Recovery and activation of hydroxymethylglutaryl coenzyme A reductase from rat small intestine

Nancy L. Young,[1] Christopher D. Saudek, Sarah A. Crawford, and Stewart L. Zuckerbrod

Department of Medicine, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

Abstract We describe a method for estimating the total activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) in the small intestine of rats. An homogenate of the whole small intestine is prepared rapidly and assayed directly to maximize the yield of enzyme and to minimize opportunity for uncontrolled change in activity. Fresh homogenate inhibits the expression of reductase in hepatic microsomes, has high HMG-CoA cleavage activity, and forms NADPH-independent metabolites which contaminate mevalonolactone isolated by thin-layer chromatography. When homogenate is preincubated, these interfering factors are decreased and reductase activity is increased. Part of this increase can be inhibited by F^-. After freezing and preincubation, total reductase activity recovered from homogenates of small intestine from 300-g male rats at the middle of their dark period is 40 nmol mevalonate per min compared to 70 from hepatic microsomes. If F^- is added at the time of homogenization, an activity of 11 nmol per min is recovered from each organ. Reductase in intestinal homogenate has an apparent K_m for S-HMG-CoA of 4 μM.—**Young, N. L., C. D. Saudek, S. A. Crawford, and S. L. Zuckerbrod.** Recovery and activation of hydroxymethylglutaryl coenzyme A reductase from rat small intestine. *J. Lipid Res.* 1982. **23:** 257–265.

Supplementary key words cholesterol synthesis • phosphatase • mevalonate • hydroxymethylglutaryl-CoA lyase • hydroxymethylglutaryl-CoA hydrolase • hydroxymethylglutaryl-CoA deacylase • hydroxymethylglutaryl-CoA cleavage enzymes.

To assess possible physiological consequences of change in both HMG-CoA reductase specific activity and organ weight, a measure of total reductase activity in the whole organ would be useful. For example, both intestinal weight and the specific activity of reductase in mucosal microsomes are increased in rats with long term insulin deficiency, and these increases may contribute to their hypercholesterolemia (1). However, a measure of total reductase activity is not currently available, and thus a quantitative evaluation of the net effect of these changes has not been attempted.

To estimate total activity, maximal yield of activity is required. Greater yields are obtained from homogenates of small intestine than from microsomes (2, 3). However, analyses have been restricted to mucosal cells isolated only from portions of ileum or jejunum (2, 3). Isolating mucosal cells has two inherent disadvantages: reductase activity changes during the procedure for isolating cells (4), and the yield of activity is reduced. Finally, previous reports on reductase in mucosal cell homogenates (2, 3) show kinetics with time and protein in the assay but do not demonstrate purity of the assay product, requirement for NADPH, substrate dose response, or absence of interfering factors.

The present report describes a method for the rapid preparation of an homogenate of whole small intestine, allowing total enzyme activity to be measured. We document the validity of the assay, and describe more fully the effects of F^- inhibitable activation and of HMG-CoA cleavage enzymes on the expression of reductase in vitro noted previously in an abstract (5).

MATERIALS AND METHODS

Animals

Wistar rats, 200–250 g (Charles River Laboratories, Wilmington, MA) were housed in a room illuminated from 6 PM to 6 AM, and fed Purina Formulab chow #5008 ad libitum. They were killed after 2 weeks at noon when males weighed ca. 300 g and females weighed ca. 250 g.

Tissue homogenates

All solutions were used at 0–5°C. Rats were anesthetized with ether. The small intestine from the pylorus to the ileocecal junction and the liver were excised, rinsed with saline, and weighed. Homogenates, 20% (w/v), were prepared in homogenizing medium (HM) with a Tissumizer® (motor SDT, shaft 18K; Tekmar Co., Cin-

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMG, 3-hydroxy-3-methylglutaric acid; MVA, mevalonic acid; MVL, mevalonolactone; TLC, thin-layer chromatography; R_f, ratio with the front; R_p, ratio with the peak; SEM, standard error of the mean; HM, homogenizing medium (composition given in text).

[1] To whom reprint requests should be addressed.

cinnati, OH) at speed 25 for 15 sec. HM contained 100 mM K_2HPO_4, 30 mM Na_2EDTA, NaOH to give pH 7.5, NaCl to give 220 mM Na^+, 10 mM dithiothreitol, and 0.2% (w/v) soybean trypsin inhibitor (Type 1-S, Sigma Chemical Co.). The concentrated homogenate was immediately diluted to 5% (w/v) with HM. To inhibit activation of HMG-CoA reductase in vitro, concentrated homogenate was diluted also into HM containing 66.7 mM NaF in place of an equal concentration of NaCl, to give a final NaF concentration of 50 mM. Total time from start of anesthesia to dilution of homogenate was less than 10 min. Portions of homogenate were fractionated (see below) and others were stored in liquid N_2.

Subcellular fractionation

All steps were performed at 0–5°C. Twenty ml of 5% homogenate was centrifuged 20 min at 9,000 *g*. The supernatant liquid was decanted and 10 ml was centrifuged 1 hr at 106,000 *g*. The Tissumizer® (shaft 10N) at speed 25 for 15 sec was used to resuspend the pellets. The low speed pellets were resuspended in HM to give a total volume of 20 ml; the high speed pellets of intestine and liver were resuspended in 1 ml and 2.5 ml HM, respectively. Fractions were assayed immediately or stored in liquid N_2.

Assay of HMG-CoA reductase and HMG-CoA cleavage activities

The method of Shapiro et al. (6) was modified as follows. Substrate-cofactor solution for one assay contained the following: *a*) 2 nmol of (3-*R*,*S*)[3-^{14}C]HMG-CoA prepared as described by Young and Berger (7) and 23 nmol of HMG-CoA (P-L Biochemicals, Inc., Milwaukee, WI) to give 5 Ci/mol; *b*) an NADPH-generating system as in (7) except that all quantities were halved; *c*) an internal recovery standard of 1 pmol (10^4 dpm) (3-*R*,*S*)[5-^{3}H]MVA (New England Nuclear, Boston, MA); *d*) NaF to give total amount after adding enzyme sample of 3.75 μmol; *e*) 5 μl HM at 5 times usual concentration; *f*) NaOH to give pH 7.5; all in a total volume of 25 μl. Fifty μl of enzyme sample was preincubated at 37°C for 30 min in a 0.5 ml polyethylene centrifuge tube. The substrate-cofactor solution was added with Vortex mixing, and incubation was continued for 15 min (liver microsomes), 30 or 60 min (intestinal homogenate prepared without or with F^-, respectively). Control samples were the complete system minus $NADP^+$, and the complete system minus enzyme. The reaction was stopped by addition of 5 μl of 6 N HCl.

To minimize cleavage of HMG-CoA by the acid, tubes were kept at −20°C or on ice unless otherwise indicated. After centrifugation (10,000 *g*, 1 min, 25°C), 10 μl of the supernatant liquid was chromatographed with butanol–acetic acid–water 7:2:3 on cellulose thin-layer sheets scored in 16 channels (System 1, reference 7) to determine the fraction of [^{14}C]HMG-CoA that was cleaved (7). The assay tube was then incubated at 37°C for 15 min to convert MVA to MVL. ^{14}C-Labeled and ^{3}H-labeled MVL in 50 μl of the supernatant liquid were isolated by chromatography with acetone–toluene 1:1 on silica gel thin-layer sheets scored in eight channels (System II, reference 7). Radiolabeled products were measured by scintillation counting (7).

HMG-CoA reductase activity was calculated from the ^{14}C-labeled products isolated with ^{3}H-labeled MVL after correcting for ^{3}H-recovery, as usual, and for NADPH-independent ^{14}C-labeled products.

HMG-CoA cleavage activity was calculated, for example in fresh mucosal homogenate without F^- (Table 2), as follows. The fraction of [^{14}C]HMG-CoA at the end of a 30-min incubation of a buffer control, 0.914, less the fraction in homogenate, 0.607, times the initial amount of substrate corrected for radiochemical purity, 23.8 nmol, gives the amount cleaved by homogenate, 7.31 nmol. This amount divided by the weight of tissue in the assay, 0.0025 g, and the assay time gives cleavage activity, 97 nmol/(min × g). Cleavage, as defined here, includes all metabolic conversions of HMG-CoA including its reduction to MVA (if NADPH was present). However, in intestinal homogenates, reductase activity was always less than 10% of cleavage activity.

Since each 50-μl sample was derived from a fixed amount of tissue (homogenate and low speed pellet from 2.5 mg of tissue, high speed pellet of small intestine and of liver from 25 and 10 mg of tissue, respectively), specific activity was expressed per g tissue. For the present application, where recovery of enzyme activity is stressed, expressing activity per g tissue is more useful than per mg protein. However, the latter can be calculated from our data for the former by dividing by protein recovery which was 143 ± 12 mg protein/g tissue in small intestine homogenate, and 23 ± 1 mg/g in liver microsomes. Total activity per organ was calculated as activity per g tissue times organ weight.

Assay of HMG-CoA reductase inhibition

Intestinal homogenate, 40 μl, and 10 μl of liver microsomes prepared without F^-, each from 2 mg of tissue, were assayed separately and together by incubation with substrate-cofactor solution in a total volume of 75 μl at 37°C for 30 min. Inhibition was calculated as the difference between the moles of MVA formed by the mixture and by intestine alone, divided by the moles of MVA formed by liver alone and then subtracted from 1. It was assumed that liver microsomes did not inhibit reductase.

RESULTS

Chromatography of [^{14}C]HMG-CoA metabolites

TLC System I separated [^{14}C]HMG-CoA from all ^{14}C-labeled metabolites and therefore was suitable for assay of cleavage activity (**Fig. 1**). In this case, both isomers of (*R*,*S*)HMG-CoA were quantitatively metabolized by intestinal homogenate prepared in Tris buffer to less polar products. However, in all subsequent experiments, where homogenate was prepared in phosphate buffer, and less tissue was incubated for a shorter time, less than 40% of [^{14}C]HMG-CoA was metabolized.

In TLC System II, most cleavage products migrated with R_f less than 0.5 (not shown). Nevertheless, some cleavage products formed by fresh intestinal homogenate migrated with R_f greater than 0.5 and were poorly resolved from MVL (R_f 0.7, **Fig. 2**). ^{14}C-Labeled products formed in the absence of added NADP$^+$ overlapped but did not comigrate with [^{3}H]MVL (**Fig. 2B**). This showed that little or no [^{14}C]MVA had been formed, and therefore that endogenous NADP$^+$ did not support [^{14}C]MVA formation. More contaminants were formed in the absence than in the presence of NADP$^+$.

When cleavage activity was decreased and reductase activity increased, as described below, the purity of [^{14}C]MVL isolated by TLC was much improved. The profile of the ^{14}C-labeled products on TLC corresponded closely with that of the ^{3}H-labeled standard as the lactone, the acid, and the amide (**Fig. 3**). The ratio of ^{14}C to ^{3}H in the peak did not decrease in consecutive TLC separations. NADPH-independent metabolites were present in small amounts (4%) and still chromatographed as in Fig. 2B.

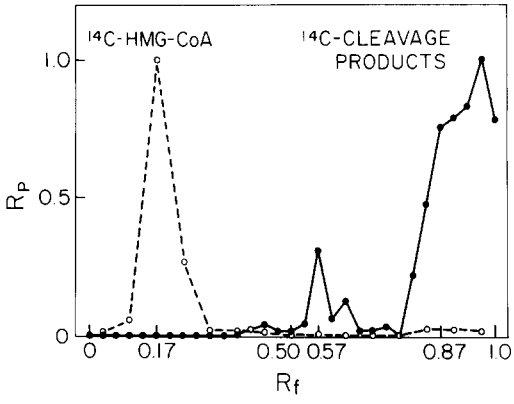

Fig. 1. Isolation of [^{14}C]HMG-CoA from its metabolites by TLC. [^{14}C]HMG-CoA (330 uM) was incubated for 1 hr at 37°C with an homogenate of 5 mg of small intestine in 50 mM Tris buffer (pH 7.5), 300 mM mannitol, in a total volume of 110 μl. After adding 5 μl of 6 N HCl and centrifuging, the supernatant liquid was chromatographed in System I. ^{14}C(dpm) in 0.5-cm sections of the TLC sheet was divided by ^{14}C(dpm) at the peak to give R_p for metabolites formed by homogenate (●) and for stock [^{14}C]HMG-CoA (○).

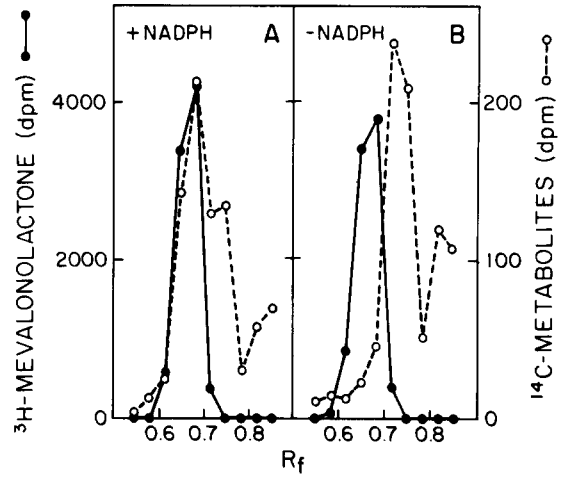

Fig. 2. Cochromatography of [^{14}C]MVL with other metabolites of [^{14}C]HMG-CoA. Intestinal homogenate (−F$^-$) was assayed as described in Materials and Methods except that freezing and preincubation were omitted. Radioactivity in 1-cm sections of TLC System II is shown.

Effect of pretreatment of intestinal homogenate on metabolism of [^{14}C]HMG-CoA

HMG-CoA cleavage activity was highest in fresh homogenate, declined slightly with storage on ice, and declined substantially after freeze-thawing and incubation at 37°C (**Table 1**). In contrast, reductase activity increased slightly with storage of homogenate on ice, but decreased over 90% with freeze-thawing. This loss was more than recovered with subsequent preincubation yielding a 4-fold increase from activity in fresh homogenate. The fraction of ^{14}C-labeled products isolated with MVL that were NADPH-independent varied from 93% after freeze-thawing to 11% after preincubation at 37°C.

Intestinal homogenate that was not preincubated in-

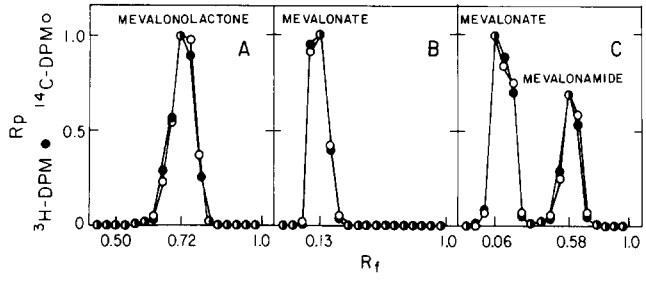

Fig. 3. Chromatography of putative [^{14}C]MVL. Intestinal homogenate (−F$^-$) was assayed as described in Materials and Methods. Panel A shows radioactivity in 0.5-cm sections of TLC System II. Material in the region R_f 0.62–0.82 was eluted with acetone and methanol, divided in half, dried with a stream of N_2, and incubated at 37°C for 15 min with either 100 μl of 0.1 N NaOH to form MVA (B) or 100 μl of concentrated ammonia to form MVA and mevalonamide (C). B and C show radioactivity in 1-cm sections of TLC System IV (cellulose sheet; butanol–ammonia–water 20:1:1 in a sandwich chamber). The ratio of ^{14}C to ^{3}H was 0.366 in MVL, 0.374 in MVA in B, and 0.379 in C, and 0.391 in mevalonamide.

TABLE 1. Effect of pretreatment of intestinal homogenate on its metabolism of [^{14}C]HMG-CoA

Pretreatment	HMG-CoA Cleavage Activity	[^{14}C]"MVL" +NAPD$^+$	[^{14}C]"MVL" −NADP$^+$	HMG-CoA Reductase Activity
	$\frac{nmol}{min \times g}$	*dpm*		$\frac{nmol}{min \times g}$
20 Min on ice	69	720	354	0.43
180 Min on ice	54	888	398	0.56
Freeze-thawed	58	348	324	0.04
Freeze-thawed + 15 min @ 37°C	32	2170	222	2.16
Absent		42	66	

A 10% (w/v) homogenate and a 15-min assay were used. Data for [^{14}C]"MVL" are for products recovered at R_f 0.61 to 0.84 of TLC System II after correcting for [^{3}H]MVL recovery.

hibited the expression of reductase in hepatic microsomes by 80–90% (**Table 2**). Inhibition declined to about 20% with a 30-min preincubation at 37°C. Associated with decreased inhibition was a decrease in HMG-CoA cleavage activity. The increase in reductase activity with preincubation was partially blocked by F^-. F^- had little or no effect on inhibitory activity, cleavage activity, or their decrease. The data in Tables 1 and 2 thus demonstrate that the increase in reductase activity with preincubation was due to the combined effects of recovery from freezing, activation of reductase by a F^--inhibitable process, and decrease in reductase inhibition activity.

The optimal time for preincubation of freeze-thawed intestinal homogenate was 30 to 60 min, when reductase activity was maximal and cleavage activity minimal.

TABLE 2. Effect of preincubation of small intestine homogenate with and without F^- on HMG-CoA reductase, reductase inhibition, and HMG-CoA cleavage activities

Preincubation Conditions	HMG-CoA Reductase Specific Activity	HMG-CoA Reductase Inhibition Activity	HMG-CoA Cleavage Specific Activity
	$\frac{nmol}{min \times g}$	%	$\frac{nmol}{min \times g}$
0 min $-F^-$	0.04 ± 0.03	81 ± 7	213 ± 41
$+F^-$	−0.01 ± 0.02	85 ± 2	179 ± 28
30 min $-F^-$	3.42 ± 0.40	19 ± 6	30 ± 8
$+F^-$	0.72 ± 0.09	17 ± 7	26 ± 4

Data are means ± SEM for small intestines from five male rats. Homogenates were freeze-thawed before preincubation at 37°C. Inhibition activity was calculated as described in Materials and Methods, assuming that liver microsomes did not inhibit reductase. Alternatively, if inhibition is calculated as moles MVA formed by the mixture of liver microsomes and small intestine homogenate divided by the sum of moles MVA formed by liver and intestine separately and then subtracted from one, inhibition is 81 and 85% without preincubation, and 17 and 13% after preincubation.

Fractionation into mucosa and muscle

The possibilities that factors in intestinal homogenate which interfered in the reductase assay orginated in muscle or resulted from freeze-thawing were tested by assaying HMG-CoA cleavage and reductase activities in mucosa and muscle separated by scraping and pretreated in various ways (**Table 3**).

After freezing and preincubation without F^-, reductase specific activity in mucosal homogenate, 5.2 nmol/(min × g), was higher than in homogenate of intact small intestine, 3.7 nmol/(min × g) (Table 3). On the other hand, total activity was higher in the latter (36 nmol/min) than in the former (25 nmol/min). Thus, although inclusion of muscle increased total cleavage activity from 98 to 206 nmol/min, it did not decrease total reductase activity. Freeze-thawing before preincubation also increased cleavage activity and did not decrease reductase activity. On the contrary, freeze-thawing appeared to increase F^--inhibitable activation of reductase in homogenate of the intact small intestine from 2.0- to 4.6-fold. The activation of reductase in mucosa after homogenization (1.3) was low compared to that in homogenate of intact small intestine (4.6). Since reductase specific activity in mucosal homogenate prepared with F^- was high before preincubation, 4.1 nmol/(min × g), it appears that activation had occurred prior to homogenization, probably during scraping. In all cases, after freezing and preincubation, less than 10% of ^{14}C-labeled products isolated with MVL were NADPH-independent.

Variation in HMG-CoA reductase activity along the length of the small intestine

In agreement with previous findings for cholesterol synthesis (8) and for reductase activity in microsomes (9), reductase activity in homogenates showed a U-shaped variation along the length of the small intestine (**Fig. 4**). Activity was highest in the short section of the proximal jejunum between the pylorus and the entrance of the bile duct.

Subcellular fractionation

Most measurable sedimentable reductase activity from small intestine was recovered in the low speed pellet (**Fig. 5**) in agreement with previous reports (2, 3, 9), in contrast to that from liver which was recovered in the high speed pellet. The yield of reductase from small intestine decreased with fractionation, while that from liver increased. Thus, the yield of activity from small intestine was maximal in homogenate, while the yield from liver was maximal in microsomes. It is evident that the expression of reductase in liver homogenate, low speed supernatant, and possibly low speed pellet was inhibited. Re-

TABLE 3. Metabolism of HMG-CoA by small intestine before and after separation of mucosa from muscle

	Weight	HMG-CoA Cleavage Activity						HMG-CoA Reductase Activity								
		Specific (−F⁻)			Total (−F⁻)			Specific (+F⁻)			Specific (−F⁻)			Total (−F⁻)			Activation		
	g	*nmol/(min × g)*			*nmol/min*			*nmol/(min × g)*			*nmol/(min × g)*			*nmol/min*			*fold*		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Mucosa	2.45	97	13	20	471	62	98	0.12	3.47	4.08	0.15	4.06	5.17	0.7	19.7	25.0	1.3	1.2	1.3
Muscle	2.51	80	17	6	397	85	31	0.13	0.71	0.83	0.09	1.04	1.10	0.5	5.2	5.5	0.7	1.5	1.3
Sum	4.96				867	148	130							1.2	24.8	30.5			
Intact	4.83	94	10	21	918	98	206	0.25	0.91	0.81	0.27	1.84	3.73	2.7	18.0	36.5	1.1	2.0	4.6

A small intestine (9.79 g) was split in half longitudinally. One-half was homogenized immediately; mucosa was scraped from the other half at 4°C; mucosa and remaining muscle were homogenized. Homogenates were assayed immediately (I) and after preincubation at 37°C for 30 min with (III) or without (II) prior freezing. Total activity was calculated for the whole small intestine. Activation is activity $-F^-$ divided by activity $+F^-$.

Fig. 4. Distribution of HMG-CoA reductase activity along the length of the small intestine. In the first experiment, each of two small intestines was divided into 10-cm sections (●, ▲), and a third into 12-cm sections (■). In the second experiment, each of five small intestines was sectioned just before the entrance of the bile duct and at 30 cm from the ileocecal junction to give three pieces (mean ± SEM given by the bars). Each section was homogenized ($-F^-$) and assayed as described in Materials and Methods.

ductase activity in all preparations, except intestinal low speed pellet, increased with freezing and preincubation, along with a decrease in cleavage activity. After freezing and preincubation, the fraction of NADPH-independent

Fig. 5. Subcellular fractionation of liver and small intestine. Homogenates (fine stipling), low speed pellet (coarse stipling), low speed supernatant (clear, center), high speed pellet (crosshatching), and high speed supernatant (clear, right) were prepared without F^- and assayed as described in Materials and Methods before (bars labeled U for untreated) and after (bars labeled T for treated) freezing and preincubating for 15 min at 37°C.

products isolated with MVL was 26% for the low speed pellet of small intestine, and less than 10% for all other preparations.

Kinetics

MVA production by 2.5 mg of homogenized intestine was linear with assay time up to 60 min (**Fig. 6A**). MVA produced in 60 min was approximately linear with amount of tissue in the assay up to 2.5 mg (Fig. 6B).

When HMG-CoA concentration was varied under special assay conditions which provided adequate [^{14}C]MVL recovery while avoiding excessive substrate cleavage, MVA production showed typical saturation kinetics with a K_m of 4.1 ± 0.2 μM (3-*S*)HMG-CoA (**Fig. 7**). When the measured decline in substrate concentration during the assay (less than 10% (*R*,*S*)HMG-CoA) was taken into account as suggested by Lee and Wilson (10), the revised estimate, 3.9 ± 0.2 μM, was not significantly different.

Variability

The coefficient of variation between triplicate assays of reductase activity in intestinal homogenate in the experiment shown in Fig. 7 averaged 2.8% and did not exceed 6.6%. The coefficient of variation among 63 rats was 25% for intestinal homogenate compared with 61% for liver microsomes.

DISCUSSION

Preparing homogenates of whole small intestine for assay of HMG-CoA reductase has several advantages. Most importantly, the yield of enzyme activity is maximized thereby permitting estimate of total activity in the organ. We recover a total activity from the small intestine of male rats weighing ca. 300 g of ca. 40 nmol/min

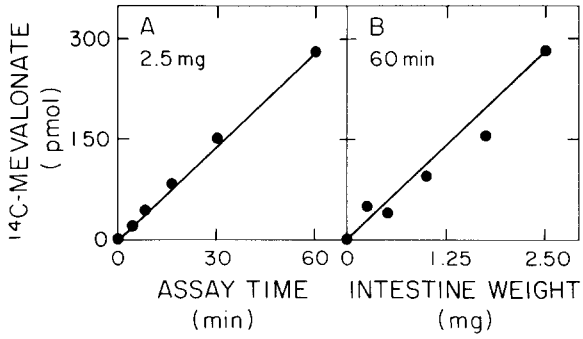

Fig. 6. Variation of MVA production with time and amount of tissue. Intestinal homogenate (−F⁻) was assayed for HMG-CoA reductase activity as described in Materials and Methods, except that assay time was varied in A, and concentration of homogenate in the preincubation and assay was varied in B.

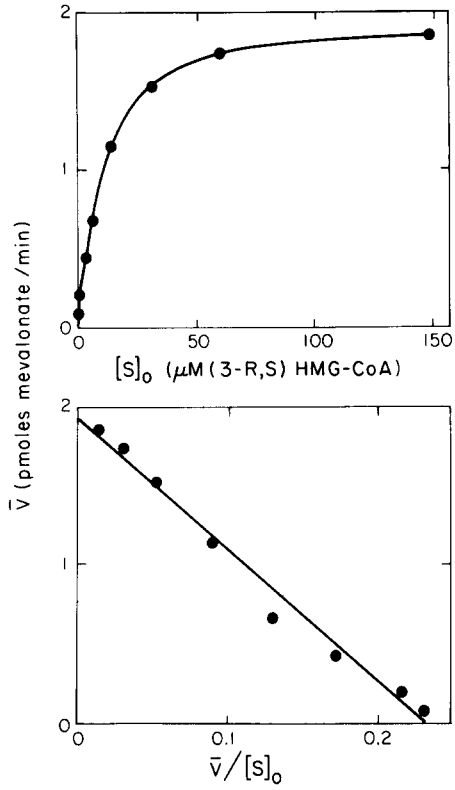

Fig. 7. Substrate dose response of MVA production. Intestinal homogenate (−F⁻) was assayed as described in Materials and Methods, except that homogenate was diluted to 1% with HM (−F⁻) after preincubation. [^{14}C]HMG-CoA concentration was from 0.4 to 150 μM and specific activity was 122 to 24 dpm/pmol, and all other components including total assay volume were doubled. Data are means of triplicate assays. Linear regression of data plotted according to Eadie-Hofstee in the bottom panel gave an estimate for K_m of 4.1 ± 0.2 μM (S)HMG-CoA and V_{max} of 1.92 ± 0.09 nmol MVA/(min × g tissue).

(**Table 4**). Inclusion of muscle does not decrease the yield (Table 3). In fact, the yield in mucosa scraped from muscle is about one-third less than in whole small intestine. The yield continues to decrease with further fractionation (Fig. 5 and references 2, 3). Panini et al. (3) measured a total activity of 15 nmol/min in homogenate of epithelial cells from the distal 50 cm of ileum. They observed successive losses of 74% and 56% with isolation of crypt cells and of microsomes, respectively, giving a final activity in crypt cell microsomes of only 1.7 nmol/min, which is 5% of the activity we recover from whole small intestine homogenate.

On the other hand, whole small intestine homogenate would be unsuitable where high reductase specific activity is required. The specific activity we see in homogenate, 28 ± 1 pmol/(min × mg protein), is much lower than that in microsomes from ileal crypt cells, 450 pmol/(min × mg) (3). Incidentally, we would predict the highest specific activity in microsomes from the most proximal jejunum (Fig. 4). Activity in homogenate is, however,

TABLE 4. Comparison of rates of cholesterol synthesis predicted from total HMG-CoA reductase activity with those of literature values from incorporation of ^3H from tritiated water

Total HMG-CoA Reductase Activity		Liver		Small Intestine	
		$+F^-$	$-F^-$	$+F^-$	$-F^-$
Males	nmol MVA/min	11 ± 1	70 ± 5	11 ± 1	39 ± 1
317 ± 5 g	mg cholesterol/hr				
n = 63	uncorrected[a]	0.04	0.27	0.04	0.15
	corrected[b]	0.12	0.77	0.05	0.19
Females	nmol MVA/min	25 ± 5	114 ± 28	12 ± 3	26 ± 3
248 ± 5 g	mg cholesterol/hr				
n = 8	uncorrected[a]	0.10	0.44	0.05	0.10
	corrected[b]	0.27	1.26	0.06	0.12
Cholesterol synthesis in vivo[c]					
Females	mg cholesterol/hr	0.53		0.26	
211 ± 5 g					
n = 4					

[a] Calculated from nmol MVA/min by assuming 6 nmol MVA/nmol cholesterol, 60 min/hr, 386.6 mg cholesterol/mmol, and 10^6 ng/mg.

[b] Corrected for 35% recovery of endoplasmic reticulum from liver and 20% inhibition of reductase activity in small intestine.

[c] Measured in Sprague-Dawley rats at 1 hr after injection of tritiated water by Turley et al. (23).

well within the range for reliable microassay, without heroic increases in the specific activity of [^{14}C]HMG-CoA. At least 1000 ^{14}C-dpm are recovered in [^{14}C]MVL, and there is less variation than in liver microsomes.

Another advantage of our method is that, by avoiding the time-consuming step of isolating mucosal cells, it reduces the opportunity for uncontrolled change from the in vivo level of enzyme activity, such as the activation that occurs during cell isolation (Table 3 and reference 4). This activation can be prevented with F^- and reversed with bicarbonate in the medium (4), but since inactivation is not controlled, the original state of activation may not be preserved. In contrast, at the time of homogenization, inactivation can be blocked with EDTA and activation can be blocked with F^- (11). In our method, intestine is homogenized in a medium with EDTA, then immediately diluted into media with and without F^-. It is highly unlikely that activation could occur within less than a minute on ice from the start of homogenization to the dilution into F^-. Thus, both activated and nonactivated reductase activity can be assayed in the same small intestine and experimental variation is reduced.

Use of intestinal homogenate, rather than microsomes, does require special care, however. Homogenate contains HMG-CoA cleavage enzymes and inhibitors of reductase activity with potential for interfering in the reductase assay in a number of ways. HMG-CoA can be excessively depleted (Fig. 1), products can be formed that contaminate isolated MVL (Fig. 2), and reductase can be inhibited over 80% (Table 2). However, these interfering factors are reduced to tolerable levels by homogenizing in phosphate buffer, preincubating, and limiting the amount of tissue and the time of assay.

It is evident that another potential problem with homogenate, the further metabolism of MVA, does not occur under our assay conditions. We include [^3H]MVA in the substrate-cofactor solution, so we can monitor and correct for loss of MVA during and after the incubation. In the non-polar region of the TLC (Figs. 2 and 3), [^3H]MVL is the only ^3H-labeled material present. Thus the NADPH-independent ^{14}C-labeled products in this region are not metabolites of MVA, and do not include Δ^2-3-CH_3-MVL, the dehydration product formed in strong acid and migrating in front of MVL (12). It is unlikely that phosphorylation of MVA, the first step in its conversion to cholesterol, could occur during the incubation. This reaction requires ATP which was not added, and Mg^{2+} which was chelated with EDTA. Finally, the recovery of [^3H]MVL after incubation with homogenate is not lower than after incubation with buffer controls.

Under the conditions we employ, the purity of isolated [^{14}C]MVL is greater than 90% (Fig. 3), reductase inhibition is less than 20% (Table 2), [^{14}C]MVA production is linear with time and weight of tissue (Fig. 6), and the K_m for (3-*S*)HMG-CoA is 4 μM (Fig. 7). This estimate is slightly lower than for reductase in intestinal

microsomes, 7 μM (9) and 21 μM (13). Low K_m is a favorable indication that substrate cleavage is not interfering in the assay (7, 14, 15) and that factors in the homogenate do not affect substrate binding any more than those in microsomes.

In this and the following report (16), we used controls without $NADP^+$ to correct for the ^{14}C-labeled contaminants isolated with MVL. It is clear from the TLC data (Fig. 2) that [^{14}C]MVA is not formed in such controls, in spite of the possible presence of $NADP^+$. However, since more contaminants are formed in the absence of $NADP^+$ than in its presence, this correction underestimates reductase activity. In properly treated homogenates, these contaminants account for less than 10% of the ^{14}C-labeled products isolated, so correction is unnecessary.

Intestinal homogenates may be stored in liquid N_2 before assay of reductase activity. Freeze-thawing drastically lowers reductase activity, but the effect is transient (Table 1). With preincubation, the yield of reductase is actually improved by prior freezing as a result of increased F^--inhibitable activation of reductase (Table 3). Cleavage activity is moderately increased by freezing, possibly due to broken mitochondria, but this increase does not impair the recovery of reductase (Table 3).

Although preincubation of intestinal preparations to increase reductase activity is common practice (1–4, 9, 13) and HMG-CoA lyase is known to be present (17), the roles of HMG-CoA cleavage enzymes (5) and of F^--inhibitable activation (4, 5) in the increase were noted only recently. The increase results from the combined effects of a 4-fold activation and about a 4-fold decrease in reductase inhibition (Table 2). Inhibition is not due to phosphorylation of either reductase or MVA by kinases since these processes require ATP which is not added and Mg^{2+} which is chelated with EDTA. The inhibition might be due to cleavage enzymes since cleavage activity declines along with reductase inhibition during preincubation. However, in this case, excessive substrate depletion by cleavage enzymes cannot account for the inhibition. Even when inhibition is 80%, the concentration of (3-*S*)HMG-CoA is greater than 30 μM, which with a K_m of 4 μM, should provide a rate of MVA formation more than 88% of maximum. On the other hand, the products of cleavage enzymes in small intestine homogenate include HMG and free CoA,[2] which are known to inhibit reductase (18, 19). Therefore, cleavage enzymes might be inhibiting reductase, not by depleting substrate in this case, but by forming inhibitory products.

Monitoring conversion of HMG-CoA to MVA as suggested by Langdon and Counsell (15) or to acetoacetate will seriously underestimate total substrate utilization in the reductase assay of intestinal homogenate. We find that intestinal homogenate forms three major ^{14}C-labeled products in addition to acetoacetate and has maximal HMG-CoA cleavage activity of 8500 nmol/(min × g tissue)[2] compared to lyase activity of 400 nmol/(min × g) (17). Although cleavage activity is reduced to 10–30 nmol/(min × g) under our reductase assay conditions, it is still higher than reductase activity of 4 nmol/(min × g). Measuring total substrate utilization by isolating all metabolites with the simple TLC method I (5, 7) is advisable for substrate-dose response experiments or when intestinal homogenate is prepared in non-standard ways.

We recover a total reductase activity in homogenate from intestine of male rats killed at mid-dark of 40 nmol/min compared to 70 nmol/min in liver microsomes (Table 4). When F^- is added at the time of homogenization, total activity from each organ is 11 nmol/min. Our reductase specific activity in liver microsomes prepared without F^-, 250 pmol/(min × mg protein), is in line with previously reported values for Wistar rats (9) when the lower activity in males compared to females (9, 20) and the decline with age (9) are taken into account. Our recovery of protein in liver microsomes, 23.6 ± 0.7 mg protein/g tissue, is similar to that reported by Eriksson (21) and Brown et al. (22) of 20 mg/g. However, according to Eriksson (21), only about 35% of the endoplasmic reticulum is recovered in liver microsomes as they are usually prepared. If corrections are made for 35% recovery of endoplasmic reticulum from liver and the 20% inhibition of reductase in intestinal homogenate, total reductase activity before activation is 31 nmol/min in liver and 14 nmol/min in small intestine, and after activation is 200 and 49 nmol/min, respectively. Thus, by these estimates in males, the total activity in small intestine is 25 to 45% of that in liver. In females, total reductase activity in small intestine is 10 to 22% of that in liver.

Our major goal has been to estimate total HMG-CoA reductase activity in small intestine. Our recovery of activity is at least 10 times that of previously published methods, but we still do not know what fraction of in vivo reductase activity we measure in vitro. HMG-CoA may not be as accessible to reductase in vesicles of endoplasmic reticulum formed during homogenization as it is to reductase in planar membranes in vivo. It is also unclear whether the activated or the unactivated level of reductase activity is the more nearly physiological. Brown et al. (22) addressed the latter question by comparing rates of cholesterol synthesis measured in vivo with reductase activity. They concluded that only the higher, activated level of activity could account for cholesterol synthesis in liver. However, these authors assumed 100% recovery of endoplasmic reticulum. Turley et al. (23)

[2] Unpublished observation by Albrecht Schnieder in our laboratory.

recently estimated rates of cholesterol synthesis in both liver and small intestine in vivo. Their data are shown with ours in Table 4 for the purposes of discussion, but it should be noted that rates of cholesterol synthesis in their rats were probably higher than in ours because they used younger rats from the faster growing Sprague-Dawley strain. Given this uncertainty, we can be confident only that at least 20% of the reductase activity is recovered in both small intestine and liver (Table 4). Studies of reductase activity and cholesterol synthesis in identically treated rats are necessary for a definite answer to these questions.

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