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In vivo regulation of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase: Immunotitration of the enzyme after short-term mevalonate or cholesterol feeding

(cholesterol biosynthesis)

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ABSTRACT **In recent studies using either a single dose of mevalonolactone administered by intragastric tube or a single meal containing 2% cholesterol, it was demonstrated that rat liver hydroxymethylglutaryl-coenzyme A reductase [mevalonate: $NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] (HMG-CoA reductase) the major regulatory enzyme in cholesterol biosynthesis, is subject to two phases of inhibition. The first phase of inhibition is explained by *in vivo* phosphorylation of the enzyme; however, the nature of the second phase of inhibition remained obscure. The present study tested two possible explanations for this second phase of inhibition—increased enzyme turnover leading to a decreased concentration of HMG-CoA reductase molecules, and further inactivation of existing enzyme molecules. The results with the technique of immunotitration of HMG-CoA reductase show that, in short-term studies conducted up to 2 hr after the administration of a single dose of mevalonolactone or up to 6 hr after a single meal of rat chow containing 2% cholesterol, the *in vivo* regulation of rat liver HMG-CoA reductase during the first half of the dark period does not occur by increased enzyme turnover but, instead, existing enzyme is further inactivated.**

In recent studies (1, 2) using either a single dose of mevalonic acid administered by intragastric tube or a single meal containing 2% cholesterol, we demonstrated that rat liver hydroxymethylglutaryl-coenzyme A reductase [mevalonate:$NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] (HMG-CoA reductase), the major regulatory enzyme in cholesterol biosynthesis, is subject to two phases of inhibition. The first phase of inhibition, observed 20 min after mevalonolactone administration or 60 min after the beginning of a single meal containing 2% cholesterol, is explained by *in vivo* phosphorylation of rat liver HMG-CoA reductase (1, 2).

However, the nature of the second phase of inhibition, observed 60 min after mevalonolactone administration or 120 min after the beginning of a single meal containing 2% cholesterol, remained obscure because the microsomal HMG-CoA reductase from treated rats was not reactivated by purified phosphoprotein phosphatase. The question remained, therefore, whether this second phase of inhibition was the result of increased enzyme turnover or, instead, was the result of further inactivation of existing enzyme. The present study examined this question by using the technique of immunotitration of rat liver microsomal HMG-CoA reductase by HMG-CoA reductase antiserum (3). This technique provides a quantitative estimate of relative changes in either enzyme concentration or enzyme activity in different physiological states.

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The results show that, in short-term studies up to 2 hr after the administration of a single dose of mevalonolactone or up to 6 hr after a single meal containing 2% cholesterol, regulation of rat liver HMG-CoA reductase, during the first half of the dark period, does not occur by increased enzyme turnover but, instead, existing enzyme is further inactivated.

MATERIALS AND METHODS

Materials. The materials used in this study were obtained from described sources (1–3). In addition, Freund's complete adjuvant and incomplete adjuvant were purchased from Difco. Rat chow containing 2% cholesterol was from U.S. Biochemicals (Cleveland, OH).

Purification of HMG-CoA Reductase. Twenty-four male Sprague–Dawley rats, which weighed 385–405 g at the time of killing, were used for enzyme purification. The rats were subjected to the following light cycle: 0300 to 1500, dark; 1500 to 0300, light; the rats were equilibrated to this cycle for a period of 10 days, prior to killing. The rats were fed a diet of ground rat chow containing 2% cholestyramine (Questran). All animals had free access to tap water.

Rat liver microsomes were prepared in buffer A (0.1 M sucrose/0.05 M KCl/0.04 M potassium phosphate/0.03 M EDTA, 10 mM dithioerythritol, pH 7.2) at 0–4°C. The livers from 25 rats were homogenized in a Waring Blendor for 10 sec at low speed and for 20 sec at high speed in buffer A (2 ml/g). The homogenate was centrifuged twice at 10,000 × *g* for 15 min. This supernatant was centrifuged at 303,000 × *g* (50.2 Ti rotor) for 45 min. After centrifugation, the soluble supernant was discarded, and the microsomal pellets were resuspended by motor-driven homogenization in buffer A (1 ml/g of liver) with a tightly fitting Teflon pestle. Centrifugation was again conducted at 303,000 × *g* for 45 min. After centrifugation, the supernatant was discarded, and the microsomal pellets were frozen by storage at −20°C as described by Beg *et al.* (4).

Thawed microsomal pellets were suspended in buffer A (15 ml per pellet) at room temperature. The suspension was homogenized with a motor-driven, tightly-fitting Teflon pestle (high speed, two strokes down and up). The homogenate was warmed at 37°C for 20 min in an atmosphere of nitrogen. The solubilized enzyme was separated from the residual microsomes by centrifugation at 303,000 × *g* for 45 min at room temperature.

The solubilized enzyme was fractionated with solid ammonium sulfate (30–50% saturation) at room temperature. The ammonium sulfate precipitate at 50% saturation was dissolved in 13 ml of 1 M KCl/0.08 M potassium phosphate/2 mM EDTA/

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; E_A, activity state of the enzyme.

30% (vol/vol) glycerol/10 mM dithioerythritol, pH 6.8, and heated at 65°C as described by Edwards *et al.* (5). The heat-treated supernatant was precipitated with ammonium sulfate (60% saturation), and the pellet was dissolved in 25 ml of buffer B (25 mM potassium phosphate/1 mM EDTA/2 mM dithioerythritol, pH 7.2). The dissolved pellet was stored overnight at 4°C in an atmosphere of nitrogen, and the following day it was applied to an agarose-hexane HMG-CoA affinity column (1.0 ml) which was equilibrated in buffer B at room temperature. The column was washed with 10 ml of buffer B and then 20 ml of buffer C (0.12 M KCl/0.04 M potassium phosphate/3 mM EDTA/2 mM dithioerythritol, pH 7.2). The enzyme was eluted from the column with buffer C (7 ml) containing HMG-CoA (0.2 mM).

The purified enzyme (380 μg) had a specific activity of approximately 9000 nmol of mevalonate formed per min per mg of protein. $NaDodSO_4$/polyacrylamide gel electrophoresis, as described by Fairbanks *et al.* (6), indicated that the enzyme was approximately 90% pure with a monomer molecular weight of 50,000 ± 2000.

HMG-CoA Reductase Antiserum. Antiserum was prepared in a New Zealand White rabbit. HMG-CoA reductase purified from the livers of cholestyramine-treated rats, as described above, was used as antigen. Purified enzyme (1 ml, 54 μg/ml) was combined with Freund's complete adjuvant (3 ml) and injected subcutaneously into several sites on the rabbit's hindquarters. The same procedure was repeated at 26 and 40 days.

Serum obtained at 40 days (after two injections) inhibited 80% of the HMG-CoA reductase present in 0.2 mg of microsomes when incubated with an equal volume of normal rat liver microsomal suspension (2 mg of protein per ml).

At 12 and 16 weeks, the injection procedure was repeated, as described above, except incomplete adjuvant was used. The antiserum used in the immunotitrations reported in the present study was harvested at 14 to 16 weeks. After each antigen injection, serum antibody titer against rat liver HMG-CoA reductase increased measurably. Ouchterlony immunodiffusion experiments with solubilized HMG-CoA reductase and the antiserum showed a single precipitation line.

It was demonstrated (data not shown) that the HMG-CoA reductase antiserum was capable of inhibiting >98% of the HMG-CoA reductase activity present in the liver microsomes for all experimental groups—i.e., control, mevalonate-treated, cholesterol-fed, or cholestryamine-fed.

Antiserum was stored in aliquots at −80°C until used.

Serum obtained from the rabbit prior to immunization with HMG-CoA reductase antigen showed no inhibition of rat liver microsomal HMG-CoA reductase activity.

The HMG-CoA reductase antiserum was characterized by two techniques. In the first technique, immunoelectrophoresis was conducted according to Grabar and Williams (7, 8). A single arc of immunoprecipitation was observed between crude rat liver HMG-CoA reductase and the rabbit antiserum. No precipitation arc was observed when immunoelectrophoresis was conducted with crude HMG-CoA reductase and serum from a control rabbit prior to immunization with homogeneous rat liver HMG-CoA reductase.

The second technique used the powerful method of crossed immunoelectrophoresis (9) as modified by Clarke and Freeman (8, 10, 11). This technique uses a combination of electrophoretic separation of proteins (from the crude rat liver extract containing HMG-CoA reductase) in an agarose gel, followed by electrophoresis perpendicular to the original separation into a gel containing the HMG-CoA reductase antiserum. The resolution of this technique is substantially greater than the technique of Grabar and Williams (7). The results (Fig. 1) show a single symmetrical peak of immunoprecipitation between crude rat liver HMG-CoA reductase and the rabbit antiserum. No immunoprecipitation peak was observed with serum from the control rabbit.

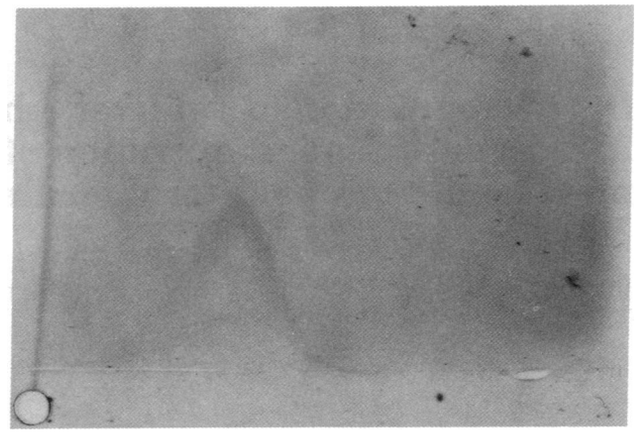

FIG. 1. Crossed immunoelectrophoresis of crude soluble extract (3 mg/ml) of rat liver microsomes (10 μl) with rabbit anti-rat HMG-CoA reductase antiserum. Crossed immunoelectrophoresis was conducted in an LKB-2117 Multiphor apparatus equipped with an LKB-2103 power supply and a Haake F3 circulating bath (10°C). The first dimension was conducted with the soluble extract for 75 min (8–10 V/cm, in 1% agarose in Tris barbiturate buffer, pH 8.4–8.6) as described (8). The cathode was at the left. Then, antiserum (0.5 ml, 58 mg/ml) was mixed with 8.5 ml of 1% agarose in the above buffer. The second dimension was then conducted for 18 hr (2 V/cm, 10°C, anode at the top). Staining was with Coomassie brilliant blue R.

Immunotitration Procedure. Immunotitration of HMG-CoA reductase activity with HMG-CoA reductase antiserum was conducted as described (3) with the following modifications: antiserum was diluted with buffer A (dithioerythritol was replaced with 10 mM dithiothreitol) containing bovine serum albumin (10 mg/ml), and control incubations contained 100 μl of buffer A containing albumin at 10 mg/ml. This technique minimizes the effect of changes in protein concentration on microsomal HMG-CoA reductase activity. Also, microsomes were preincubated at 37°C for 20 min prior to the addition of antiserum, so that HMG-CoA reductase would be in a maximally activated state before exposure to antibody.

Physiological Experiments. Male Sprague–Dawley rats (100–150 g at the time of killing) were maintained on a lighting cycle of dark 0400 to 1600 and light 1600 to 0400 for a minimum of 1 week prior to the experiment. All animals had free access to tap water.

For the mevalonolactone experiment, mevalonolactone (100 mg, dissolved in 1 ml of distilled water) was administered by intragastric tube 20, 60, or 120 min prior to sacrifice at 1000 (mid-dark). Control animals received 1 ml of distilled water only by intragastric tube. Rat liver microsomes were prepared in buffer A as described (1).

For the 1- and 2-hr cholesterol experiments, rats were given food (rat chow) only between 0800 and 0900 for 3 days prior to the day of the experiment. On the day of the experiment, the experimental group received a single meal of rat chow containing 2% cholesterol from 0800 to 0900 (2 hr prior to sacrifice) or from 0900 to 1000 (1 hr prior to sacrifice). All animals were killed at 1000 (mid-dark). Control animals received rat chow only, on the same schedule as the cholesterol fed animals.

For the 6-hr cholesterol experiments, rats were deprived of food at 1600 on the day immediately preceding the experiment (beginning of the light period). Then, at 0400 (12 hr later), the rats were given a single meal of rat chow containing 2% cho-

lesterol. All animals were then killed 6 hr later at 1000 (mid-dark). Control animals received rat chow only, on the same schedule as the cholesterol-fed animals.

Rat liver microsomes for all three groups of cholesterol-fed rats, as well as control rats, were prepared as described (2).

For the cholestyramine feeding experiments, rats were fed 2% cholestyramine (Questran) in ground rat chow (Wayne Lab Blox) ad lib for 5 or 10 days prior to sacrifice at 1000 (mid-dark). Controls received rat chow only.

RESULTS

Fig. 2 shows the results of an experiment in which mevalonolactone was administered by intragastric tube to rats. The animals were sacrificed 20, 60, or 120 min after mevalonolactone administration. There was a time-dependent decrease in the enzyme activity inhibited as a function of antiserum dilution when equivalent amounts of rat liver microsomes were immunotitrated with HMG-CoA reductase antiserum. It was shown in an earlier investigation (3) that this plot—i.e., units of activity inhibited (nmol/min) vs. antiserum dilution—is capable of showing changes in the activity state of the enzyme (E_A). The results show a time-dependent progressive inhibition of the activity of existing HMG-CoA reductase enzyme molecules in the microsomes. Table 1 shows the quantitative aspects of this comparison. E_A decreased with time after mevalonolactone treatment: at 20 min, $E_A = 0.83$; at 60 min, $E_A = 0.30$; at 120 min, $E_A = 0.16$. Thus, by 120 min, the enzyme was only 1/6th as active as in the controls.

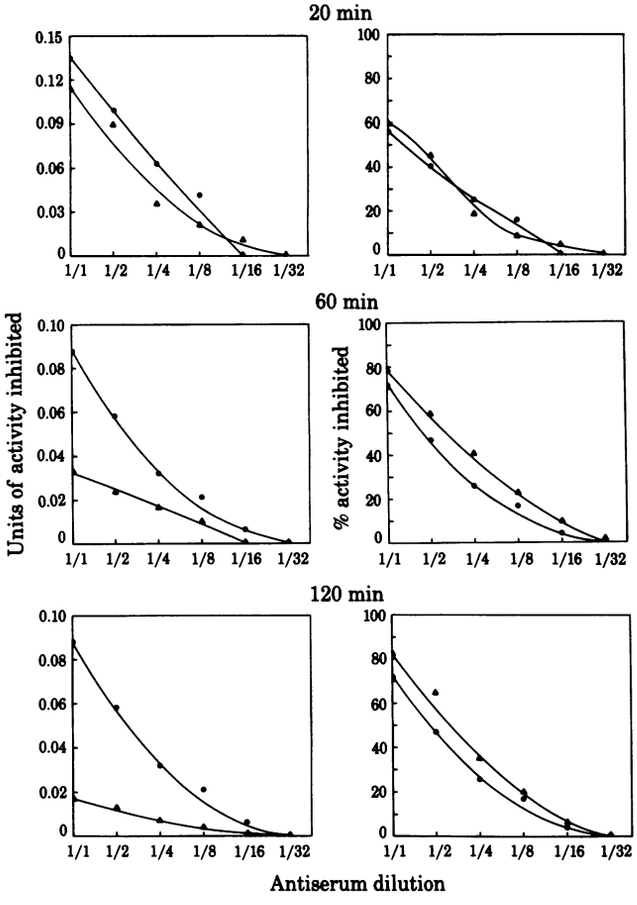

FIG. 2. Immunotitration of liver microsomal HMG-CoA reductase from rats after the administration of a single dose of mevalonolactone (100 mg, dissolved in 1 ml of distilled water) by intragastric tube (▲). The animals were killed 20, 60, or 120 min after mevalonolactone administration. Control rats (●) received 1 ml of distilled water by intragastric tube and were sacrificed at the same time intervals. The final protein concentrations were: 20 min treated, 0.85 mg/ml; 60 min treated, 1.0 mg/ml; 120 min treated, 1.05 mg/ml; control, 0.9–1.1 mg/ml. The observed percentage inhibition for the treated animals was normalized to the control microsomal protein concentration for each experiment. Thus, these plots show the percentage of HMG-CoA reductase activity inhibited at an equivalent microsomal protein concentration (3). In addition, units of HMG-CoA reductase activity (nmol/min) inhibited are plotted as a function of antiserum dilution. The observed specific activities for the microsomes were: 20 min treated, 0.95 nmol of mevalonate formed per min per mg of protein; 60 min treated, 0.39; 120 min treated, 0.20; control, 1.15–1.25.

Fig. 2 also shows the percentage of enzyme activity inhibited plotted as a function of antiserum dilution. It was shown previously (3) that this plot is capable of showing changes in the concentration of enzyme molecules (E_C). There was no significant change in the number of HMG-CoA reductase enzyme molecules present in the liver microsomes of rats at 20, 60, or 120 min after the intragastric administration of mevalonolactone. The quantitative aspects of this comparison are shown in Table 1. The theoretical value for no change in enzyme concentration is $E_C = 1.00$.

Table 1 shows that E_A essentially accounts for the observed ratio of the specific activities of the microsomes of mevalonate-treated animals to those of control animals.

Therefore, the results in Fig. 2 and Table 1 demonstrate that, within the time-period examined, mevalonolactone treatment does not result in increased enzyme turnover (decreased concentration of enzyme); instead, existing enzyme molecules are inactivated.

Fig. 3 shows the results of an experiment in which rats were fed a single meal of rat chow containing 2% cholesterol. The animals were killed 1, 2, or 6 hr after the feeding, liver microsomes were isolated by ultracentrifugation, and immunotitration of microsomal HMG-CoA reductase was performed. There was a time-dependent decrease in the units of enzyme activity inhibited at equivalent antiserum dilutions, when equivalent amounts of rat liver microsomes were immunotitrated with HMG-CoA reductase antiserum. Thus, the results show a time-dependent progressive inhibition of the activity of HMG-CoA reductase enzyme molecules present in the microsomes. Table

Table 1. HMG-CoA reductase relative concentration and activity in various physiological states

Condition	*n*	E_C	E_A	$E_C E_A$	Ratio of specific activities
After mevalanolactone					
20 min	3	0.92	0.83	0.76	0.75
60 min	4	0.95	0.30	0.29	0.28
120 min	3	0.92	0.16	0.15	0.14
After cholesterol					
1 hr	3	1.00	0.82	0.82	0.79
2 hr	3	0.99	0.55	0.54	0.54
6 hr	3	1.01	0.20	0.20	0.21
After cholyestyramine					
5 days	2	1.02	2.42	2.47	2.56
10 days	2	2.33	1.95	4.54	4.40

n, Number of experiments used for calculating average values for E_C, E_A, and the specific activities; E_C, relative enzyme concentration factor, defined for a given antiserum dilution as E_C = % inhibition untreated/% inhibition treated; E_A, relative enzyme activity factor, defined for a given antiserum dilution as E_A = units enzyme activity inhibited in treated/units enzyme activity inhibited in untreated. Experimental points between 10% and 90% inhibition were used to calculate average values for E_C and E_A. The ratio of average specific activities is given for treated/untreated.

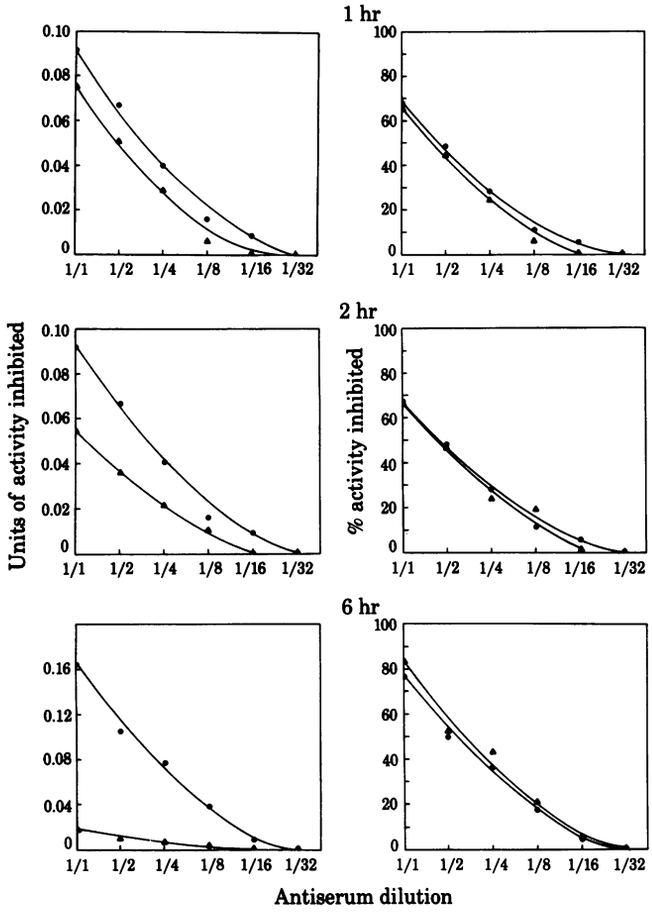

FIG. 3. Immunotitration of liver microsomal HMG-CoA reductase from rats fed a single meal of rat chow containing 2% cholesterol (▲). The animals were killed 1, 2, or 6 hr after the single meal was begun. Control rats (●) were fed normal rat chow. The final protein concentrations were as follows: 1 hr treated, 0.92 mg/ml; 2 hr treated, 0.90 mg/ml; 6 hr treated, 0.87 mg/ml; control, 0.85–0.94 mg/ml. The observed percentage inhibition for the treated animals was normalized to the control microsomal protein concentration for each experiment. In addition, units of HMG-CoA reductase activity (nmol/min) inhibited are plotted as a function of antiserum dilution. The observed specific activities for the microsomes in this experiment were as follows: 1 hr treated, 0.74 nmol of mevalonate formed per min per mg of protein; 2 hr treated, 0.54; 6 hr treated, 0.10; control, 1.21–1.23.

1 shows the quantitative aspects of this comparison. By 6 hr, the enzyme was only 1/5th as active as in the controls.

In addition, Fig. 3 shows the results obtained when the percentage of enzyme activity inhibited is plotted as a function of antiserum dilution. There was no significant change in the number of HMG-CoA reductase enzyme molecules present in the liver microsomes of rats at 1, 2, or 6 hr after cholesterol feeding. The quantitative aspects of this comparison are shown in Table 1. The values are indistinguishable from the theoretical value for E_C of 1.00 (no change in enzyme concentration compared to control animals).

Furthermore, Table 1 shows that E_A entirely accounts for the observed ratio in the specific activities between the microsomes of cholesterol-fed animals and of control animals.

Therefore, the results in Fig. 3 and Table 1 demonstrate that, under the experimental conditions and time periods tested, the feeding of a single meal containing 2% cholesterol does not result in increased enzyme turnover (decreased concentration of enzyme); instead, existing enzyme molecules are inactivated.

We also examined the effect of feeding rat chow containing 2% cholestyramine. Cholestyramine interferes with cholesterol absorption by sequestering bile acids in the small intestine. After rats were on the cholestyramine diet for 5 days, there was no significant increase in the concentration of HMG-CoA reductase enzyme. However, the enzyme was activated. At 10 days, however, there was an increase in both the number of enzyme molecules and the enzyme molecules were activated.

An immunotitration experiment, using microsomes from control animals and microsomes from animals killed 60 min after a single intragastric dose of mevalonate, was conducted for the purpose of directly comparing the analytical method described above (for calculation of relative changes in E_C and E_A) (3) and that described by Edwards *et al.* (5). [This method of Edwards *et al.* is similar to the method used by Pollock (12) and by Richmond (13).] A straight line is derived from points in the low range of inhibition when enzyme activity is plotted as a function of antiserum concentration. If identical protein concentrations are used for the samples being compared, the ratio of the slopes of the lines is the same as the ratio of enzyme activities (E_A). The ratio of the end points (the *X* intercepts of the derived straight lines) is the same as the ratio of enzyme concentrations in the two samples.

Data from this experiment gave an E_A (using the graphical method described here and in ref. 3) of 0.176; by using the ratio of the slopes as in ref. 5, $E_A = 0.168$. Calculated according to our method (3), $E_C = 0.949$; by using the ratio of the *X* intercepts as in ref. 5, $E_C = 0.966$. Therefore, both methods of analysis give essentially the same result—i.e., 60 min after a single dose of mevalonate the observed inhibition of HMG-CoA reductase is caused by enzyme inactivation and not by increased enzyme turnover. The method of linear extrapolation at low inhibition (5), in order to be reliable, requires a large number of experimental points at relatively low inhibition, whereas the method described here and previously (3) can rely on points in the range of 10–90% inhibition.

Another corollary is derived from the fact that E_C and E_A remained relatively constant for a given immunotitration, whether calculated by the linear extrapolation method (5) using points at low inhibition or by the semilogarithmic method described here and previously (3), which uses points from 10% to 90% inhibition. This finding demonstrates that the antigenieity of the various forms of HMG-CoA reductase remained constant during the various nutritional manipulations.

DISCUSSION

In an earlier study (3), we described the immunotitration of HMG-CoA reductase, the major regulatory enzyme in cholesterol biosynthesis, by HMG-CoA reductase antiserum. This technique provides a method for the quantitative estimate of relative changes in either enzyme concentration or enzyme activity in different physiological states, even in crude samples such as liver microsomal membranes.

In a recent study (1) on the regulation of HMG-CoA reductase, using the intragastric administration of a single dose of mevalonolactone, we found two phases of inhibition of rat liver microsomal reductase. The first phase, observed 20 min after mevalonolactone administration, was completely reversed by preincubation of the microsomes with purified phosphoprotein phosphatase. This finding demonstrated that phosphorylation of the enzyme was an early *in vivo* regulatory event after the intragastric administration of mevalonolactone. However, the second phase of inhibition, observed 60 min after mevalonolactone administration, was not reversed by phosphoprotein phosphatase preincubation of the microsomes.

In a related investigation (2), we demonstrated a similar phenomenon when rats received a single meal containing 2% cho-

lesterol. The first phase of inhibition, observed 60 min after the beginning of cholesterol feeding, was completely reversed by preincubation of the microsomes with purified phosphoprotein phosphatase. Again, this finding demonstrated that phosphorylation of the enzyme was an early *in vivo* regulatory event after the single meal containing 2% cholesterol. The second phase of inhibition of HMG-CoA reductase was observed 120 min after the beginning of cholesterol feeding, and this inhibition was not reversed by phosphoprotein phosphatase preincubation of the microsomes.

The question remained, therefore, whether this second phase of inhibition was the result of increased enzyme turnover or of inactivation of existing enzyme.

The results reported here demonstrate that in these short-term experiments the second phase of inhibition is due not to increased enzyme turnover but is due to inactivation of existing enzyme. The molecular mechanism for the further inactivation of the enzyme is not known.

The immunotitrations of microsomal HMG-CoA reductase reported here after mevalonolactone administration are consistent with the results obtained by Edwards *et al.* (5). Also, the studies of Tanabe *et al.* (14), Higgins and Rudney (15), and Edwards and Gould (16) are consistent with the concept that cholesterol feeding may inhibit the activity of preexisting enzyme.

In an earlier study (3), we demonstrated that regulation of HMG-CoA reductase by changes in the concentration of enzyme molecules (E_C) was an important regulatory factor in the diurnal rhythm of the enzyme and also in the long-term regulation of the enzyme after 7 days of cholesterol feeding. Thus the experiments reported here, in which increased enzyme turnover was not an important regulatory event, were all short-term regulatory interventions, performed during the first half of the dark period with animals that were always sacrificed at mid-dark.

This experimental design is proving to be an excellent model for examining the molecular events involved in the short-term *in vivo* regulation of HMG-CoA reductase, the major rate-limiting enzyme in cholesterol biosynthesis.

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