at 24-hr and 72-hr time periods but showed a slight (24%) decrease at the 48-hr period. The 2-fold increase in the combined specific activity of reductase compared to normal rats at 72 hr appeared to offset the 55% decrease in cell protein content, thereby sustaining the total enzyme units.

Examination of formalin-fixed and hematoxylin–eosin-stained sections from the mid-intestines of 72-hr APP-treated rats revealed a reduction in the number and size of villus structures when compared to either normal or fasted controls (data not shown). This suggests that the changes in epithelial cell protein content observed during APP treatment may signify changes in cell number.

DISCUSSION

The results of studies on localization of sterol synthetic activity in the small intestine have been contradictory. Earlier studies by Dietschy and Siperstein (3) and Shefer et al. (6) indicated that the undifferentiated crypt cells were the locus of both

TABLE 4. Distribution of alkaline phosphatase in ileal epithelial cells after various treatments

Treatment	Alkaline Phosphatase (U/mg protein ±SEM)			
	UV	MV	LV	C
Normal	2.14 ± 0.19	0.89 ± 0.09	0.31 ± 0.04	0.14 ± 0.02
24-hr fed	1.02 ± 0.19	0.44 ± 0.09	0.17 ± 0.03	0.07 ± 0.01
48-hr fed	1.19 ± 0.10	0.61 ± 0.06	0.19 ± 0.05	0.05 ± 0.01
72-hr fed	2.11 ± 0.37	1.00 ± 0.29	0.39 ± 0.10	0.09 ± 0.02
24-hr fasted	2.16 ± 0.56	0.60 ± 0.07	0.23 ± 0.04	0.14 ± 0.02
48-hr fasted	1.60 ± 0.15	0.73 ± 0.09	0.27 ± 0.02	0.13 ± 0.03
72-hr fasted	1.94 ± 0.21	0.76 ± 0.05	0.47 ± 0.05	0.19 ± 0.05
24-hr APP-treated	1.14 ± 0.08	0.52 ± 0.03	0.32 ± 0.05	0.13 ± 0.02
48-hr APP-treated	0.13 ± 0.02	0.08 ± 0.01	0.06 ± 0.004	0.04 ± 0.004
72-hr APP-treated	0.11 ± 0.006	0.05 ± 0.007	0.03 ± 0.002	0.004 ± 0.001
72-hr cholestyramine-fed	2.09 ± 0.13	0.86 ± 0.09	0.35 ± 0.04	0.12 ± 0.01

The protocols for various treatments are described in Materials and Methods. The APP-treated rats were injected with APP at a level of 20 mg/kg body weight in 0.154 M NaCl, 0.025 M phosphate, pH 2.5. Both fed and fasted controls received the same volume of solvent. Each value is mean ± SEM of six different experiments (rats).

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TABLE 5. Distribution of HMG-CoA reductase in ileal epithelial cells after various treatments

Treatment	HMG-CoA Reductase (U/mg protein ±SEM)			
	UV	MV	LV	C
Normal	79 ± 10	141 ± 9	215 ± 23	248 ± 30
24-hr fed	133 ± 17	202 ± 15	300 ± 18	328 ± 32
48-hr fed	127 ± 14	186 ± 10	240 ± 25	300 ± 13
72-hr fed	109 ± 6	143 ± 16	193 ± 9	203 ± 16
24-hr fasted	62 ± 8	68 ± 7	87 ± 7	117 ± 10
48-hr fasted	29 ± 4	33 ± 5	48 ± 6	65 ± 8
72-hr fasted	17 ± 2	26 ± 3	36 ± 5	34 ± 6
24-hr APP-treated	145 ± 14	172 ± 17	189 ± 14	184 ± 10
48-hr APP-treated	175 ± 21	202 ± 28	205 ± 29	219 ± 23
72-hr APP-treated	347 ± 27	343 ± 31	378 ± 45	404 ± 24
72-hr cholestyramine-fed	733 ± 35	773 ± 48	773 ± 45	759 ± 56

The experimental conditions are as described in the legend to Table 4. Each value is the mean ± SEM of six different experiments (rats).

activities while the mature villus cells were relatively inactive. Muroya, Sodhi, and Gould (7), on the other hand, reported that the villus cells were as active as crypts in their capacity to synthesize digitonin-precipitable sterols. In the above studies, the villus and crypt cells were isolated by a differential scraping technique (3) which we found to be quite injurious to the cell membrane as judged by the ability of the cells to exclude the dye, trypan blue. Others have presented evidence showing that the scraping technique severely damages intestinal cells, resulting in lower enzymatic activities (7–9).

In view of the above, the method developed by Weiser (9) to isolate intestinal epithelial cells sequentially in a villus-to-crypt gradient appeared very promising. Using a modification of this method, Merchant and Heller (8) reported that the villus cells showed higher HMG-CoA reductase activity than did the crypts. Their results on the distribution of sterol synthetic activity agreed essentially with those of Muroya et al. (7). However, in a recent paper, Shakir, Sundaram, and Margolis (20), using cells isolated by the original Weiser method, reported that incorporation of tritiated water into cellular lipids, including sterols, was 3- to 4-fold higher in crypt cells than in the upper villus cells.

The results reported here are in agreement with those of Shakir et al. (20), Dietschy and Siperstein (3), and Shefer et al. (6). Our results clearly show that HMG-CoA reductase activity of rat ileum is predominantly located in the less differentiated lower villus and crypt cells. This distribution of reductase is the inverse of that of the known micro-villus membrane marker, alkaline phosphatase. Our results also demonstrate that the villus-to-crypt cell gradient in reductase activity is not due to proteolytic inactiva-

TABLE 6. Changes in epithelial cell protein content and enzyme activities of rat ileum after various treatments

Treatment	Total Ileal Epithelial Cell Protein		Alkaline Phosphatase		HMG-CoA Reductase	
	mg ± SEM	Mean % of Normal	Sp. Activity Mean % of Normal	Total Units Mean % of Normal	Sp. Activity Mean % of Normal	Total Units Mean % of Normal
Normal	84.0 ± 8.1	100	100	100	100	100
24-hr fed	87.4 ± 5.8	104	48	50	140	146
48-hr fed	96.7 ± 8.5	115	63	73	122	140
72-hr fed	126.0 ± 16.6	150	109	163	94	141
24-hr fasted	87.0 ± 8.0	104	91	95	46	48
48-hr fasted	72.5 ± 4.5	86	81	70	24	21
72-hr fasted	71.6 ± 5.3	85	100	85	16	14
24-hr APP-treated	79.4 ± 6.4	95	63	60	100	95
48-hr APP-treated	54.5 ± 9.6	65	9	6	117	76
72-hr APP-treated	38.1 ± 4.5	45	4	2	213	96
72-hr cholestyramine-fed	84.2 ± 3.8	100	101	101	441	441

Epithelial cells were isolated from a 50-cm segment of ileum proximal to ileo-cecal junction as described in Methods. At least six rats were used in each treatment and control. Total enzyme units were obtained by adding the units in each cell type. Combined specific activity was calculated by dividing the mean of total units by the mean of total cell protein. Both fed and fasted rats received intraperitoneal injections of saline–phosphate, pH 2.5, as described in Methods.

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tion of the enzyme in the mature villus cells. This distribution of enzymes persisted in fed and fasted rats injected with control saline–phosphate. Both cholestyramine feeding and APP treatment abolished the gradient in reductase activity.

The logic of the presence of higher HMG-CoA reductase activity in crypt cells, in view of the greater need for cholesterol in these growing cells, is intuitively obvious. However, the fact that both APP treatment and cholestyramine feeding enhance the enzyme activity in the cells from villus tips to a level found in the crypt cells suggests a rigorous physiological control of reductase activity in the mature villus cells in the normal rat. The nature of the physiological regulator(s) is not clear. The present results, as well as those from past studies (10–12), suggest that the regulator could be intracellular cholesterol, or bile acids, or a combination of the two. Thus, the "derepression" of HMG-CoA reductase activity in the absorptive villus cells on cholestyramine feeding could be due to a decreased exposure of these cells to lumenal bile acids and/or decreased absorption of lumenal cholesterol. In the APP-treated rats, the derepression could be attributed to decreased delivery of plasma cholesterol to these cells. However, APP treatment in combination with fasting may also alter intestinal bile acid flux. It is also possible that APP treatment affects the synthesis and assembly of chylomicrons and very low density lipoproteins known to take place in the microsomes of the villi (21). In a recent study, Gebhard and Cooper (22) reported that HMG-CoA reductase activity of canine intestinal mucosa in short-term organ culture could be inhibited by the addition to the medium of bile salts, cholesterol, or its oxygenated derivatives. Addition of canine lipoproteins was without effect, probably due to the short (6 hr) time period of the study.

Attempts to localize HMG-CoA reductase activity to any one particular subcellular fraction were not successful. It is possible that cross-contamination among organelles, due to mucus or other factors, is unavoidable in intestinal cell fractionation. While we did not characterize our subcellular fractions with marker enzymes, the inapplicability of such an approach in intestinal cell fractionation has been documented (6, 23). However, this experiment was designed primarily to establish the validity of using the enzyme activity in whole homogenates as a representative value. This is shown by the similarity of the relative enzyme activities in whole homogenates and in "microsomes" as well as by the almost complete recovery of the homogenate enzyme activity in various subcellular fractions in all cell types.

We have attempted to correlate our data with the reports in the literature of the contribution of plasma cholesterol and lumenal cholesterol and bile acids to the regulation of sterol synthesis in ileal epithelial cells. As shown in **Table 7,** alterations in the combined specific activity of HMG-CoA reductase in ileal cells closely followed changes in the rate of ileal sterol synthesis cited from literature. These comparisons support the concept of the rate-limiting nature of HMG-CoA reductase in the sterol synthetic pathway of rat ileum.

Our results lead us to stress the point that these qualitative changes in the rates of sterol synthesis and the combined specific activity of reductase do not necessarily translate into analogous quantitative

TABLE 7. Comparison of overall rate of sterol synthesis and combined HMG-CoA reductase specific activity in rat ileum

Experimental Treatment	Control	Sterol Synthesis	HMG-CoA Reductase
24-hr fasted	Normal		↓54%
48-hr fasted	Normal	↓40–50% (24)	↓76%
72-hr fasted	Normal		↓84%
96-hr fasted	Normal	↓80% (12)	
72-hr APP-treated (20 mg/kg)	Normal		↑2-fold
72-hr APP-treated (20 mg/kg)	72 hr fasted		↑13.3-fold
96-hr APP-treated (50 mg/kg)	Normal	↑2-fold (12)	
96-hr APP-treated (50 mg/kg)	Pair-fed	↑10.5-fold (12)	
Cholestyramine-fed	Normal	↑2 to 4-fold (24)	↑4.4-fold
24-hr fed and saline-injected	Normal		↑40%
48-hr fed, stressed	Normal	↑42% (24)	
Crypts	Upper villi	↑3 to 4-fold (20)	↑3 to 4-fold

Reference numbers to published data are given in parentheses. Unreferenced values are from this study. Symbols: ↑ indicates an increase and ↓ a decrease in response to treatment. For the definition of combined specific activity, see legend to Table 6. Fasted rats in our studies were also injected with saline–phosphate, pH 2.5, as described in Methods.

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changes in total reductase activity and, by inference, total sterol synthetic capacity of the ileum. Changes in the cell protein content become a very significant factor in determining the total enzyme activity of the ileum. This is illustrated in the case of fed controls injected with control saline–phosphate and, more dramatically, in the case of APP-treated animals. Thus, even though there was a 2-fold stimulation of the combined specific activity of HMG-CoA reductase compared to normal rats, total enzyme units were unchanged (Table 6).

The decrease in cell protein content of ilea from APP-treated rats was not due to a loss of cells during the initial incubation of intestines in Buffer A. Nor was there excessive loss of protein into the supernatant fraction when the cells collected in Buffer B were pelleted for resuspension in fresh buffer (data not shown). These results raise some interesting possibilities. Is a specific cell type, e.g., the mature villus cells, being selectively lost from the ilea of the APP-treated rats? This could explain the loss of alkaline phosphatase while all of reductase is retained. Alternatively, there may be a uniform loss of cells of all cell types, with concomitant stimulation of HMG-CoA reductase and inhibition of alkaline phosphatase. Another possibility is that there may be a loss of biochemical differentiation in epithelial cells from the ilea of APP-treated rats while morphological differentiation is retained. This has been observed in some gastric and colonic diseases such as gastric atrophy and familial polyposis, and in neoplastic lesions (25, 26).

Treatment with APP has been used as a powerful tool to investigate the role of plasma lipoprotein cholesterol in the regulation of sterol synthesis in several extrahepatic tissues (12, 15, 16, 27, 28). In these studies, it has been assumed that the liver is the primary, if not the sole, target of APP, and that its effect on sterol synthesis in extrahepatic tissues is indirect and caused only through the lowering of plasma lipoprotein cholesterol. The effects of APP on intestinal cell protein content, intestinal morphology, as well as alkaline phosphatase activity demonstrated in this paper, call for a reevaluation of this assumption. Treatment with APP is known to inhibit incorporation of formate and adenine into the nucleic acids in the intestines of tumor-bearing mice (29) and to lower substantially the intestinal transport of glucose in rats (30). In addition, APP is taken up by the small intestine of mice minutes after intraperitoneal injection and is rapidly converted into nucleotides and incorporated into nucleic acids (31). In view of this, the effects of APP on ileal enzymes and sterol synthesis might reflect the direct action of this compound on ileal cell metabolism.

These data illustrate the profound effect of three major parameters on the regulation of HMG-CoA reductase and, by inference, on sterol synthesis in the small intestine. These are: fasting and, conversely, the level of alimentation; the uptake of bile acids and cholesterol from the lumen; and the level of lipoproteins in the blood. The interplay of these factors would thus determine the levels of sterol synthesis and its rate-limiting enzyme in the small intestine.

This work was supported by grants NIAMDD, #12402 and NHLBI, 20428 from the National Institutes of Health. We thank Mrs. Isabelle Namanworth for her technical assistance and Dr. Alfred L. Britt for the preparation of intestinal sections for histopathology.

Manuscript received 8 January 1979; accepted 10 May 1979.

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