

In Vivo Effect of Cholesterol Feeding on the Short Term Regulation of Hepatic Hydroxymethylglutaryl Coenzyme A Reductase during the Diurnal Cycle*

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Light-dark-cycled rats were fed a 3% cholesterol-supplemented diet at the beginning of the dark phase. Cholesterol-fed and control animals were taken at intervals throughout the following 12 h and the microsomal and solubilized hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase was isolated. Immunotitrations of this microsomal and solubilized enzyme were performed with a monospecific antibody to 3-hydroxy-3-methylglutaryl coenzyme A reductase. In contrast to the specific activity of the enzyme, which differs extremely during the diurnal cycle, the immunotitrations obtained from cholesterol-fed and control animals, yielded in identical antisera equivalence points. On the other hand, when the enzyme was phosphorylated *in vitro*, the antisera equivalence points corresponded to the alterations of the specific activity. In contrast to the results published by Higgins and Rudney ((1973) *Nature New Biol.* 246, 60-61), our data prove that even the *in vivo* short term changes in enzyme activity are due to changes in the quantity of enzyme rather than to a modulation of the catalytic activity.

The activity of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), the rate-limiting enzyme in cholesterol biosynthesis in liver (for review, see Ref. 1), can be regulated by a variety of physiological conditions including ingestion of cholesterol and the diurnal cycle. In general, the activity of HMG-CoA reductase¹ may be regulated by the rate of synthesis or degradation of enzyme protein or by reversible alterations of the active site of the enzyme. The very rapid turnover time of the enzyme, 2 to 4 h (2), would enable quite fine and rapid control to be exerted by regulation of the enzyme synthesis and degradation, as argued by Mitropoulos and Venkatesan (3). Since Beg *et al.* (4) have reported that hepatic HMG-CoA reductase was inactivated *in vitro* when microsomes were incubated with cytosol in the presence of ATP and Mg²⁺, it has been tried intensively to show the modulation of HMG-CoA reductase by phosphorylation-dephosphorylation to be a mechanism working *in vivo* too (5, 6). But this point has to be further elucidated. There are many authors, on the other hand, who have demonstrated that HMG-CoA reductase is modulated *in vivo* in different nutritional states and, at the nadir and peak of the diurnal rhythm, by changes in the quantity of the enzyme and not be altera-

tions of the catalytic activity (7-9). So, Beg *et al.* argue that long term physiological modulation involves changes in the quantity of the enzyme, whereas short term regulation involves reversible phosphorylation (5). The studies of Higgins and Rudney concerning both the diurnal cycle and the cholesterol feeding also reveal two effects modulating HMG-CoA reductase: an immediate inhibition of activity independent of protein synthesis and a subsequent inhibition of protein synthesis (10).

We present data using immunotitrations with a monospecific antibody to HMG-CoA reductase which provide evidence that even short term *in vivo* regulation after ingestion of cholesterol-supplemented diet as well as changes of enzyme activity during the diurnal cycle are due to a change of the quantity of enzyme protein only.

MATERIALS AND METHODS

Animals—Female Sprague-Dawley rats used for isolation of HMG-CoA reductase were housed for at least 10 days in a light-controlled room (dark cycle 3:00 a.m. to 15:00 p.m.). Approximately same aged animals (150 to 200 g) were kept on standard diet or at the beginning of the dark phase on a 3% cholesterol-supplemented standard diet.

Preparation of Microsomes—Excised livers were washed in ice-cold homogenization medium containing 10 mM mercaptoethanol, 300 mM sucrose, and homogenized in the same medium using 1 g of liver/2-ml solution for 15 s in a Waring blender, followed by 10 strokes with a motor-driven Teflon pestle in a Potter-Elvehjem-type glass homogenizer. The homogenate solution was diluted 2-fold with homogenization medium and centrifuged at 0 °C for 20 min at 12,000 × g, and the resulting supernatant was centrifuged at 0 °C for 60 min at 100,000 × g. The microsomal pellets were suspended in washing solution (1 ml/g of liver) containing 40 mM KH₂PO₄, 1 mM EDTA, 5 mM dithioerythritol, 200 mM sucrose, pH 7.5, recentrifuged at 0 °C for 60 min at 100,000 × g, and were stored in the centrifuge tube in liquid N₂ until needed. Microsomes from an average of 1.5 rat livers occupied each ultracentrifuge tube.

Solubilization and Purification of the Solubilized Enzyme—Microsomes were thawed at room temperature for 40 min, (all further operations were carried out at room temperature unless specified otherwise) and homogenized in buffer (1 ml/microsomal pellet) containing 100 mM KH₂PO₄, 100 mM KCl, 10 mM EDTA, 10 mM dithioerythritol, 50% glycerol, pH 7.2. This homogenate solution was incubated for 60 min at 37 °C. In order to get the solubilized enzyme, the solution was diluted 3-fold with medium containing 100 mM KH₂PO₄, 100 mM KCl, 10 mM EDTA, 10 mM dithioerythritol, pH 7.2, homogenized once more, and centrifuged at 100,000 × g for 60 min. The supernatant was removed and the protein precipitating between 35% and 50% ammonium sulfate was solvated in 0.5 ml of buffer containing 100 mM KH₂PO₄, 100 mM KCl, 10 mM EDTA, 10 mM dithioerythritol, 36% glycerol, pH 7.2/microsomal pellet. This solution was heated for 8 min at 65 °C and centrifuged at 100,000 × g for 30 min. The supernatant was separated from denatured protein, glycerol was added to give a final concentration of 50% and was stored at -20 °C until needed without significant loss of activity. This solution was diluted 10-fold with dilution buffer which contained 50 mM KH₂PO₄, 100 mM sucrose, 10 mM EDTA, 10 mM dithioerythritol, pH 7.2. The

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¹ The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; SDS, sodium dodecyl sulfate.

diluted enzyme was applied to a Blue Sepharose-CL-6B column (5 mg of protein/ml column volume), equilibrated with chromatographic buffer A containing 50 mM KH_2PO_4 , 100 mM sucrose, 10 mM EDTA, 10 mM dithioerythritol, 5% glycerol, pH 7.2. The column was washed with this chromatographic buffer A and then eluted with a linear gradient of KCl from 0 to 1 M in buffer A. All fractions containing more than 40 units of enzyme activity were pooled and dialyzed against chromatographic buffer B containing 40 mM KH_2PO_4 , 4 mM EDTA, 5 mM dithioerythritol, pH 7.0. The dialyzed material was applied to a HMG-CoA-Agarose-Hexan column (5 to 10 mg of protein/2 ml column volume) and equilibrated with buffer B. The column was washed with buffer B containing 50 mM KCl, followed by the elution of HMG-CoA reductase with buffer B containing 50 mM KCl and 200 μM DL-HMG-CoA. The HMG-CoA reductase was dialyzed against 0.05% NaCl, lyophilized, and stored at -20°C .

Antibody Production—Rat liver HMG-CoA reductase was purified to homogeneity by the method described above. Three intramuscular injections (0.05 mg of protein) at 2-week intervals were given to a rabbit, the first injection as an emulsion with complete, the other two with incomplete Freund's adjuvant. Blood was withdrawn after 8 weeks of the initial injection, was allowed to clot, and the crude antiserum was used for immunotitrations. A control, nonimmune serum was made from blood which was withdrawn from the same rabbit just before the enzyme injection.

In Vitro Inactivation of HMG-CoA Reductase—Inactivation of the enzyme was performed according to the method of Kleinsek *et al.* (7). Briefly, the first 100,000 $\times g$ microsomal pellet was recovered from standard diet-fed rat liver, as described above, and was homogenized in buffer containing 100 mM KH_2PO_4 , 100 mM KCl, 1 mM EDTA, 10 mM dithioerythritol, pH 7.0, at a protein concentration of about 40 mg/ml. This microsomal suspension was made up to 5 mM ATP, 10 mM MgCl_2 , 50 mM KF, and incubated for 60 min at 37°C with 1 ml of 100,000 $\times g$ supernatant (cytosol)/1.5 ml of microsomal suspension.

Immunotitration—Microsomes were thawed and suspended at 0°C in 100 mM KH_2PO_4 , 100 mM KCl, 1 mM EDTA, 10 mM dithioerythritol, pH 7.0. For immunotitration of the solubilized enzyme, the thawed microsomes were suspended and incubated as mentioned above. Immunotitrations were carried out using constant amounts of enzyme activity and increasing amounts of antibody. The equivalence point is defined as the number of enzyme units inactivated by 1 μl of antibody. There was no neutralizing activity of antiserum presorbed against purified HMG-CoA reductase. This demonstrates that non-specific cross-reactions do not occur or interfere with the antigen-antibody reaction (data not shown). The control antiserum likewise had no neutralizing activity.

Assay of the HMG-CoA Reductase—The assay was performed according to the method of Shapiro *et al.* (17). The enzyme solution was preincubated for 20 min at 37°C in a total of 230 μl of assay buffer containing 435 mM KH_2PO_4 , pH 7.2, 43.5 mM EDTA, 43.5 mM glucose 6-phosphate, 4.35 mM NADP, 6.5 units of glucose-6-phosphate dehydrogenase, 10.4 mM dithioerythritol, 0.1 mg of bovine serum albumin. The enzyme reaction was started by adding 20 μl of substrate solution containing 1 mM DL-HMG-CoA, 7.7 μM DL-3-hydroxy-3-methyl[3- ^{14}C]glutaryl coenzyme A (51.3 mCi/mmol). After incubation for 20 min at 37°C , 100 μl of a mixture containing 750 mM mevalonolactone and 200 mM HMG-acid were added and then 100 μl of 6 M H_2SO_4 . The mixture was incubated for 10 min at 50°C to complete lactonization. After centrifugation to remove the protein, mevalonolactone and HMG-acid were separated by thin layer chromatography in benzene-acetone (1:1) on Kieselgel plates (45 g of Kieselgel HF suspended in 95 ml of H_2O , spread 0.5 mm on glass plates, dried, and activated at 100°C for 60 min) using an aliquot of the clear supernatant. Mevalonolactone appeared as a light spot on the plate upon illumination with UV light (360 nm). The area was scraped out and counted in dioxane scintillation solution. At a substrate concentration of 95.5 μM , the enzyme assay in all cases was linear with respect to time and to protein concentration (in the range used) and the substrate conversion did not exceed 20%. Protein was determined by the method of Lowry *et al.* (12). One unit of enzyme is defined as the amount of protein which converts 1 nmol of HMG-CoA to mevalonic acid/min.

RESULTS AND DISCUSSION

A typical purification of hepatic microsomal HMG-CoA reductase is summarized in Table I. The specific activity of the purified enzyme from four different preparations was

23,000 $\pm$ 6,000 nmol/mg $^{-1}$ min $^{-1}$. The homogeneity was checked by both the polyacrylamide gel electrophoresis with and without SDS (Fig. 1). The antiserum produced was characterized by Ouchterlony double diffusion analysis. The antiserum was monospecific when tested against enzyme on different purification steps and there was a reaction of identity (Fig. 1). Microsomes of light-dark-cycled rats fed a standard diet or a 3% cholesterol-supplemented standard diet at the beginning of the dark phase were isolated at 2-h intervals throughout the 12 h of the dark phase. The course of the

TABLE I

Purification of HMG-CoA reductase from rat liver microsomes

Hepatic microsomes were prepared from 90 rats killed at midpoint of the 12-h dark cycle. Details of the purification procedure are described under "Materials and Methods."

Purification step	Protein mg	Specific activity nmol/mg $\times$ min	En- zyme units nmol/ min	Yield %
Microsomes	8225	0.68	5593	100
Solubilized enzyme	1160	4.13	4790	86
$(\text{NH}_4)_2\text{SO}_4$ precipitate	311	9.50	2954	53
Heat-treated enzyme	89.4	29	2592	46
Blue Sepharose pool	2.3	509	1170	21
HMG-CoA-agarose pool	0.03	18,429	553	10

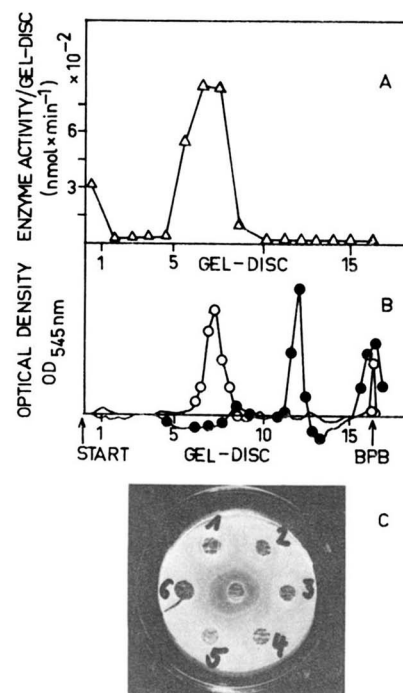


FIG. 1. Purified rat liver HMG-CoA reductase. Polyacrylamide gel electrophoresis was performed according to the method of Maizel (16). In A, 8 μg of enzyme was electrophoresed on polyacrylamide gel electrophoresis without SDS, the gel was sliced in 5-mm segments and assayed for reductase activity. In B, 8 μg of enzyme was electrophoresed on polyacrylamide gel electrophoresis with (●—●) and without (○—○) SDS. Staining was performed with 0.2% Coomassie blue, and the optical density was recorded with a microdensitometer. The position of bromophenol blue (BPP) is shown. The enzyme preparation shows co-migration of enzyme activity with protein. The single staining protein band has R_F values of 0.45 for polyacrylamide gel electrophoresis without SDS and 0.71 for polyacrylamide gel electrophoresis with SDS. In C, Ouchterlony double diffusion analysis of an antiserum prepared against purified HMG-CoA reductase is shown. The center well contains 20 μl of antiserum; well 1, 1 unit of microsomal enzyme; well 2, 1.5 units of solubilized enzyme; well 3, 0.6 unit of $(\text{NH}_4)_2\text{SO}_4$ fraction; well 4, 3.5 units of heat-treated enzyme; wells 5 and 6, 4 and 0.1 unit of purified enzyme.

specific activity of the microsomal and solubilized HMG-CoA reductase is outlined in Fig. 2. In cholesterol-fed rats, the enzymic activity declines rapidly and is depressed compared to the standard diet group between 2 and 4 h. The enzymic activity of the control animals fed a standard diet shows the characteristic pattern of the diurnal rhythm. In contrast to the results of Higgins and Rudney (10), immunotitrations of the microsomal and the solubilized enzyme of the controls and cholesterol-fed animals resulted in identical equivalence points (Fig. 3, Table II). So, both the increase and the decrease of reductase activity is the result of increased and decreased number of reductase molecules.

On the other hand, if reductase is inactivated *in vitro* by ATP/Mg²⁺, the equivalence point will be lowered considerably and will correspond well with the loss in enzyme activity (Table III). As argued by Kleinsek *et al.* (7), this indicates that the inactivated HMG-CoA reductase retains the binding domain(s) for antibody interaction, so that it is possible to distinguish whether the enzyme is regulated by the phosphatase-kinase short term regulation *in vivo*. Our results show that the *in vitro* inactivation of the enzyme results in a decreased catalytic activity of pre-existing active enzyme, whereas the *in vivo* inactivation during the diurnal cycle and the cholesterol feeding is completely due to alteration in the number of enzyme molecules. The difference between the findings of Higgins and Rudney (10) and ours might find an explanation in the enzyme purification, and consequently, the antibody used by those authors for the immunoprecipitation experiments.

Recently, Beg *et al.* (5) suggested the phosphorylation-dephosphorylation of HMG-CoA reductase to be an important physiological *in vivo* short term regulation mechanism. These authors found a few hundred counts out of 2 mCi (4×10^9 dpm) of the applied ³²P to be bound as immunoprecipitate

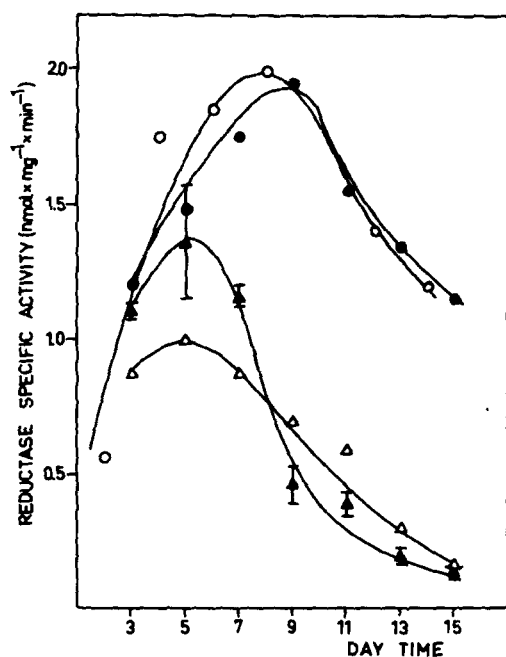


FIG. 2. Effect of cholesterol feeding on the specific enzymic activity of microsomal and solubilized (but microsomes have not been separated) HMG-CoA reductase in rat liver. ○—○, normal diet, microsomal HMG-CoA reductase; ●—●, normal diet, solubilized HMG-CoA reductase; △—△, cholesterol-fed, microsomal HMG-CoA reductase; ▲—▲, cholesterol-fed, solubilized HMG-CoA reductase. For each point of each curve, three rat livers were pooled and the mean of three determinations is marked. For clearness, standard deviation brackets are given only for one curve.

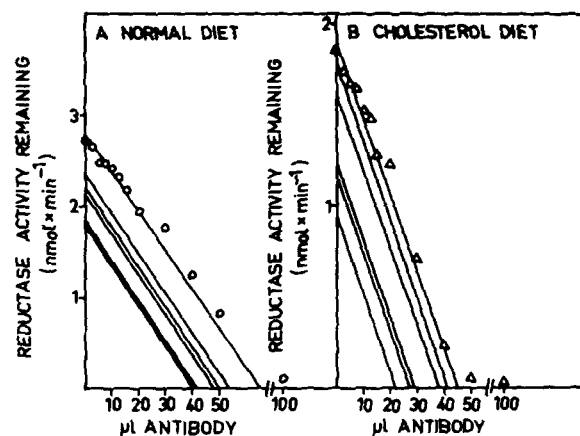


FIG. 3. Neutralization of HMG-CoA reductase activity with antiserum. Immunotitrations were carried out in a 200- μ l volume; solubilized enzyme (0.47 to 2.75 units) was preincubated with 1:16 antiserum dilutions for 30 min at 37 °C in 40 mM KH₂PO₄, 10 mM EDTA, 30 mM KCl, 20 mM NaCl, 1 mM dithioerythritol, pH 7.0. The immunoprecipitate was sedimented at 12,000 $\times$ g and the supernatant was assayed for reductase activity. For clearness, only the symbols for one immunotitration are shown. In A, the immunotitration curves (from left to right) correspond to the daytime values 13, 5, 3, 11, 7, 9 h of Fig. 2. In B, the immunotitration curves (from left to right) correspond to the daytime values 13, 11, 3, 9, 5, 7 h of Fig. 2.

TABLE II

Immunotitrations of microsomal and solubilized HMG-CoA reductase from animals fed a normal or a 3% cholesterol-supplemented diet during the dark phase of the diurnal cycle

Time during the diurnal cycle	Equivalence points (enzyme units inactivated/ μ l antiserum)			
	Standard diet		3% cholesterol diet	
	Microsomal	Solubilized	Microsomal	Solubilized enzyme
<i>h</i>				
3.00		0.68	0.66	0.68
4.00	0.67			
5.00		0.68	0.69	0.68
6.00	0.67			
7.00		0.68	0.68	0.67
8.00	0.68			
9.00		0.68	0.69	0.67
10.00	0.67			
11.00		0.68	0.68	0.67
12.00	0.68			
13.00		0.68	0.68	0.67
14.00	0.67			

TABLE III

Effect of the *in vitro* phosphorylation of HMG-CoA reductase on the equivalence point and the specific activity

	Equivalence point	Specific activity
	enzyme units inactivated/ μ l antibody	nmol/mg $\times$ min
A. Microsomal enzyme at diurnal peak	0.64	0.89
B. ATP/Mg ²⁺ -inactivated microsomal enzyme at diurnal peak	0.11	0.15
Ratio: B/A	0.171	0.168

of HMG-CoA reductase. It can not be completely excluded that this might be due to unspecific binding and phosphorylation. Just as low incorporation rates of ³²P into HMG-CoA reductase were published by Keith *et al.* (14) and Gil *et al.* (15) for *in vitro* conditions. Additionally, as was argued by Brown *et al.* (9), a phosphorylation process of the enzyme

during its isolation also should be considered.

Very recently, Ness *et al.* (13) report on the impossibility of showing a covalent ^{32}P label of HMG-CoA reductase after inactivation of the purified enzyme with ATP/ Mg^{2+} and a cytosolic and microsomal inactivator. The inactivation of the enzyme was found to be independent of the incubation time, to be reversed by dilution and dialysis, and the enzyme could not be reactivated by various phosphatases. We were unable to show a covalent ^{32}P -binding to the microsomal and solubilized HMG-CoA reductase, too.

Therefore, from data published so far on binding of ^{32}P to HMG-CoA reductase, it is not unequivocally proved that a phosphorylation-dephosphorylation of HMG-CoA reductase has relevance physiologically in the short term regulation of cholesterol biosynthesis. Our results suggest even short term regulation of HMG-CoA reductase after cholesterol feeding to be the consequence of the altered number of the enzyme molecules.

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