3-Hydroxy-3-methylglutaryl coenzyme A reductase from rat intestine: subcellular localization and in vitro regulation[1]

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Abstract The subcellular localization of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in rat intestine was reinvestigated. Highly enriched fractions of endoplasmic reticulum and mitochondria were prepared from mucosal cells. The highest specific activity of HMG-CoA reductase was located in the endoplasmic reticulum fraction with recovery of 25% of the total activity. The mitochondria had low specific activity and low recovery of reductase activity relative to whole homogenate (2–5%). Despite attempts to maximize cell lysis, much of the activity (about 60%) was recovered in a low speed pellet which consisted of whole cells, nuclei, and cell debris as determined by light microscopy. Taken together, the evidence strongly suggests that much of the cellular HMG-CoA reductase activity is present in the endoplasmic reticulum fraction and that mitochondria have little or no intrinsic HMG-CoA reductase. The in vitro regulation of intestinal microsomal HMG-CoA reductase was studied. The intestine possesses a cytosolic HMG-CoA reductase kinase-phosphatase system which appears to be closely related to that present in the liver. Intestinal reductase activity in microsomes prepared from whole mucosal scrapings was inhibited 40–50% by the presence of 50 mM NaF in the homogenizing buffer. It was less susceptible to the action of the kinase than liver reductase. The effects of NaF were reversed by incubation with partially purified intestinal or liver phosphatases. These results suggest that the kinase-phosphatase system could play a role in the regulation of intestinal sterol and isoprene synthesis in vivo.—**Field, F. J., S. K. Erickson, M. A. Shrewsbury, and A. D. Cooper.** 3-Hydroxy-3-methylglutaryl coenzyme A reductase from rat intestine: subcellular localization and in vitro regulation. *J. Lipid Res.* 1982. **23:** 105–113.

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The liver and intestine together account for approximately 90% of the body's cholesterol production (1). The rate-controlling enzyme for cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) which catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid (2). HMG-CoA reductase activities from intestine and liver exhibit similarities and differences. They have similar pH optima (3), and are inhibited by cholesterol or oxygenated sterols in vitro (4–7). However, the effects of dietary cholesterol on intestinal cholesterol synthesis in vivo are still controversial (8–10). Although reductase activity in both organs has a diurnal rhythm, that in intestine is of lower amplitude (11, 12).

One potentially important difference between liver and intestine is the reported subcellular localization of the enzyme. Hepatic HMG-CoA reductase is localized to the endoplasmic reticulum (13) while in the rat, intestinal HMG-CoA reductase activity has been recovered in both mitochondrial and microsomal fractions (3, 14, 15). Such a difference is of potential physiologic importance. The substrate for the enzyme, HMG-CoA, is strictly compartmentalized because the mitochondrial membrane is impermeable to CoA and its derivatives (16). Thus in liver, mitochondrial HMG-CoA cannot directly contribute to mevalonate formation. If reductase is present in intestinal mitochondria, HMG-CoA formed there could be converted directly to mevalonate. This mevalonate could cross the mitochondrial membrane and contribute to isoprene and cholesterol biosynthesis along with the mevalonate formed by HMG-CoA reductase in the endoplasmic reticulum. Therefore, a dual localization of HMG-CoA reductase in the intestine would have important implications for the regulation of intestinal isoprene and cholesterol biosynthesis.

Regulation of the activity of HMG-CoA reductase is believed to be dependent upon both the amount of enzyme protein present and on the level of its catalytic activity. Beg, Allman, and Gibson (17), Bove and Hegardt (18), and Nordstrom, Rodwell, and Mitschelen (19) have shown that incubation of hepatic microsomes with Mg^{2+} and ATP decreases the activity of HMG-CoA reductase. This activity can be further depressed by the addition

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonolactone.

[1] A portion of this reseach was presented at the Annual Meeting of the American Association for the Study of Liver Disease in 1979 (30).

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of an inactivator protein or kinase present in the cytosol (17). Reactivation of the reductase can be accomplished by incubating Mg^{2+}–ATP-treated microsomes with an activator or phosphatase also present in the cytosol (19). The reactivation is inhibited by the presence of NaF (19). Inactivation of HMG-CoA reductase with Mg^{2+}–ATP and inhibition or reactivation by NaF suggest that phosphorylation–dephosphorylation is involved in regulation of hepatic HMG-CoA reductase activity. Recently, Beg, Stonik, and Brewer (20) have demonstrated that the enzyme protein is phosphorylated. Whether this system plays a role in the regulation of intestinal HMG-CoA reductase is unknown.

The purpose of this investigation was twofold. First, because the question of the subcellular localization of HMG-CoA reductase in the intestine is an important one which has not been completely resolved, improved methods for isolating microsomes enriched in endoplasmic reticulum and for isolating purified mitochondria from rat intestine were developed. Both HMG-CoA reductase and a variety of specific organelle markers were monitored. Second, once the subcellular localization of HMG-CoA reductase in intestine was demonstrated to be microsomal, the in vitro regulation of the enzyme was investigated.

MATERIALS

D,L-3-Hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (40–60 mCi/mmol) and *R,S*-[5-^{3}H]mevalonic acid (DBED salt, 1–5 Ci/mmol) were obtained from New England Nuclear. Indoxyl acetate, glucose-6-phosphate dehydrogenase, nucleotide adenine diphosphate, soybean trypsin inhibitor, chicken trypsin inhibitor, α-antitrypsin inhibitor, and sodium succinate were obtained from Sigma. Orcinol was obtained from Baker and was recrystallized before use. 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) was purchased from Eastman Chemicals. All other chemicals were reagent grade. Mylar-backed silica gel G chromatography sheets were purchased from Eastman Kodak.

Animals

Male Sprague-Dawley rats were housed in a windowless room with illumination from 7 AM to 5 PM. They were allowed Purina rat chow and water ad libitum. They weighed 250–300 g at time of killing.

METHODS

Subcellular fractionation

Animals were killed by decapitation between 8 AM and 9 AM. Subcellular fractions were prepared from either mucosal scrapings or from isolated cells. In both instances the entire length of intestine from the ligament of Trietz to the ileocecal valve was used. Cells were obtained by a modification of the method of Weiser (21). The cells were harvested by filling the intestine with Weiser's buffer B and exchanging the buffer every 15 min until the entire mucosa was collected. Five to six changes of buffer were used. The cells were washed with phosphate-buffered saline, pH 7.4, and collected by centrifugation at 500 *g* for 10 min at 4°C.

The subcellular fractionation schemes are outlined in **Fig. 1.** An enriched endoplasmic reticulum fraction was prepared by a modification of the method of Shirkey, Chakraborty, and Bridges (22) (Fig. 1a). The isolated cells or intestinal mucosal scrapings were homogenized by 15 strokes at moderate speed with a Potter-Elvehjem motor-driven homogenizer in 2 ml of 75 mM sucrose, 10 mM dithiothreitol and 1 mg of soybean tyrpsin inhibitor. Immediately after homogenization, the solution was made isotonic with 15 ml o 0.04 M KH_2PO_4 buffer, pH 7.4, containing 0.05 M KCl, 0.03 M EDTA, 0.3 M sucrose, and 1 mg of trypsin inhibitor (Buffer A). The total homogenate contained 0.36–0.40 g of protein. The homogenate was centrifuged at 10,000 *g* for 20 min and the resulting supernatant was centrifuged at 105,000 *g* for 1 hr. The recovered pellet (P_1) and the supernatant (S_1) were kept at 4°C until ready for assay. To ensure maximal recovery of the microsomal fraction, the pellet from the 10,000 *g* centrifugation was diluted with 35 ml of Buffer A and rehomogenized with 30 strokes of the Potter-Elvehjem homogenizer. This was centrifuged at 450 *g* for 10 min. The supernatant was saved and the pellet was treated twice in the same manner. The three supernatants were combined and centrifuged at 10,000 *g* for 20 min. The resulting supernatant was centrifuged at 105,000 *g* for 1 hr. The pellet (P_2) was combined with P_1 and the supernatant (S_2) was combined with supernatant S_1 from above.

The mitochondrial fraction was prepared by a modification of the methods of Freedman, Weiser, and Isselbacher (23) and Mircheff and Wright (24) as outlined in Fig. 1b. Isolated intestinal cells were harvested as described above. The cells were homogenized with 15 strokes of a Dounce homogenizer (loose fitting pestle) in 15 ml of buffer containing 0.1 M sucrose, 0.05 M KCL, 0.04 M KH_2PO_4. 0.03 M EDTA, pH 7.4, and 1 mg of soybean trypsin inhibitor (Buffer B). The ho-

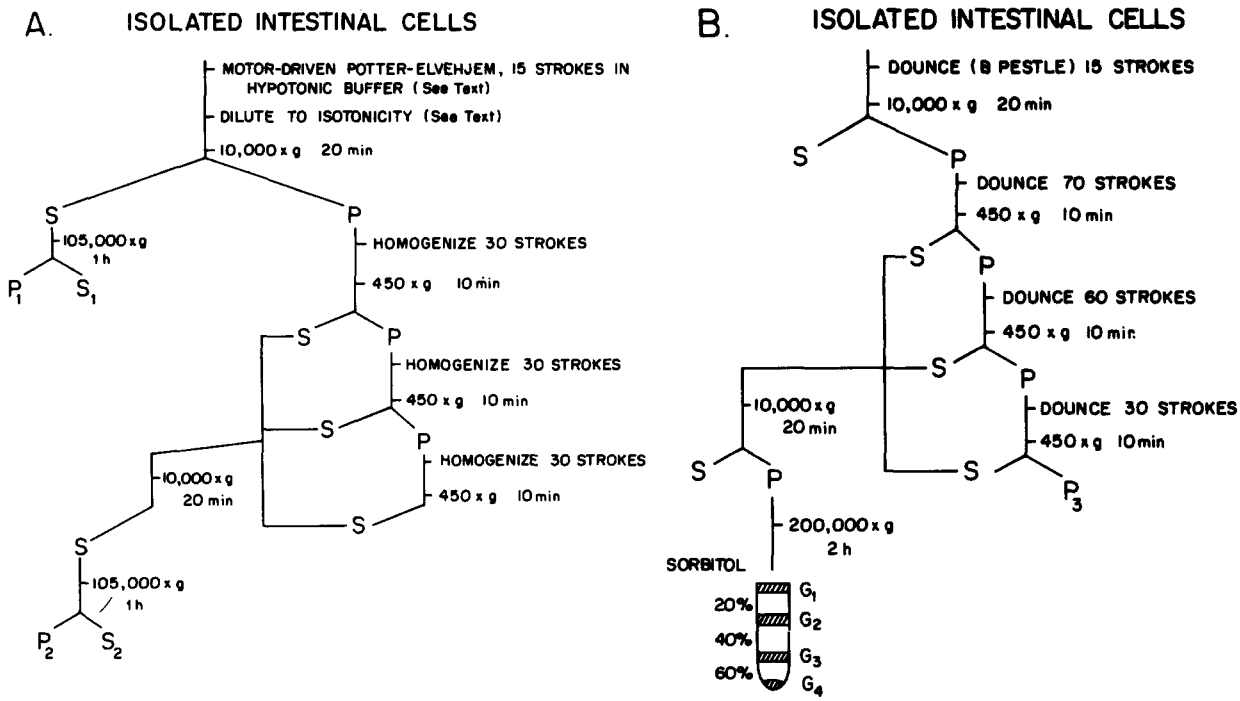

Fig. 1. Scheme for preparation of microsomes (A) and mitochondria (B) from rat intestine. A: Preparation of intestinal endoplasmic reticulum. Endoplasmic reticulum from rat small intestine was prepared by modifying the technique of Shirkey et al. (22) (See Methods). Combined pellets P_1 and P_2 represent endoplasmic reticulum, whereas S_1 and S_2 are the combined cytosolic fractions. B: Preparation of intestinal mitochondria. Mitochondria were isolated from the small intestine by a modification of the techniques of Mircheff and Wright (24) and Freedman et al. (23) (See Methods). P_3 is a pellet contaminated with intact cells, nuclei, and mucous obtained by low-speed centrifugation of the whole homogenate. Pellets G_2 through G_3 represent an enriched mitochondrial fraction.

mogenate was centrifuged at 10,000 *g* for 20 min and the resulting pellet was rehomogenized in 40 ml of buffer with 70 strokes of a Dounce homogenizer. This was centrifuged at 450 *g* for 10 min. The supernatant was saved and the pellet was treated twice in the same manner using 60 strokes and 30 strokes, respectively. The final pellet (P_3) was saved for assay. The three supernatants were pooled and centrifuged at 10,000 *g* for 20 min. The pellet obtained was resuspended in 6 ml of 60% sorbitol and homogenized by 10 strokes with a motor-driven Potter-Elvehjem homogenizer. Four ml was layered on top of 1 ml of 60% sorbitol solution in a Beckman SW 41 tube. This was overlaid with 4 ml of 40% sorbitol followed by 3 ml of 20% sorbitol. The discontinuous density gradient was placed in a Beckman SW 41 rotor and centrifuged at 200,000 *g* for 2 hr at 4°C. Four fractions were collected by careful aspiration starting at the top of the gradient and were resuspended in 30 ml of Buffer B. The fractions were centrifuged at 58,000 *g* for 2 hr and the four pellet fractions (G_1, G_2, G_3, G_4) were assayed for reductase immediately.

Assay of HMG-CoA reductase

HMG-CoA reductase was assayed essentially as previously described (6). Each assay contained 0.05 to 0.15 mg of protein, 25 mM glucose-6-phosphate, 3 mM NADP, 70 mM NaCl, 0.174 units of glucose-6-phosphate dehydrogenase, 15 mM dithiothreitol, 0.2 mg of trypsin inhibitor, 0.04 M KH_2PO_4, 0.05 M KCl, 0.25 M sucrose, 0.03 M EDTA, pH 7.2, in a final volume of 0.170 ml. The mixture was incubated at 37°C for 10 min and the assay was started by addition of [^{14}C]HMG-CoA (15 nmol, sp. act. 15–20,000 dpm/nmol). Longer preincubation did not alter the results. Assays were terminated at 20 min by the addition of 0.025 ml of concentrated HCl. D,L-[5-^{3}H]mevalonic acid, 2 nmol (20,000 dpm/nmol), was added as internal standard and the mixture was allowed to stand overnight to insure complete lactonization. The samples were then extracted with diethyl ether after addition of Na_2SO_3, the ether extracts were taken to dryness under N_2, and the residue was taken up in acetone and plated on silica gel G thin-layer chromatography sheets. The chromatograms were developed with benzene–acetone 1:1 (v/v) (3). The chromatograms were scanned and the region corresponding to MVA was scraped and added to 10 ml of Aquasol. The radioactivity was determined in a Beckman liquid scintillation counter. All activities were corrected for quenching and isotope spill-over. Activity was expressed as pmol of mevalonate produced/mg protein per min.

In both the homogenate and microsomes, mevalonate formation was linear with respect to both time (between 10 and 20 min) and protein concentration (between 0.05 and 0.4 mg). The assay was zero order with respect to HMG-CoA concentration (7).

Preparation of activators and inactivators from cytosols

Rats were killed by decapitation and their livers and small intestines were immediately excised. Liver was homogenized in a motor-driven Potter-Elvehjem glass–teflon homogenizer in two volumes of buffer containing 0.1 M sucrose, 0.05 M KCl, 0.02 M EDTA, and 0.04 M KH_2PO_4, pH 7.2. The homogenate was centrifuged at 12,000 *g* for 15 min and the resulting supernatant was centrifuged at 105,000 *g* for 1 hr. Intestinal microsomes were prepared from mucosal scrapings essentially as described by Shirkey et al. (22) except that all buffers contained 75 μg/ml each of soybean trypsin inhibitor, chicken trypsin inhibitor, and α-antitrypsin inhibitor (protein inhibitor mix), and the buffer was the same as for the preparation of liver cytosol (see above).

The 105,000 *g* supernatants from both tissues were fractionated exactly as described by Nordstrom et al. (19) through the acetone and $(NH_4)_2SO_4$ precipitations and freeze-thawing steps to obtain partially purified inactivators (kinases) and partially purified activators (phosphatases).

Inactivation and reactivation experiments

Hepatic and intestinal microsomes were prepared as described for the preparation of cytosols above. Microsomes containing inactivated HMG-CoA reductase were prepared in the same manner except that the homogenizing buffers contained 50 mM NaF.

The activation and inactivation experiments were performed as described by Nordstrom et al. (19) except that all buffers for the intestinal incubations contained the protease inhibitor mix described above.

Assay of subcellular organelle markers

Succinate INT dehydrogenase was assayed according to the method of Pennington (25). Aryl esterase was assayed using indoxyl acetate (26). RNA was determined by the method of Ceriotti (27). Protein was estimated according to Lowry et al. (28) using bovine serum albumin as the reference standard.

RESULTS

Subcellular localization of intestinal HMG-CoA reductase activity

It had been reported previously that intestinal HMG-CoA reductase was distributed equally in the microsomal and mitochondrial fractions (3, 14, 15). Because such a distribution would have important implications for understanding the regulation of the enzyme and, thus, the regulation of isoprene and sterol biosynthesis in the intestine, this problem was re-examined.

Subcellular fractionation of rat intestine has been difficult (29) with a major problem being gross microsomal contamination of the different organelle fractions. In agreement with previous reports (3, 14, 15), intestinal homogenates fractionated by standard differential ultracentrifugation techniques resulted in organelle fractions that were heterogenous with respect to HMG-CoA reductase activity. However, the fractions were also heterogenous with respect to specific organelle markers. This raised the possibility that mitochondrial reductase activity might be the result of microsomal or whole cell contamination.

To study this problem further, more refined techniques for the preparation of intestinal organelles were used. Initially,the techniques of Mircheff and Wright (24) and Freedman et al. (23) for intestinal subcellular fractionation were combined. Starting with mucosal scrapings, little reductase activity was recovered in the mitochondrial pellet (30). Most of the membrane-associated HMG-CoA reductase activity was recovered in the microsomal fraction or in a low speed pellet which contained large numbers of unbroken cells. However, the total recovery of reductase activity was low, and 80% of this activity was in the 105,000 *g* supernatant along with other microsomal membrane-bound enzymes such as glucose-6-phosphatase. Further experimentation revealed that these findings were artifactually induced by the histidine–imidazole buffer utilized in the procedures of Freedman et al. (23) and Weiser (21).

The microsomal fraction enriched in endoplasmic reticulum was isolated using a modification of the method of Shirkey et al. (22) (Fig. 1a). Beginning with isolated cells, a hypotonic sucrose solution was used for homogenization followed immediately by readjustment of the solution to isotonicity with a phosphate, KCl, EDTA, sucrose buffer. Microsomes were then isolated by differential ultracentrifugation. The microsomal fraction obtained ($P_1 + P_2$) was enriched about twofold in reductase, RNA, and aryl esterase (**Table 1**). It was free of contamination with mitochondria as estimated by succinate INT dehydrogenase activity. It was enriched twofold in HMG-CoA reductase activity with an overall recovery of 25%. In contrast to the findings with the histidine–imidazole buffer system, virtually no reductase or membrane marker activities were recovered in the cytosol ($S_1 + S_2$), (Table 1).

The mitochondrial fraction was prepared using the differential ultracentrifugation technique of Mircheff and Wright (24) coupled with the discontinuous density

TABLE 1. Subcellular distribution of HMG-CoA reductase

Fraction	HMG-CoA Reductase pmol MVA·min^{-1}·mg protein^{-1}	%[a] Rec	Succinate INT Dehydrogenase μmol INT·mg protein^{-1}·15 min^{-1}	% Rec	RNA μg·mg protein^{-1}	% Rec	Aryl Esterase[b] μmol·min^{-1}·mg protein^{-1}	% Rec
Homogenate	76 ± 7	100	1243 ± 115	100	36 ± 5	100	349	100
Microsomes (P_1 + P_2)	113 ± 24	25 ± 6	276 ± 140	2 ± 1	157 ± 25	40 ± 10	1117	37
Mitochondria (G_2 + G_3)	15 ± 1	2 ± 1	4495 ± 320	49 ± 6	40 ± 12	13 ± 3	660	18
Cytosol (S_1 + S_2)	6 ± 2	3 ± 1	235 ± 98	38 ± 5	32 ± 10	25 ± 7	44	4
Nuclei & Whole Cells (P_3)	183 ± 35	72 ± 7	1944 ± 153	42 ± 2	32 ± 8	21 ± 8	761	47

[a] Percent recovery.
[b] One determination.
Intestinal cells were isolated and subcellular fractions were prepared as described in Methods. The results represent the average of three determinations. Overall recovery of protein relative to the whole homogenate was 66%; of HMG-CoA reductase activity, 67%; of succinate INT dehydrogenase, 74%; and of RNA, 56%. The specific activities were ±S.E.

gradient described by Freedman et al. (23), but substituting a phosphate, KCl, EDTA buffer instead of the histidine–imidazole buffer. A fraction highly enriched in mitochondria was obtained that had markedly reduced cross-contamination as estimated by marker enzymes (Table 1). The mitochondrial fraction was enriched over threefold in succinate INT dehydrogenase activity. It was depleted in endoplasmic reticulum markers. It had little detectable HMG-CoA reductase activity and only 2–5% of the total was recovered in this fraction. To ensure that total potential mitochondrial reductase activity was measured, the mitochondria were frozen and thawed to release matrix enzymes; no increase in activity was observed. These results provide strong evidence that reductase is not a mitochondrial enzyme in intestinal mucosal cells. Any HMG-CoA lyase present in the mitochondria, which would have interfered with the assay, should have been inhibited since EDTA is known to inhibit this enzyme and was present in the buffer (31). Cleavage enzymes (32, 33) are also unlikely to have interfered under the conditions of the assay.

The total yield of reductase was 67% (Table 1). This agreed well with the recoveries of total protein, RNA, and succinate dehydrogenase activity. A large amount of reductase activity was consistently recovered in the low speed pellet designated P_3 (Fig. 1b; Table 1). Examination by light microscopy showed that the pellet contained intact cells, nuclei, and amorphous material. Attempts to separate this fraction further were unsuccessful.

Taken together, these results suggest strongly that intestinal mitochondria contain no reductase and that, as in other tissues, the endoplasmic reticulum is likely to be the major site of the enzyme. Previous reports suggesting the presence of a mitochondrial enzyme may have been due to failure to exclude the unbroken cells and trapped microsomes from the mitochondrial fraction.

Regulation of intestinal microsomal HMG-CoA reductase

It has been shown that incubation of rat liver microsomes with Mg^{2+} and ATP decreases the activity of HMG-CoA reductase (17–19, 34). The reductase activity can be fully restored to initial levels by incubating these microsomes with cytosol. This reactivation is completely inhibited by 50 mM NaF (17–19, 34). These and more direct experiments (20) have been interpreted as suggesting that HMG-CoA reductase can be regulated by a phosphorylation–dephosphorylation mechanism. To ascertain whether similar regulatory mechanisms exist in the intestine, the effects of Mg^{2+}–ATP, NaF, and partially purified kinase and phosphatase fractions on the intestinal enzyme were investigated. In addition, partially purified kinase and phosphatase fractions were isolated from intestinal cytosol and characterized.

The HMG-CoA reductase kinase–phosphatase system in intestine

To ascertain whether such a system is present in intestine, HMG-CoA reductase kinase and the phosphatase were partially purified from intestinal cytosol by the method of Nordstrom et al. (19) developed for liver. The resulting material was assayed for its ability to activate (phosphatase) and inactivate (kinase) rat liver microsomes. Intestinal cytosol clearly possesses the capabilities both to inactivate liver reductase in the presence of Mg^{2+}–ATP and to reactivate "phosphorylated" liver reductase (**Table 2**). The partially purified kinase and phosphatase preparations from liver and intestine were equally potent per mg protein added.

In other experiments (not shown) the phosphatase activities from liver and intestine showed very similar elution volumes on Sephadex G-75 gel filtration with apparent molecular weight estimates corresponding to about 30,000 daltons. From all of the above evidence, it is likely that the kinase–phosphatase systems of liver and intestine are similar if not identical.

Interaction of the kinase–phosphatase system with intestinal HMG-CoA reductase

Before studying the in vitro regulation of intestinal microsomal HMG-CoA reductase in detail, the specific

TABLE 2. Evidence for an HMG-CoA reductase kinase–phosphatase system in intestine

Sample	% Control Activity
1. (a) Control (10)[a]	100
(b) Prepared in 50 mM NaF (10)	23 ± 7
2. +Mg–ATP:	
(a) + Liver kinase (7)	38 ± 11
(a) + Intestinal kinase (7)	42 ± 12
3. Dephosphorylation:	
(b) + Liver phosphatase (9)	62 ± 11
(b) + Intestinal phosphatase (9)	46 ± 8

[a] The number in parentheses is the number of determinations.

Liver microsomes were prepared in the presence and absence of 50 mM NaF as described in Methods. Cytosol was prepared from the intestine, and partially purified kinase and phosphatase fractions were prepared as described in Methods. Values are expressed as the % control activity (no NaF added to preparation buffer) ± S.E. The control value for liver microsomes was 0.98 ± 0.11 (5) ηmol MVA · min^{-1} · mg $protein^{-1}$. The kinase preparations were added at 100 μg/assay; the phosphatase preparations, at 230 μg/assay. P_0 = relative to (a); P_1 = relative to (b). In all cases, P_0 was <0.01 and P_1 was <0.01 using Students' t test for paired samples.

activities of microsomal reductase from different types of preparations were compared. Microsomes were prepared from intestinal mucosal scrapings and from isolated cells. There were no significant differences in HMG-CoA reductase activity from the two preparations: 0.16 ± 0.02 nmol MVA min^{-1} mg $protein^{-1}$ (avg. of nine determinations) for scrapings versus 0.17 ± 0.02 nmol MVA min^{-1} mg protein $^{-1}$ (avg. of nine determinations) for isolated cells. For studying the in vitro regulation of the enzyme, mucosal scrapings were routinely used.

TABLE 3. In vitro regulation of rat intestinal HMG-CoA reductase

Sample	% Control Activity
1. (a) Control (7)	100
(b) Prepared in 50 mM NaF (7)	51 ± 7[a]
2. + Mg–ATP:	
(a) + Liver kinase (5)	98 ± 11[b]
(a) + Intestinal kinase (5)	169 ± 41[b]
3. Dephosphorylation:	
(b) + Liver phosphatase (7)	136 ± 22[b]
(b) + Intestinal phosphatase (8)	107 ± 12[b]

[a] $P < 0.01$ using Students' t test for paired samples.

[b] Not statistically significantly different from control.

Microsomes were prepared from intestine in the presence or absence of 50 mM NaF as described in Methods. Partially purified kinase and phosphatase fractions were prepared from liver and intestine as described in the text. The number of determinations is given in parentheses. The kinase and phosphatase preparations were added at 100 μg/assay or 230 μg/assay, respectively. Values are expressed as the % control value (no NaF added to preparation buffer) ± S.E. The control value for intestinal HMG-CoA reductase was 0.12 ± 0.05 nmol MVA · min^{-1} · mg $protein^{-1}$.

Preparation of intestinal microsomes in the presence of 50 mM NaF resulted in recovery of 51 ± 7% of the total (fully activated) reductase activity (**Table 3**). This is in contrast to the liver enzyme where only 10–20% of the activity can be recovered (18, 19, 35). The NaF-suppressed reductase activity could be restored to control levels by incubation with either intestinal or hepatic phosphatases (Table 3). The amount of the reactivation was dependent on the amount of phosphatase added whether the enzyme was of intestinal or liver origin (**Fig. 2**). Interestingly, addition of Mg^{2+}–ATP and either the liver or intestinal kinase preparation to intestinal microsomes failed to inactivate intestinal reductase (Table 3), despite the fact that these same kinase preparations were very effective with the liver enzyme (Table 2). Taken together, these results suggest that intestinal HMG-CoA reductase is less susceptible to inactivation by kinase-mediated phosphorylation than the liver enzyme.

DISCUSSION

Liver and intestine are the two major sites of cholesterol biosynthesis in the body (1). Previous reports had suggested that some fundamental differences in this process might exist between the two organs. One reported

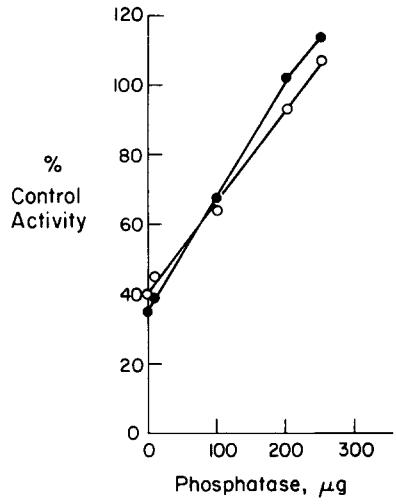

Fig. 2. Dependence of restoration of HMG-CoA reductase activity on amount of intestinal or liver phosphatase. Intestinal microsomes were prepared in the presence of 50 mM NaF as described in Methods. Phosphatases from liver or intestinal cytosol were also prepared as described in Methods. Intestinal microsomes were activated with varying amounts of the two phosphatases for 40 min followed by assay for HMG-CoA reductase activity as described in the text.

difference was that HMG-CoA reductase activity in the intestine was present both in mitochondria and in microsomes. Because such a difference could result in very different regulation of reductase activity and of overall cholesterol and isoprene biosynthesis, it was important to resolve this question.

Subcellular fractionation of intestinal cells is difficult. No single method is available which isolates all of the organelles in highly purified form. Initially, preparation of organelle fractions using differential centrifugation and discontinuous density gradients on sorbitol in histidine–imidazole buffer was attempted (30). Although these fractions appeared to be highly enriched in mitochondria and endoplasmic reticulum, a large proportion (80%) of the total homogenate HMG-CoA reductase activity was recovered in the 105,000 *g* supernatant or cytosol. It later became clear that the use of histidine–imidazole buffer resulted in solubilization of reductase as well as partial inactivation of the enzyme. Therefore, a second method for preparation of the organelles was devised. In this procedure, intestinal cells were isolated (21), one portion was used to prepare microsomes enriched in endoplasmic reticulum, and a second portion was used for preparation of purified mitochondria. Enriched fractions of each organelle were obtained in good yield. The enrichment of fractions was monitored by assaying recognized organelle markers. In contrast to previous reports (3, 14, 15), little detectable HMG-CoA reductase was recovered in the mitochondria. The small amount of reductase activity recovered in this organelle could be accounted for by microsomal contamination. Since previous workers did not characterize their mitochondrial and microsomal fractions biochemically, the differences reported here are most likely explained by contamination of mitochondria prepared by simple ultracentrifugation with microsomes, whole cells, and debris. We found it was essential to use a sucrose density gradient in order to obtain mitochondria which, under the light microsocope, were free of whole cells and nuclei.

The P_3 pellet consisting of whole cells and nuclei and cell debris was resistant to further fractionation. In preliminary work, preparation of a nuclear fraction relatively enriched in DNA also showed enriched reductase activity; however, this preparation still contained whole cells and probably microsomes. Nevertheless, the possibility that there may be some reductase activity in nuclei cannot be rigorously excluded. The less than perfect correspondence between reductase and aryl esterase could be due to differences in enzyme stability or to differences in localization within microsomes (i.e., rough vs. smooth endoplasmic reticulum or Golgi). deDuve (36) points out that absolute correspondence is rare and that there may be heterogeneity even within an organelle.

The low activity and low recovery of reductase activity in the mitochondria is unlikely to be due to HMG-CoA cleavage enzymes. Although HMG-CoA lyase is present in intestine (31), it and cleavage enzymes (32) are inhibited by buffers containing phosphate and EDTA. Both of these were present in the assays used. Moreover, microsomal reductase activity was not affected by the presence of mitochondria. Thus, failure to detect mitochondrial reductase activity cannot be explained by the presence of enzymes that compete for HMG-CoA as a substrate.

The subcellular fraction with the highest specific activity of HMG-CoA reductase was the microsomal fraction which was also enriched in endoplasmic reticulum. Although we have not been able to carry out a complete subcellular localization of HMG-CoA reductase in intestine, three firm conclusions can be drawn from these studies. First, reductase in the intestine is not present in a highly purified mitochondrial fraction. Second, the enzyme is present in endoplasmic reticulum, and third, because of problems with yield and organelle distribution, for physiologic studies of intestinal reductase, the use of the whole homogenate may be preferable.

In vitro regulation of hepatic HMG-CoA reductase by a phosphorylation–dephosphorylation mechanism mediated by a cytosolic kinase–phosphatase system(s) has been established (17–19, 33, 34). From the present work it is clear that a kinase–phosphatase system is also present in intestinal cytosol, and that this system appears to be very similar to that of the liver. The intestinal enzymes can be partially purified using the same procedures as for the liver enzymes. Biochemically, they behave similarly when assayed in the liver microsomal reductase system. The reductase activity of microsomes prepared in the presence of 50 mM NaF can be activated to control levels by partially purified preparations of either liver or intestinal phosphatases. However, intestinal reductase (isolated from the total mucosa) appears resistant to inactivation by partially purified kinase preparations from either organ at least when the same conditions for inactivation are employed as described for the liver system. These findings, together with the observation that NaF only inhibits 50% of the intestinal reductase activity, suggest that the intestinal enzyme may be less sensitive to regulation by activation–inactivation. Preliminary immunochemical studies demonstrated that the hepatic and intestinal enzymes are cross-reactive but there were quantitative differences in their immunotitration curves.[3] Thus there may be intrinsic differences in the two enzymes. Alternatively, the intestinal mucosa is composed

[3] Erickson, S. K., P. A. Edwards, F. J. Field, A. D. Cooper, and A. M. Fogleman. Unpublished observations.

of different cell types, crypt and villus cells, which are at very different stages of differentation. Thus it is possible that the susceptibility to regulation of reductase activity by activation–inactivation is different in the two cell types. This could also cause the observed quantitative differences. Such potential differences will be the subject of future investigations. The demonstration that the HMG-CoA reductase kinase–phosphatase system is indeed present in the intestine raises the possibility that it may play an important role in regulating intestinal reductase activity and therefore, isoprene and sterol biosynthesis in this organ. A very recent study is consistent with this interpretation (37).

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