Localization of 3-hydroxy-3-methylglutaryl CoA reductase and 3-hydroxy-3-methylglutaryl CoA synthase in the rat liver and intestine is affected by cholestyramine and mevinolin

Andrew C. Li,[*] Richard D. Tanaka,[1,*] Kimberly Callaway,[†] Alan M. Fogelman,[*] and Peter A. Edwards[*,**]

Division of Cardiology,[*] Department of Medicine; School of Public Health,[†] Division of Nutrition; Department of Biological Chemistry;[**] University of California Los Angeles, Los Angeles, CA 90024

Abstract In the normal fed rat, both 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase and HMG-CoA reductase are found in high concentrations in hepatocytes that are localized periportally. The majority of the liver cells show little or no evidence of either enzyme. Addition of cholestyramine and mevinolin to the diet resulted in all liver cells showing strong positive staining for both HMG-CoA reductase and HMG-CoA synthase. These two drugs increased the hepatic HMG-CoA reductase and HMG-CoA synthase activities 92- and 6-fold, respectively, and also increased the HMG-CoA reductase activity in intestine, heart, and kidney 3- to 15-fold. We used immunofluorescence and avidin–biotin labeled antibody to localize HMG-CoA reductase in the rat intestine. In rats fed a normal diet, the most HMG-CoA reductase-positive cells were the villi of the ileum > jejunum > duodenum. Crypt cells showed no evidence of HMG-CoA reductase. Addition of cholestyramine and mevinolin to the diet led to a dramatic increase in the concentration of HMG-CoA reductase in the apical region of the villi of the ileum and jejunum and in the crypt cells of the duodenum. Hence these two drugs affected both the relative concentration and distribution of intestinal HMG-CoA reductase. ▇▇ Cholestyramine and mevinolin feeding induced in the liver, but not intestine, whorls of smooth endoplasmic reticulum that were proximal to the nucleus and contained high concentrations of HMG-CoA reductase. Administration of mevalonolactone led to the rapid dissolution of the hepatic whorls within 15 min, at a time when there is little or no change in the mass of HMG-CoA reductase. We conclude that the whorls are present in the livers of rats fed cholestyramine and mevinolin because the cells are deprived of a cellular product normally synthesized from mevalonate.— Li, A. C., R. D. Tanaka, K. Callaway, A. M. Fogelman, and P. A. Edwards. Localization of 3-hydroxy-3-methylglutaryl CoA reductase and 3-hydroxy-3-methylglutaryl CoA synthase in the rat liver and intestine is affected by cholestyramine and mevinolin. *J. Lipid Res.* 1988. **29**: 781–796.

Supplementary key words crypt cells • ileum • jejunum • duodenum • villus cells • periportal cells • immunocytochemistry

3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (EC 1.1.1.34) is a key regulatory enzyme in the biosynthesis of cholesterol, ubiquinone, and dolichol (reviewed in 1 and 2). The activity of the enzyme in rat liver microsomes can be modulated over 1000-fold by diet; the activity is low following administration of cholesterol (2) or mevalonolactone (3) or high after feeding diets supplemented with cholestyramine and mevinolin (4). Cholestyramine is a bile acid sequestrant (5) and hence interrupts the enterohepatic circulation, while mevinolin is a competitive inhibitor of HMG-CoA reductase (6). These changes in HMG-CoA reductase activity result from alterations in the rates of synthesis and degradation of the hepatic enzyme (7, 8).

Cholestyramine and mevinolin are hypocholesterolemic drugs that appear to lower plasma cholesterol levels in dogs and man by affecting both cholesterol synthesis (2) and the expression of the LDL-receptor in the liver (9, 10). The hypocholesterolemia results from increased levels of the LDL-receptor and the resulting increased clearance of plasma LDL (9, 10). There are few studies that report on the effects of these two drugs on the levels of extrahepatic reductase. Of the extrahepatic tissues, the small intestine is particularly active in cholesterol biosynthesis (11). However, the relative importance of the different intestinal cell types in cholesterogenesis is controversial (12–16). Recently Singer et al. (17) have used immunocytochemistry to show that ileal HMG-CoA reductase shows a con-

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Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoproteins; FITC, fluorescein isothiocyanate.

[1]Current address: The Squibb Institute for Medical Research, Princeton, NJ.

centration gradient along the villus–crypt axis with the villi containing maximal enzyme levels. Ileal HMG-CoA reductase levels were shown to increase after feeding cholestyramine and mevinolin (17). We have used immunocytochemistry to determine which intestinal cells in the duodenum, jejunum, and ileum contain significant amounts of HMG-CoA reductase. We have also determined whether the concentration of HMG-CoA reductase in the duodenum, jejunum, and ileum is affected by dietary cholestyramine and mevinolin.

Singer et al. (18) recently demonstrated that HMG-CoA reductase in rat liver was localized in periportal cells. We have now assessed the distribution of HMG-CoA synthase, (EC 4.1.3.5) an enzyme coordinately regulated with HMG-CoA reductase (19–22), in the livers of rats fed a normal diet and diets supplemented with cholestyramine and mevinolin. We demonstrate that HMG-CoA synthase, like HMG-CoA reductase, is concentrated in periportal cells of livers of normal fed rats and is induced in all hepatocytes after addition of cholestyramine and mevinolin to the diet.

Addition of either cholestyramine and mevinolin to rat diets (18) or mevinolin to UT-1 Chinese hamster ovary cells in culture (23) leads to proliferation of smooth endoplasmic reticulum that is enriched with HMG-CoA reductase. Recently Pathak, Luskey, and Anderson (24) have proposed that, in UT-1 cells, HMG-CoA reductase is synthesized along the outer nuclear membrane that is itself the precursor for the proliferative smooth endoplasmic reticulum. In the current studies we demonstrate that in rat liver the proliferative whorls of smooth endoplasmic reticulum that are found proximal to the nucleus are destabilized very rapidly following the administration of mevalonolactone. These results are consistent with the proposal that the proliferating smooth endoplasmic reticulum forms after the administration of cholestyramine and mevinolin because of the depletion of some product that is normally derived intracellularly from mevalonate.

MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: fluorescein isothiocyanate (FITC) conjugated to goat anti-rabbit IgG (F(Fab')$_2$ fragment specific) from Cappel Laboratories (Cochranville, PA); avidin–biotin–glucose oxidase complex (ABC-GO) Kit from Vector Laboratories (Burlingame, CA); osmium tetroxide from Ted Pella Inc. (Tustin, CA); goat anti-rabbit IgG coupled to colloidal gold (GAR gold) from Janssen Life Science Products (Piscataway, NJ); glutaraldehyde, sodium cacodylate, Epon–Aral-

dite, Lowicryl K4M from Polyscience (Warrington, PA); bovine serum albumin (fraction V), reagent grade, from Miles Scientific (Naperville, IL); Hydrofluor, National Diagnostics (Sommerville, NJ); cholestyramine (Questran) from Mead Johnson (Evansville, IN). Mevinolin was a generous gift from A. Alberts, Merck Sharp and Dohme (Rahway, NJ). The sources of all other materials have been described previously (4, 7).

Animals

Male Sprague-Dawley rats weighing 200 g were housed under 12 hr light–dark cycles and were fed ad libitum. Rats were fed either normal rat chow or normal rat chow supplemented with 5% cholestyramine for 2 days, followed by 5% cholestyramine and 0.1% mevinolin for 2 days. The rats were killed between the 6th and 8th hour of the dark period, the diurnal high of hepatic HMG-CoA reductase activity (7). The liver, kidney, heart, and small intestine were removed and chilled on ice in buffer A (0.25 M sucrose, 15 mM EDTA, 15 mM EGTA, 5 µM leupeptin, 5 µg/ml of aprotinin, and 0.5 mM phenylmethylsulfonylfluoride, pH 7.4). All subsequent procedures were carried out at 4°C. The small intestine was flushed with isotonic saline and its length was measured. The average length was 115 cm. Lengths of 10 cm were excised from different regions of the small intestine representing the duodenum, jejunum, and ileum and used in further studies.

Preparation of microsomes

Each organ was homogenized in buffer A (10 ml/g) at 4°C with a motor-driven, loose-fitting, glass–Teflon Potter-Elvejhem homogenizer. Cell debris and mitochondria were removed by two 15-min successive centrifugations at 16,000 g. The supernatant was then centrifuged at 100,000 g for 1 hr. The supernatant was discarded and the pellets were stored at −70°C.

Enzyme assays

HMG-CoA reductase and HMG-CoA synthase were assayed as previously described (7, 20).

Sources of antibodies

The preparation of rabbit anti-HMG-CoA reductase serum has been described (7). Cytoplasmic HMG-CoA synthase was purified from livers of rats fed cholestyramine and mevinolin (22). Antibodies raised against this latter homogeneous protein precipitated HMG-CoA synthase activity from solution and gave a single band when used in immunoblots (22). Purified HMG-CoA reductase (200 µg) or HMG-CoA synthase (500 µg) was coupled to CNBr-activated Sepharose 4B as described by the suppliers (Phar-

macia, Uppsala, Sweden). Affinity-purified antibodies were isolated from these columns; antisera was repeatedly passed over the appropriate bound antigen and the column was then washed with phosphate-buffered saline containing 0.2 M KCl (pH 7.4) until the absorbance at 280 nm was 0. Affinity-purified antibodies were then eluted in 0.1 M glycine (pH 3.0) and collected in tubes containing sufficient 0.1 M Tris (pH 8.3) to increase the pH to 7.2. These antibodies were stored at −20°C.

Immunocytochemistry: immunofluorescence

The method was essentially that of Singer et al. (18). Briefly, fresh unfixed tissues were rapidly frozen in liquid nitrogen. Five-micron sections were cut on a cryostat at −20°C, picked up on glass slides, fixed in acetone for 10 min, and air dried. The sections were then rehydrated in phosphate-buffered saline, and later incubated in 4% bovine serum albumin in the same buffer for 10 min to block any nonspecific binding. The sections were reacted with either HMG-CoA reductase antiserum or nonimmune serum (diluted 1:50) for 1 hr at room temperature in the albumin–saline buffer. The slides were washed in phosphate-buffered saline for 30 min with several buffer changes, followed by incubation of the FITC conjugated to goat anti-rabbit IgG [F(ab')$_2$ fragment specific] (1:20 dilution) in the albumin–saline buffer for 1 hr in the dark at room temperature. The slides were washed extensively in phosphate-buffered saline and the coverslips were mounted on a glycerol–phosphate–saline medium (pH 8.4) and viewed under a Nikon Optiphot microscope with an episcopic-fluorescence attachment.

Avidin–biotin–glucose oxidase complex technique (ABC-GO)

Immunofluorescence gave no signal for HMG-CoA synthase in either the liver or intestine. This result might be due either to a low titre of the antibody or to low levels of HMG-CoA synthase protein. A more sensitive technique, utilizing avidin–biotin (ABC-GO technique) was therefore used. The preparation of sections was as described above for immunofluorescence. Staining was exactly according to the procedure recommended by Vector Laboratories for their Vectastain ABC-GO kits. Sections were incubated for 18 hr at 4°C with affinity-purified anti-HMG-CoA synthase (6.8 μg/ml) or anti-HMG-CoA reductase (2.3 μg/ml). Controls were incubated with nonimmune IgG at the same protein concentration. Samples were then incubated with a biotinylated second antibody and then a preformed avidin and biotinylated glucose oxidase macromolecular complex exactly as described by Vector Laboratories.

Immunoelectron microscopy

Tissues were processed according to Roth et al. with some minor modifications (25). The samples were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer containing 0.1 M sucrose (pH 7.4) for 2 hr at 4°C. The samples were briefly rinsed in the sodium phosphate buffer before being rinsed with 0.5 M NH$_4$Cl in 0.1 M sodium phosphate and 0.1 M sucrose (pH 7.4) for 2 hr at 4°C. The samples were washed again in phosphate buffer and then dehydrated in increasing concentrations of ethanol at progressively lower temperatures until reaching 100% ethanol at −20°C. The samples were infiltrated in Lowicryl K4M–ethanol solution and stored overnight in 100% Lowicryl. Samples were placed in gelatin capsules with fresh Lowicryl and polymerized by a diffuse ultraviolet fluorescent lamp for 24 hr at −20°C and 3 days thereafter at room temperature. Tissues were also prepared for standard electron microscopy (26). Briefly the samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 2 hr at 4°C. The fixed materials were washed overnight in the sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 1 hr at 4°C. The samples were dehydrated in increasing concentrations of ethanol and propylene oxide before being embedded in an Epon–Araldite mixture. "Silver" thin sections were collected on Formvar-coated single-slot copper grids and stained with uranyl acetate and lead citrate. Samples were viewed and photographed with a Philips 300 electron microscope at 60 kV.

Immunogold labeling

Lowicryl blocks were sectioned and silver sections were collected on single-slot nickel grids that had freshly made carbon-coated Formvar films (25). The grids were floated on droplets of phosphate-buffered saline (pH 7.4) to rehydrate the section and were then incubated in the same buffer containing 4% bovine serum albumin. The sections were incubated with either the affinity-purified HMG-CoA reductase antibody or nonimmune IgG at a concentration of 65 μg/ml for 20 hr at 4°C. The grids were washed in phosphate-buffered saline droplets and then reacted with the collodial gold particles (5 nm) conjugated to goat-antirabbit IgG for 1 hr at room temperature. The grids were rinsed in phosphate-buffered saline and double-distilled water before staining with uranyl acetate.

Morphometric analysis

Morphometric analysis was determined by point-counting volumetry where $V_V = A_A = L_L = P_P$ and where V_V, A_A, L_L, and P_P represent the volume, area, line, and point, respectively (27). For each animal, three
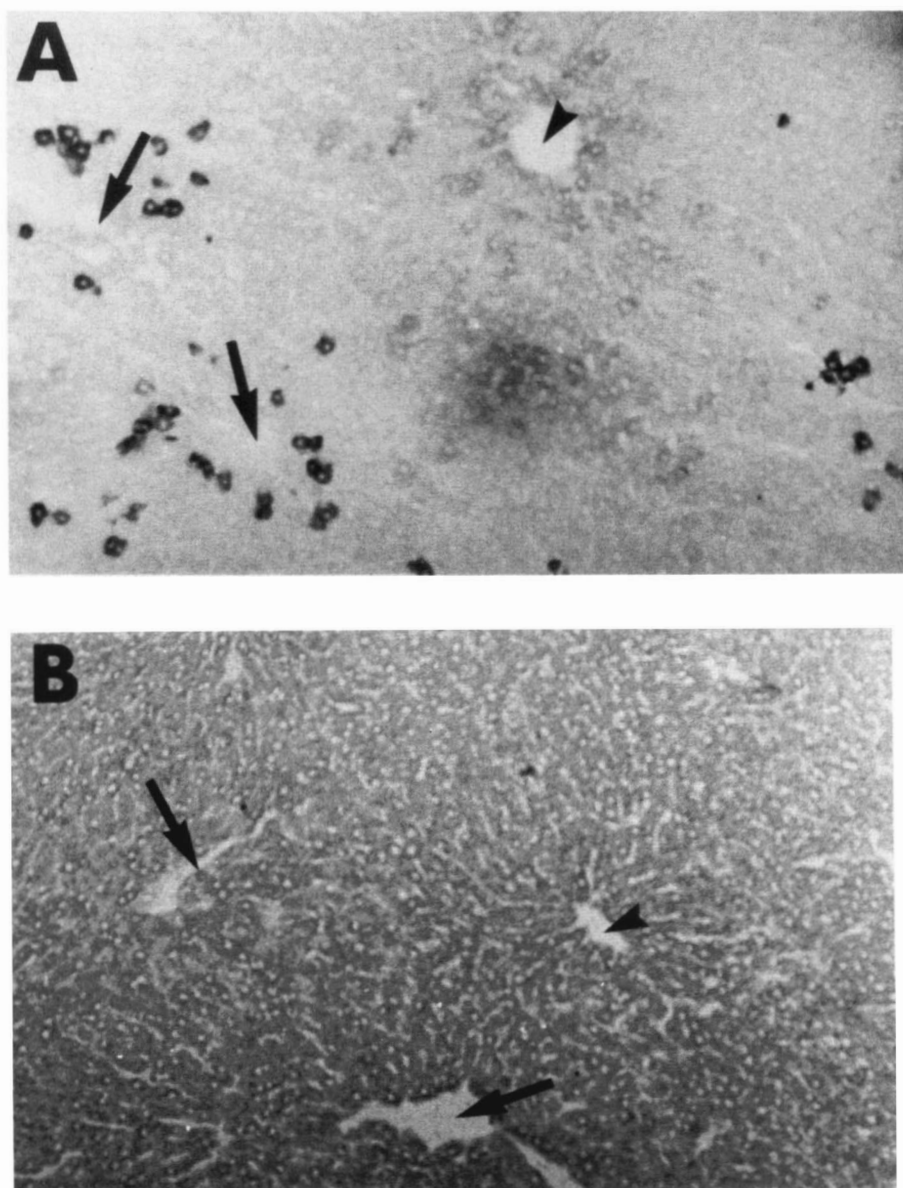
Fig. 1. Localization of hepatic HMG-CoA reductase and HMG-CoA synthase. Rats were fed a normal diet (A and C) or a diet supplemented with cholestyramine and mevinolin (B and D). Antibodies to HMG-CoA reductase (A and B) or HMG-CoA synthase (C and D) were used together with avidin–biotin–glucose oxidase to identify cross-reacting antigens. The solid arrows and the arrowheads show the periportal triads and central veins respectively. Magnification × enlargement = 98.6.

liver samples were randomly selected and a total of 30 pictures per animal at a primary magnification at 10,000 x were taken. The error of probability was $P < 0.05$.

RESULTS

Enzyme activity and immunocytochemistry

Addition of cholestyramine and mevinolin to normal rat chow resulted in increased activities of HMG-CoA reduc-

tase in the liver, duodenum, jejunum, ileum, heart, and kidney **(Table 1).** The results indicate that the two drugs induce the enzyme in a number of tissues. The liver showed the greatest increase (92-fold) with the intestinal enzyme increasing approximately 9-fold (Table 1). The heart and kidney reductase increased 3- and 15-fold, respectively. HMG-CoA synthase in the liver increased 6-fold under these conditions (20) (data not shown).

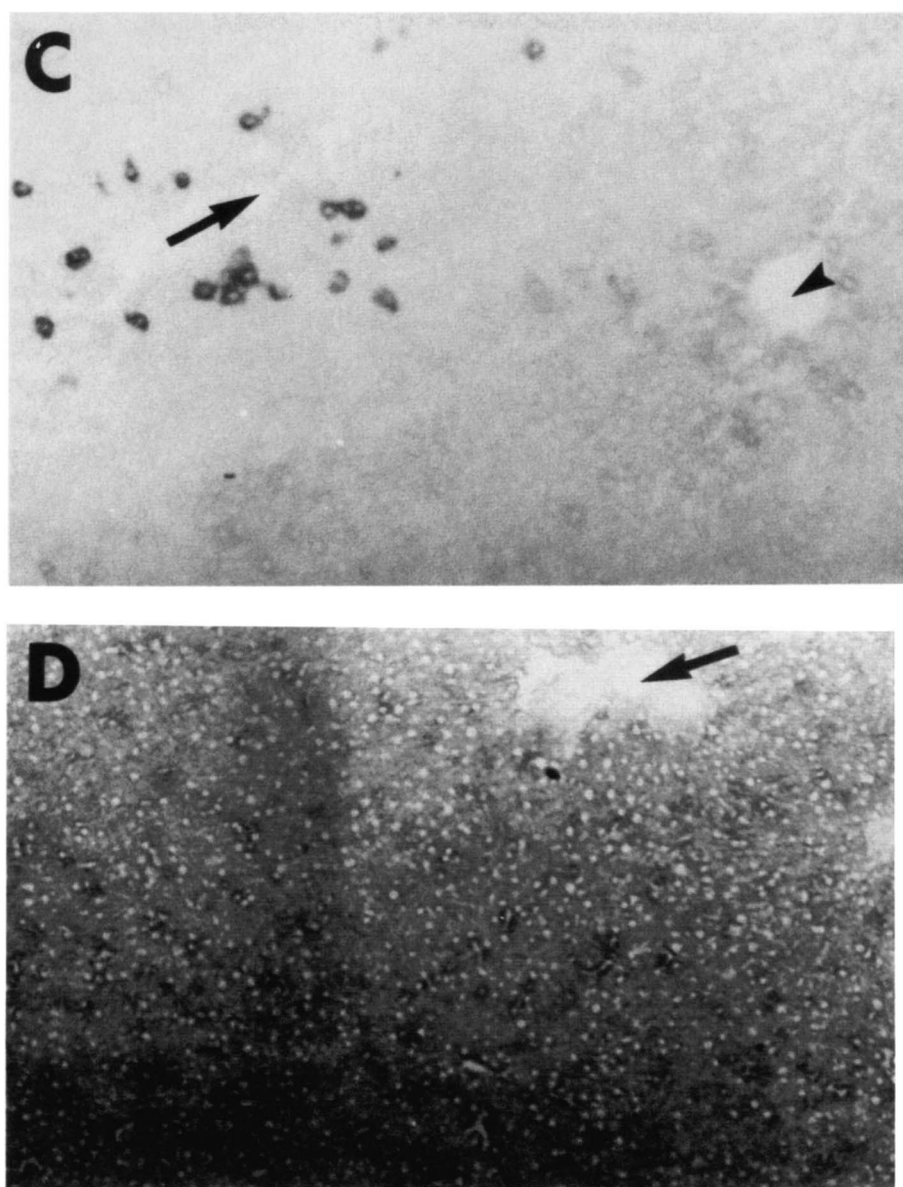In agreement with Singer et al. (18), HMG-CoA reductase in normal livers was concentrated in a few hepatocytes

Fig. 1.

that were localized in the periportal region **(Fig. 1A)**. A novel finding was that HMG-CoA synthase was also concentrated in a few cells localized in the periportal region (Fig. 1C). It is not known whether the same liver cells contain elevated levels of both HMG-CoA reductase and HMG-CoA synthase. When rats were fed cholestyramine and mevinolin, all the hepatocytes showed a strong positive signal for both HMG-CoA reductase (Fig. 1B) and HMG-CoA synthase (Fig. 1D). However, HMG-CoA synthase was still found to be unequally distributed in the liver with a few cells showing very high enzyme levels (Fig. 1D). No staining was seen when normal rabbit serum IgG was used (data not shown).

These studies demonstrate that the concentrations of both HMG-CoA reductase and HMG-CoA synthase, and hence presumably the relative rates of cholesterol biosynthesis, are not uniform in all hepatocytes of normal fed rats. The results obtained after feeding cholestyramine and mevinolin indicate that all hepatocytes are *capable* of expressing both HMG-CoA reductase and HMG-CoA synthase at high levels under the appropriate stimulus.

Preliminary results indicated that the induction of hepatic microsomal HMG-CoA reductase by cholestyramine and mevinolin was fairly specific. Microsomes isolated from control and experimental animals were analyzed on SDS-polyacrylamide gels under denaturing conditions. Analy-

TABLE 1. Effect of cholestyramine and mevinolin on the activity of HMG-CoA reductase in different tissues

| Tissue | HMG-CoA Reductase Activity | | Fold Increase |
	Normal Diet	CM Diet[a]	
	nmol mevalonate min^{-1} mg^{-1}		
Liver	0.237 ± 0.17	21.83 ± 9.0	92
Duodenum	0.012 ± 0.011	0.035 ± 0.02	2.9
Jejunum	0.012 ± 0.005	0.108 ± 0.06	9
Ileum	0.012 ± 0.009	0.117 ± 0.04	9.9
Heart	0.015 ± 0.007	0.051 ± 0.03	3.3
Kidney	0.002 ± 0.001	0.033 ± 0.03	15

Tissues were removed at the sixth hour of the dark period from either five control animals or four rats fed cholestyramine and mevinolin. Microsomes were prepared and assayed for HMG-CoA reductase activity as described in Materials and Methods.

[a] Animals were fed cholestyramine and mevinolin as described in Materials and Methods.

sis of the gels after staining for protein indicated that only one microsomal protein (HMG-CoA reductase; $M_r = 97000$) showed a significant increase in mass (data not shown).

Previous attempts to determine either the relative concentration of HMG-CoA reductase or the relative rate of cholesterogenesis in different intestinal cells have led to discrepant results (12–16). These latter published studies have relied on the physical separation of upper villi, middle villi, lower villi, and crypt cells (12–16). We have therefore used immunocytochemistry, a technique that does not require cell separation, to determine the relative concentration of HMG-CoA reductase in intestinal cells under normal and inducing conditions. In rats fed a normal diet the most HMG-CoA reductase-positive cells were in the middle and upper villus of the ileum (Fig. 2A), jejunum (Fig. 2B), and duodenum (Fig. 2C). In three different studies the order of intensity was ileum > jejunum > duodenum (Fig. 2A–C). Control rabbit antisera showed no staining (data not shown).

When rats were fed diets supplemented with cholestyramine and mevinolin, there was a significant increase in HMG-CoA reductase staining in the upper and middle villi of the ileum and jejunum (Fig. 2D,E) consistent with the increase in enzyme activity (Table 1). The ileum was the most intensely stained region of the intestine (Fig. 2D and data not shown)). Crypt cells of the ileum and jejunum showed no positive staining (Fig. 2D,E). In contrast, the crypt cells of the duodenum became intensely stained after administration of the two drugs (Fig. 2F). Under this drug regimen the villi of the duodenum did not show significantly increased levels of HMG-CoA reductase protein (compare Figs. 2C and 2F). All these drug-induced changes

in HMG-CoA reductase were observed in three separate studies. No staining was seen when normal rabbit serum IgG was used (Fig. 2G and data not shown).

All the results shown in Fig. 2 are representative of studies in which five rats on each diet were examined. For each rat, four different sections were removed from the ileum, jejunum, and the duodenum. Each section (12 per rat) was analyzed and five or six photographs were taken. Although quantitation of the staining in Fig. 2 is difficult, it is obvious that dietary cholestyramine and mevinolin both increase the signal in HMG-CoA reductase positive cells and that the distribution of the HMG-CoA reductase in villi and crypts changes between the ileum and duodenum (Fig. 2 and data not shown). The increased HMG-CoA reductase signal that is observed after cholestyramine and mevinolin administration is also consistent with increased enzyme activity (Table 1).

Immunofluorescence (Fig. 3A) and antibody–avidin–biotin (Fig. 2) showed that HMG-CoA reductase in all HMG-CoA reductase-positive villi cells was concentrated in the apical portion of the cell. Goblet cells showed no HMG-CoA reductase staining (Fig. 3A). No immunofluorescent staining was observed when normal rabbit antibody was used (Fig. 3B).

Electron microscopy

Proliferation of the hepatic smooth endoplasmic reticulum occurs when rats are fed cholestyramine and mevinolin (18) (Fig. 4A). In the current study we used electron microscopy to demonstrate that the induced smooth endoplasmic reticulum forms whorls of membrane that are located proximal to the nucleus (Fig. 4A). These whorls constitute approximately 12% of the cytoplasm of all the hepatocytes of rats fed cholestyramine and mevinolin (Table 2). We have never observed these whorls of smooth endoplasmic reticulum in the livers of rats fed a normal diet. Immunoelectron microscopy studies showed that, in rats fed cholestyramine and mevinolin, these whorls contain high concentrations of HMG-CoA reductase (Fig. 4B). As expected, the concentration of gold particles was reduced over 95% when control antisera were used (Fig. 4C) or when the anti-HMG-CoA reductase was preincubated with a purified proteolytic 52,000-dalton fragment of the intact 97,000-dalton HMG-CoA reductase (data not shown). We conclude that the presence of gold particles is a measure of the concentration and location of HMG-CoA reductase. In agreement with Keller et al. (28) we have demonstrated with immunoelectron microscopy that HMG-CoA reductase is also found in hepatic peroxisomes (data not shown). Whorls of smooth endoplasmic reticulum were not found in the intestinal villi cells of rats fed either a normal diet or diets supplemented with cholestyramine and mevinolin
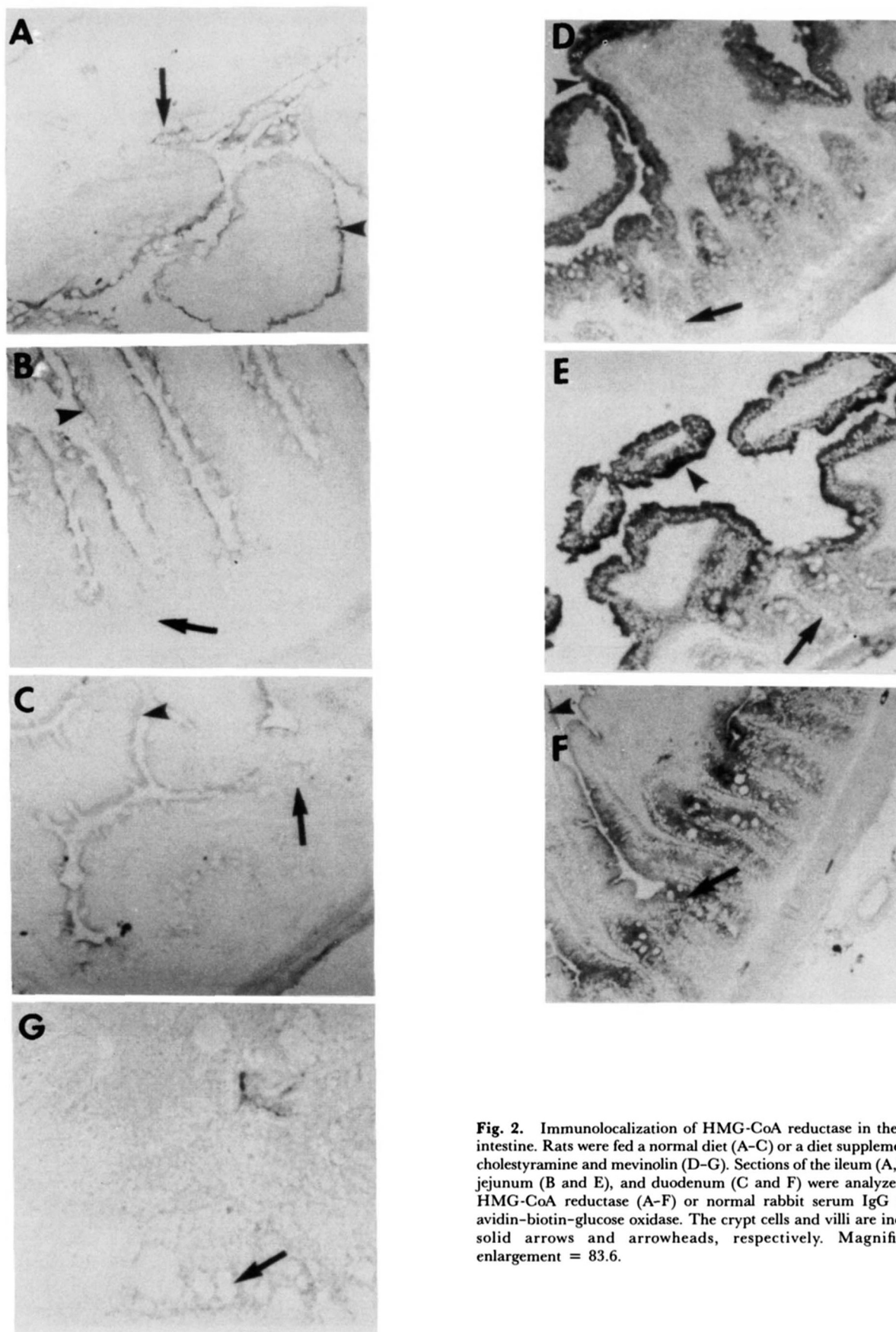
Fig. 2. Immunolocalization of HMG-CoA reductase in the rat small intestine. Rats were fed a normal diet (A–C) or a diet supplemented with cholestyramine and mevinolin (D–G). Sections of the ileum (A,D and G), jejunum (B and E), and duodenum (C and F) were analyzed by anti-HMG-CoA reductase (A–F) or normal rabbit serum IgG (G) using avidin–biotin–glucose oxidase. The crypt cells and villi are indicated by solid arrows and arrowheads, respectively. Magnification × enlargement = 83.6.
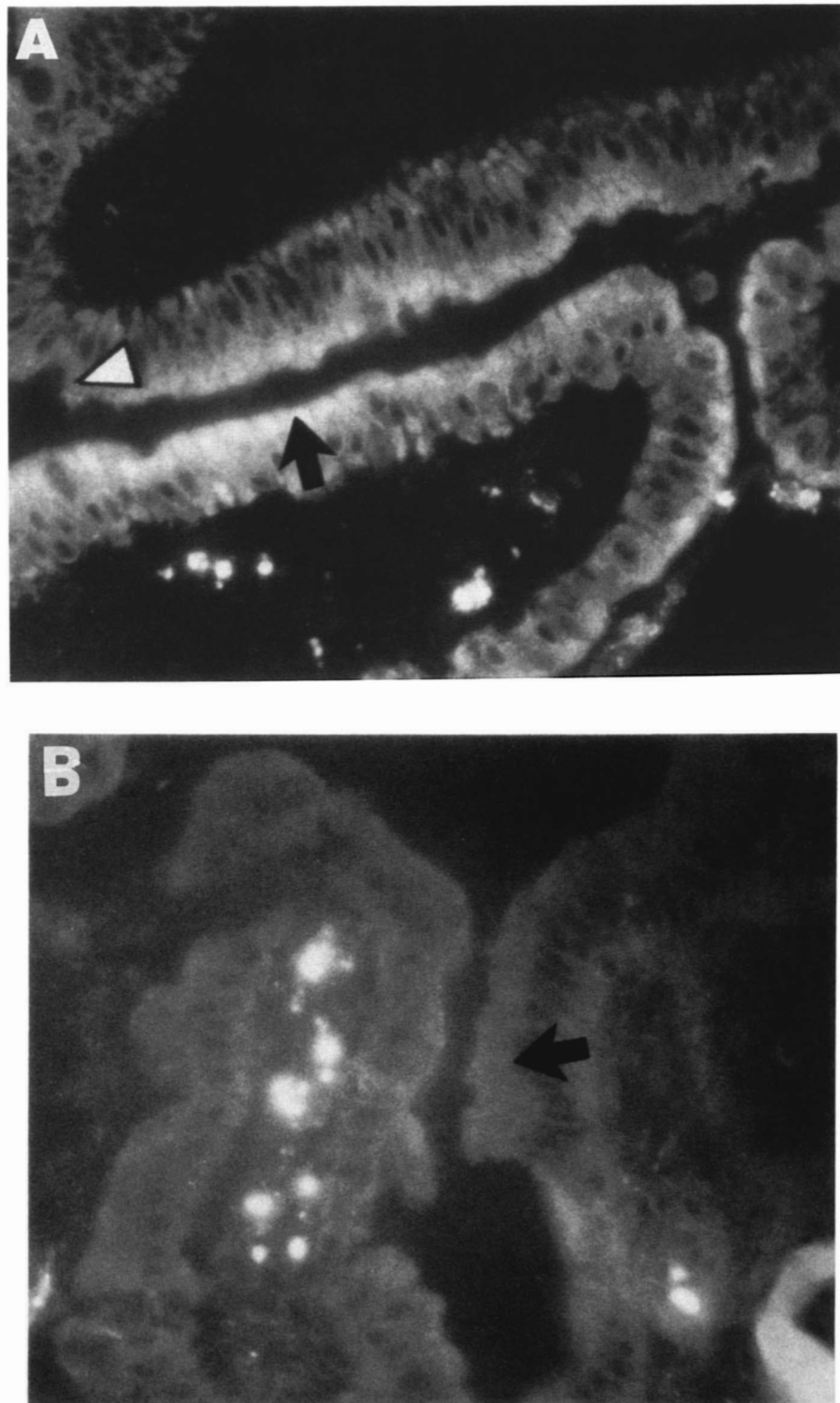
Fig. 3. Immunofluorescence microscopy of rat ileal HMG-CoA reductase. Rats were fed cholestyramine and mevino-lin. Anti-HMG-CoA reductase (A) or normal rabbit serum IgG (B) were incubated with ileal sections as described for immunofluorescent studies. Goblet and villi cells are indicated by an open arrowhead and closed arrow, respectively. Magnification × enlargement = 1020.

(data not shown). Hence the whorls of smooth endoplasmic reticulum containing HMG-CoA reductase may only occur in cells in which HMG-CoA reductase is expressed at very high levels.

Effect of mevalonolactone on both the stability of the whorls and on the distribution of hepatic HMG-CoA reductase

The appearance of either whorls of endoplasmic reticulum in rat liver (18) (Fig. 4) or crystalloid endoplasmic reticulum in UT-1 cells (24) occurs after the addition of competitive inhibitors (e.g., mevinolin or compactin) of HMG-CoA reductase. It has not yet been established whether the appearance of these membranes is either a response to the massive cellular overproduction of the HMG-CoA reductase (29, 30) so that sufficient membrane is available for the insertion of the enzyme or whether they are a result of cellular deprivation of a product, possibly cholesterol, normally derived from mevalonate. Such a product may be required for the normal transport and dispersion of these membranes away from their site of synthesis at the nuclear membrane (24).

Administration of mevalonolactone to intact rats fed the diet containing cholestyramine and mevinolin resulted, within 15 min, in a decrease in the cytoplasmic volume of the whorls from 12% to 1% (Table 2). At this time (15 min) we observed large amounts of random smooth membranes proximal to the nucleus (Fig. 5). These membranes might represent the structures that form immediately after the dissolution of the whorls of smooth endoplasmic reticulum. No whorls were apparent 45 min after treatment (Table 2). At this time the cells could not be distinguished from the cells of a normal liver when studied by electron microscopy. Presumably the membranes that originally made up the whorls were at this time dispersed throughout the cell cytoplasm. As expected, the mitochondrial volume did not change under these conditions consistent with a specific effect of mevalonolactone on cholesterogenic enzymes (Table 2).

Preliminary immunoelectron microscopy studies carried out 45 min after mevalonolactone administration to rats showed that HMG-CoA reductase was dispersed in the cell (data not shown). With this technique it was not possible to determine unequivocally whether the enzyme was still localized on smooth membranes resulting from dispersion throughout the cytoplasm of the membranes that originally made up the whorls (Fig. 5). Alternatively, the enzyme might have been in a soluble non-membrane form. In order to more clearly determine the cellular localization of HMG-CoA reductase after mevalonolactone administration and to demonstrate that the membranous whorls disappeared before significant loss of HMG-CoA reductase activity or

enzyme protein, the following experiment was performed. Mevalonolactone was administered to rats and after various times both the microsomes and cytosol were obtained. **Fig. 6A** shows that, 3 hr after mevalonolactone administration, there was greater than 80% loss of HMG-CoA reductase protein as determined by immunoblotting. The lower band in Fig. 6A results from degradation of the HMG-CoA reductase protein during processing of the samples. Densitometric scanning of the immunoblot showed that enzyme mass declined less than 40% 45 min after mevalonolactone administration (Fig. 6B). The rate of loss of microsomal HMG-CoA reductase activity and microsomal enzyme mass was similar (Fig. 6B). In two other studies both microsomal enzyme mass and activity declined less than 40%, 60 min after mevalonolactone administration (data not shown). One or 3 hr after mevalonolactone treatment the cytoplasmic volume of the smooth endoplasmic membrane whorls had declined from 11.3 ± 5.9% (n = 3), to 0% (n = 2) and 0% (n = 2), respectively. Hence the smooth endoplasmic reticulum membranous whorls have disappeared visually at a time when more than 60% of the HMG-CoA reductase is still membrane-bound and enzymatically active.

Mevalonolactone administration for up to 3 hr did not result in solubilization of HMG-CoA reductase since the liver cytoplasm (100,000 g supernatant) contained less than 0.1% of the HMG-CoA reductase mass or activity. In preliminary immunoelectron microscopy studies, no evidence for an increase in peroxisomal HMG-CoA reductase was observed after mevalonolactone administration. We conclude that mevalonic acid administration leads to dissolution of the membranous whorls but that the enzyme initially remains bound to the membranes which become dispersed throughout the cytoplasm.

Since the cytoplasmic volume occupied by the whorls of membrane is reduced by over 90% within 15 min and 100% by 60 min of mevalonolactone treatment at a time when there is less than a 40% decline in total HMG-CoA reductase activity or enzyme mass (Fig. 6), we conclude that the accumulation of the whorls of endoplasmic reticulum (Fig. 4) is a result of acute cellular deprivation of a product, possibly cholesterol, derived intracellularly from mevalonate. The finding that administration of mevalonolactone leads to the rapid biosynthesis of sterols and to a rapid dissolution of the whorls (Table 2; Fig. 5) is consistent with this hypothesis.

Previous biochemical studies have shown that administration of mevalonate leads to increased degradation of HMG-CoA reductase (31) by a pathway that appears in part to be lysosomal (32). However, in the current immunoelectron microscopic studies we were unable to observe immunoreactive HMG-CoA reductase in lysosomes or autophagic vacuoles nor was there any increase in the
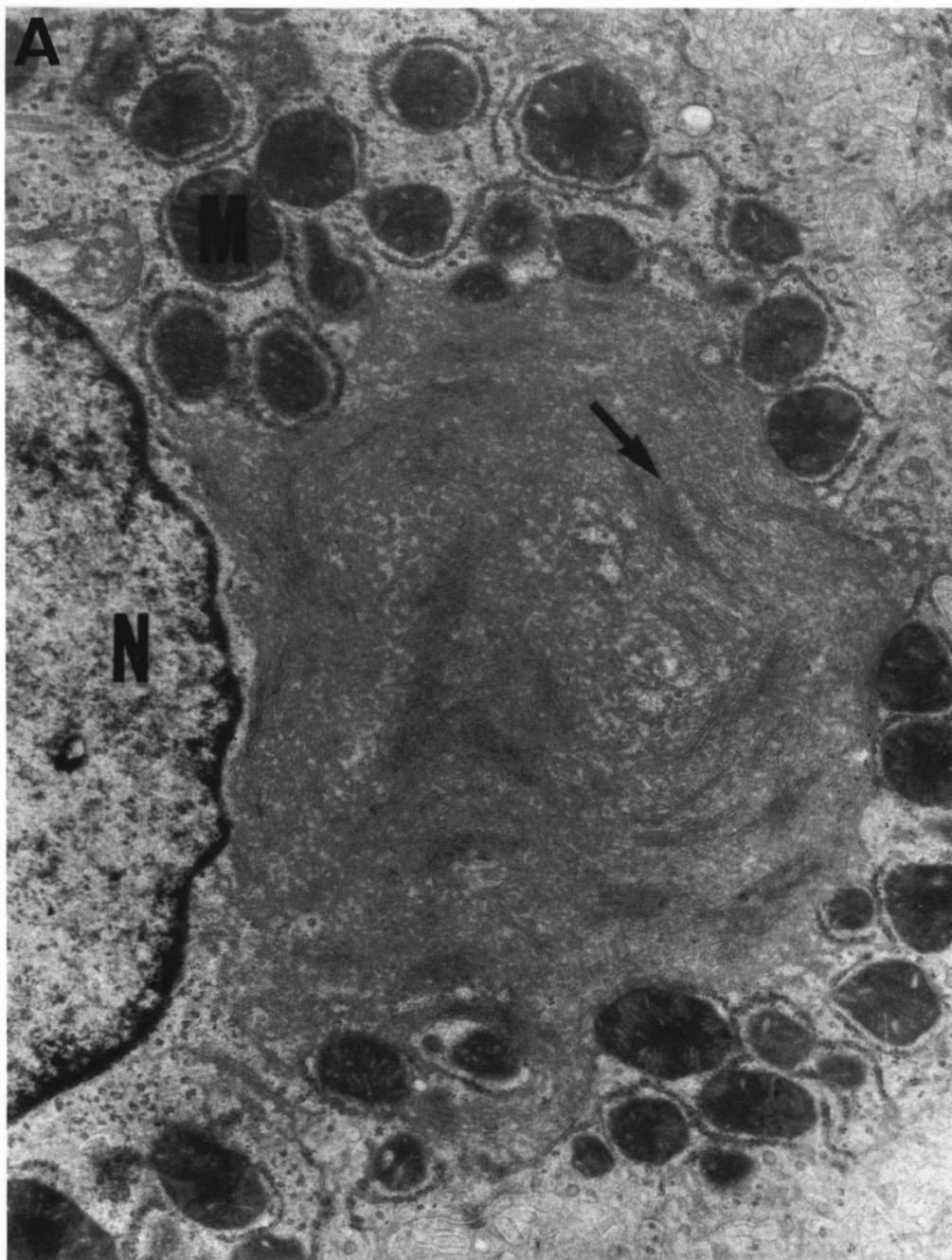
Fig. 4.

number of autophagic vacuoles following mevalonate administration (data not shown). The exact mechanism(s) involved in degradation of HMG-CoA reductase remains unknown.

We also used immunofluorescence to determine whether administration of mevalonolactone to rats fed cholestyramine and mevinolin was equally effective in reducing the level of HMG-CoA reductase protein in all hepatocytes. When animals were fed cholestyramine and mevinolin, all hepatocytes showed a strong immunofluorescent signal **(Fig. 7A)**. No effect of the mevalonolactone on liver immunofluorescence was observed after 15 min since all hepatocytes still showed a strong HMG-CoA reductase-positive signal (data not shown). After 45 min a few cells in the periportal region had significantly lower immunofluorescence (Fig. 7B). After 3 hr a large number of cells in the periportal region no longer were positive for HMG-CoA reductase (Fig. 7C). However, large number of hepato-
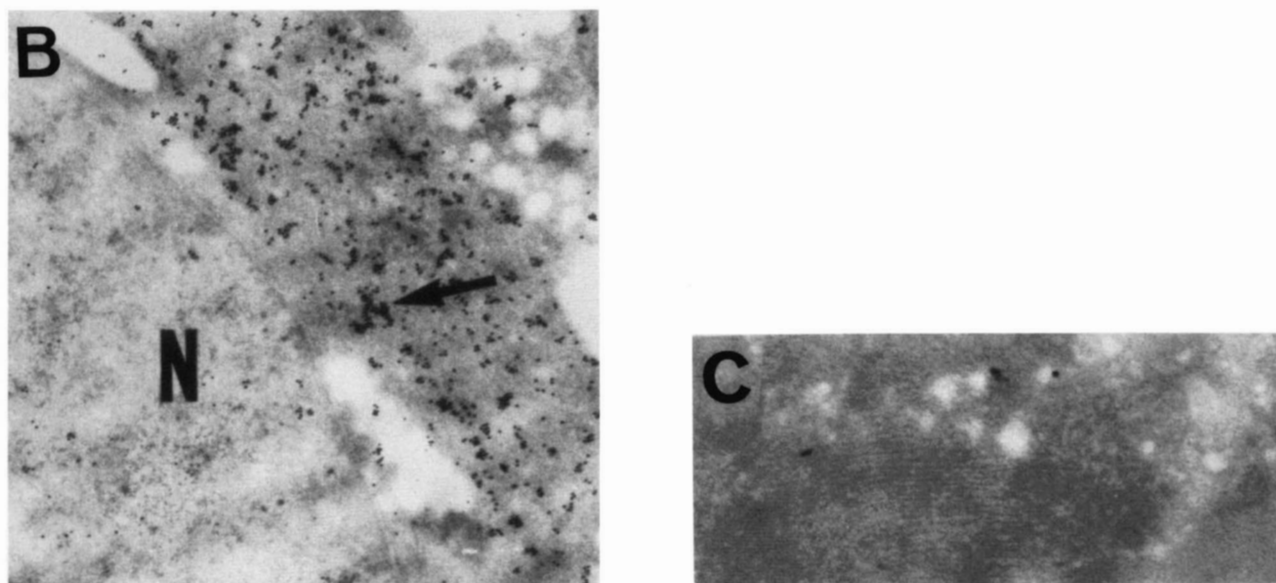
Fig. 4. Electron and immunoelectron microscopic analysis of the livers of rats fed cholestyramine and mevinolin. A: The whorls of smooth endoplasmic reticulum (solid arrow) adjacent to the nucleus (N) are shown. Mitochondria (M) are also indicated. B: Immunoelectron microscopy using antigen-purified antibody to HMG-CoA reductase followed by a colloidal gold adduct (solid arrow) of goat anti-rabbit IgG is shown. The nucleus is indicated (N). C: Immunoelectron microscopy of membrane whorls using nonimmune serum. Magnification × enlargement = 20,714 (A) and 24,743 (B, C).

cytes distal to the periportal region still showed strong positive HMG-CoA reductase fluorescence 3 hr after mevalonolactone administration (Fig. 7C) indicating that the effect of mevalonolactone on HMG-CoA reductase levels is not equal throughout the liver. The enzyme in these latter HMG-CoA reductase-positive cells must be present in membranes that are not organized into whorls of smooth endoplasmic reticulum since such whorls are not present in the livers of animals treated with mevalonolactone for 45 min (Table 2), 1 hr, or 3 hr (data not shown).

DISCUSSION

We used immunocytochemistry and immunofluorescence to demonstrate that specific liver cells located periportally contain high concentrations of HMG-CoA reductase and HMG-CoA synthase. The majority of the liver cells of rats fed a normal diet contain, according to these assays, no detectable HMG-CoA reductase or HMG-CoA synthase. Singer et al. (18) have previously reported on a similar pattern of rat liver staining for HMG-CoA reductase. In the latter studies, approximately 27% of the hepatocytes were positive for HMG-CoA reductase. Taken together, these results suggest that in specific periportal hepatocytes of normal fed rats, the coordinately regulated enzymes HMG-CoA synthase and HMG-CoA reductase (21, 22) are in-

duced by normal physiological processes to levels significantly greater than in the majority of the hepatocytes. An interesting question raised by the current study is whether, in normal rats, the specific periportal cells that show high levels of HMG-CoA reductase and HMG-CoA synthase might be preferentially involved in lipoprotein or bile acid biosynthesis.

Dietary cholestyramine and mevinolin led to increased activity and mRNA levels of HMG-CoA reductase and HMG-CoA synthase (20, 22). Under these conditions all hepatocytes expressed both enzymes at high levels (Fig. 1). Mevinolin would be expected to inhibit HMG-CoA reductase activity and hence cholesterogenesis. However, the increased activity of HMG-CoA synthase that is observed

TABLE 2. Effect of mevalonolactone treatment on the hepatic volume of both the closely packed, flattened cisternae of smooth endoplasmic reticulum whorls and mitochondria

Treatment[a]	Percent of Total Cytoplasmic Volume	
	Mitochondria	Whorls
None	20.7 ± 2.6	12.4 ± 2.4
Mevalonolactone, 15 min	18.1 ± 2.2	1.0 ± 0.04
Mevalonolactone, 45 min	24.1 ± 3.2	0

[a]Rats were fed cholestyramine and mevinolin and then given mevalonolactone (1 mg/g body weight) 15 or 45 min before they were killed.
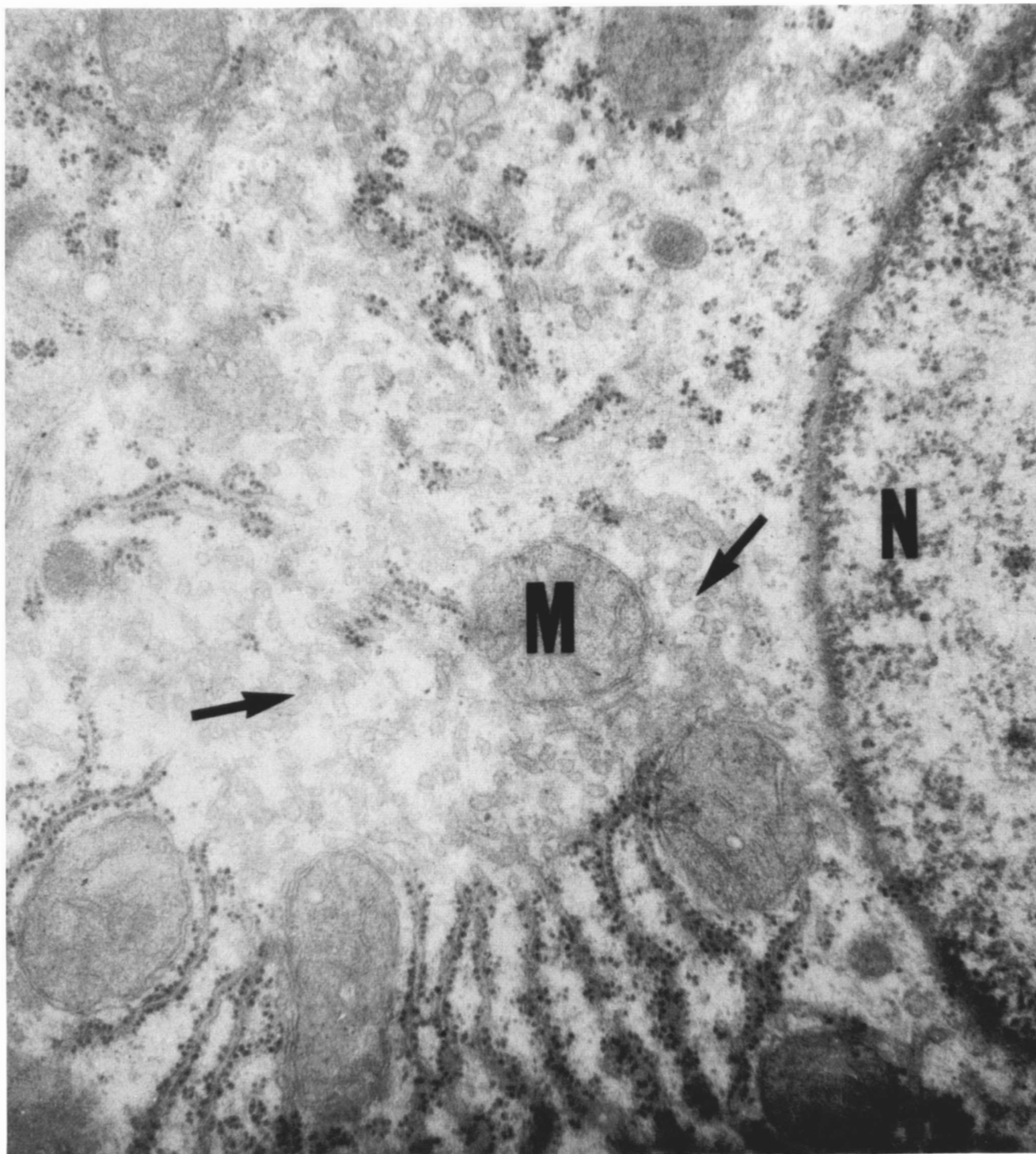
Fig. 5. Electron microscopic appearance of rat liver 15 min after mevalonolactone (1 mg/g body weight) was given to a rat fed a diet supplemented with cholestyramine and mevinolin. The arrows indicate excessive smooth endoplasmic reticulum adjacent to the nucleus (N). M indicates mitochondria. Magnification × enlargement = 74,454.
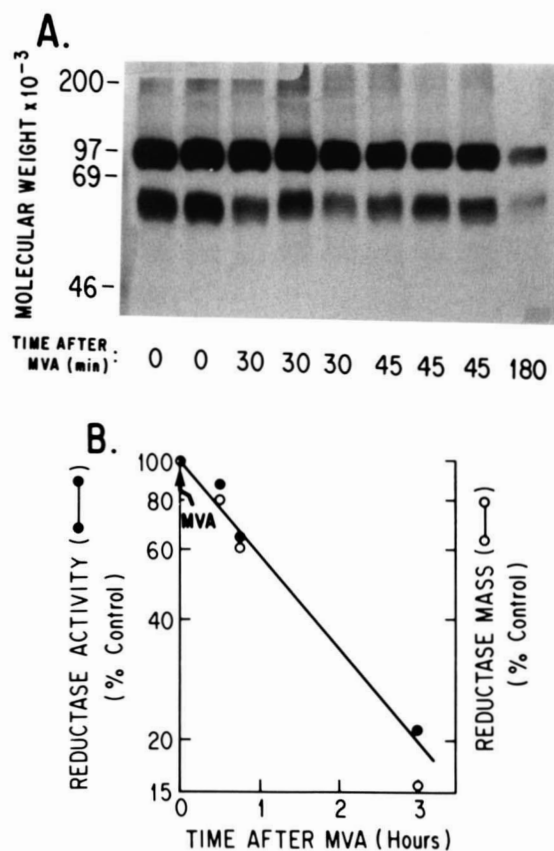
Fig. 6. Effect of mevalonolactone administration on hepatic microsomal HMG-CoA reductase. Rats were fed cholestyramine and mevinolin and then given mevalonolactone (MVA) (1 mg/g body weight) at D6. Hepatic microsomes (100 μg) from each rat were analyzed by immunoblot (30). The undegraded HMG-CoA reductase protein has an $M_r = 97,000$. The smaller protein is a proteolytic fragment of HMG-CoA reductase. B: Changes in HMG-CoA reductase mass were determined from densitometric scanning of (A) after shorter exposure times. Microsomal enzyme activities were measured as described in Materials and Methods.

(20–22) might result in the biosynthesis of sufficient HMG-CoA to compete with mevinolin for the active site on HMG-CoA reductase (21). Such a competition could lead to the production of small amounts of mevalonate and derived isoprenoids.

Like the liver, the intestinal epithelial cells do not all contain equivalent amounts of HMG-CoA reductase. In the normal fed animal there is a gradient of enzyme both down the villus–crypt axis and in the intestine with ileum > jejunum > duodenum. We were unable to localize HMG-CoA synthase in intestinal cells, possibly because of a low concentration of this soluble enzyme.

Previous studies have reported on the relative rates of cholesterol synthesis or HMG-CoA reductase activity down the villus–crypt axis and in different parts of the intestine (12–16). However, these studies have been contradictory. In

some studies the highest HMG-CoA reductase activity or rate of cholesterogenesis was reported to be in either the crypt cells (12, 14) or lower villus (13) with the lowest levels in the upper villi (12–14). In contrast Muroya, Sodhi, and Gould (15) found no difference between villus and crypts and Merchant and Heller (16) reported that both HMG-CoA reductase activity and the rate of cholesterogenesis were highest in the villus and lowest in the crypt cells. These different results may have resulted from the difficulties associated with physically separating and isolating different intestinal epithelial cells.

The current studies are not complicated by techniques involving separation of different cell types. The present results indicate that in the normal fed rat the upper villi cells contain the highest levels of HMG-CoA reductase. This is in agreement with the work of Merchant and Heller (16) and the recent studies of Singer et al. (17). The current studies, in agreement with those of Singer et al. (17), demonstrate that the enzyme is strikingly concentrated in the apical region of the villus cells. Such an observation is consistent with the concentration of endoplasmic reticulum in the apical portion of these cells (33).

Addition of cholestyramine and mevinolin to the diet led to a striking increase in the concentration of HMG-CoA reductase in the middle and upper villi of the ileum and jejunum. In contrast, these drugs induced HMG-CoA reductase in the crypt cells of the duodenum. The reasons for these differences are not readily apparent since in the normal fed rat the villus cells were the only HMG-CoA reductase-positive cells in all regions of the small intestine (Fig. 2).

Cholestyramine and mevinolin are hypocholesterolemic agents (9, 10). Liver and intestine are the major organs involved in cholesterol and lipoprotein biosynthesis (11). The current studies and those of Singer et al. (17, 18) demonstrate that these drugs have profound effects on both hepatic and extrahepatic tissues. Nonetheless, the magnitude of the increase in HMG-CoA reductase activity is greatest in the liver and it is this organ and not the intestine that responds by inducing stacks of endoplasmic reticulum forming whorls in which HMG-CoA reductase is incorporated. In vivo this regular pattern of tubules stacked on top of each other to form whorls is seen in normal steroidogenic tissue of the adrenal cortical cells (34) and testicular interstitial cells (35) of fetal guinea pigs. Neaves (36) reported that the formation of such membrane stacks was related to the levels of plasma testosterone in the breeding season of rock hyrax. It is not known whether these membranes in steroidogenic tissues contain high levels of HMG-CoA reductase.

The rapid dissolution of the hepatic whorls of smooth endoplasmic reticulum within 15 min of mevalonolactone
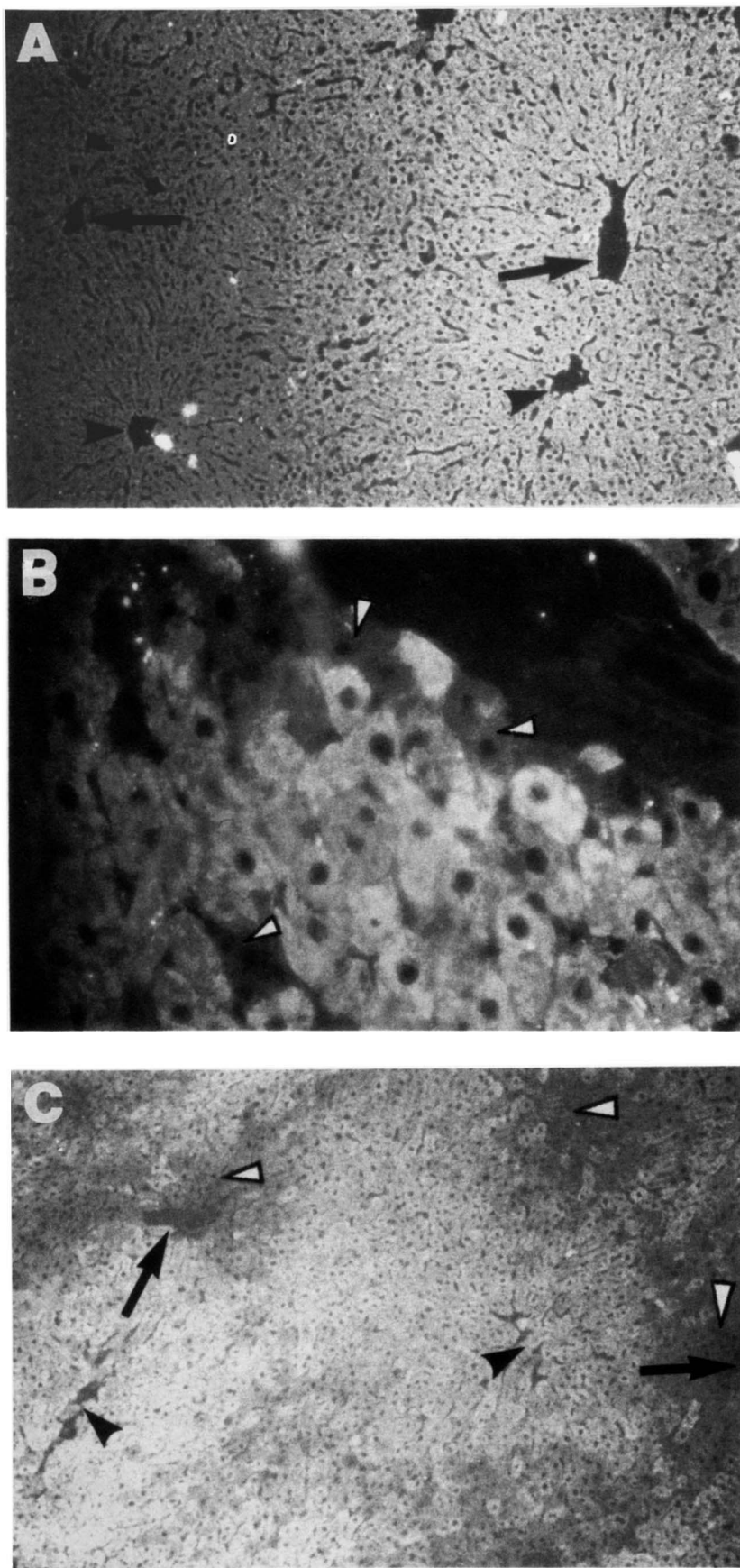
Fig. 7. Effect of mevalonolactone on the hepatic localization of HMG-CoA reductase. Rats were fed cholestyramine and mevinolin (A) and then given mevalonolactone (1 mg/g body weight) by stomach intubation 45 min (B) or 3 hr (C) before the livers were removed and analyzed for HMG-CoA reductase by immunofluorescence. The periportal is indicated by a solid arrow and the central vein by a solid arrowhead. Areas showing reduced immunofluorescence are indicated by open arrowheads. Magnification × enlargement: 84.8 (A,C) and 847.6 (B).

administration (Table 2, Fig. 5) under conditions where there is little change in the mass of HMG-CoA reductase (Fig. 6) indicates that these membranes may accumulate because of deprivation of a product derived from mevalonate. Orci et al. (23) have previously studied the stability of crystalloid endoplasmic reticulum in UT-1 cells, a cell line with multiple copies of the HMG-CoA reductase gene (37). Orci et al. (23) showed that delivery of cholesterol, in low density lipoproteins, to these cells resulted in a loss of HMG-CoA reductase activity after 4 to 8 hr that was followed, after 8 to 24 hr, in a loss of the crystalloid endoplasmic reticulum. It was proposed that the UT-1 cells may accumulate their crystalloid endoplasmic reticulum membranes because of a lack of cholesterol in these membranes (23). The current studies demonstrate that the ordered whorls of stacked membranes are extremely sensitive to mevalonolactone, or a product derived from this compound. The rapid dissolution of the whorls occurs in the absence of any large change in HMG-CoA reductase and at a time when many hepatocytes are still HMG-CoA reductase-positive as judged by immunofluorescence (Fig. 7). Hence, our studies are consistent with the proposal that cholesterol is required for the normal transport and movement of endoplasmic reticulum containing HMG-CoA reductase away from its site of synthesis at the nucleus (23, 24). Our studies are not consistent with the proposal that the membrane whorls form in order to accommodate the large amounts of HMG-CoA reductase produced either by dietary cholestyramine and mevinolin (7) or as a result of gene amplification and expression of HMG-CoA reductase (24, 37). ∎

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