Fine Structure of the Cyclic Rhythm of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase. Differential Effects of Cholesterol Feeding and Fasting†

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ABSTRACT: Rat liver microsomal 3-hydroxy-3-methylglutaric acid (HMG)-CoA reductase (mevalonate:NADP-oxidoreductase (acylating CoA), EC 1.1.1.34) undergoes striking cyclic variations which are dependent on protein synthesis. Distinct activity peaks are observed at about midnight and 1:45 a.m. (EST). Cycloheximide prevents both the initial (6 p.m. to midnight) rise in HMG-CoA reductase activity and the second rise which occurs between 12:30 a.m. and 1:45 a.m. A rapid, 35–45% decrease in HMG-CoA reductase activity is observed from midnight to about 12:30 a.m. After the second cyclic peak at about 1:45 a.m., reductase activity declines, reaching basal levels in midmorning. In rats fasted for 36 hr reductase activity decreases 20 to 25-fold. Nevertheless, the cyclic rhythm with both peaks in activity persists in rats fasted for 36 hr. This suggests that cyclic variations in amino acid and tryptophan intake are not responsible for the cyclic rhythm in HMG-CoA reduction. Short duration (10–18 hr) cholesterol feeding resulted in an overall decline in reductase activity from 6 p.m. to midnight. Activity in rats fasted 10–18 hr increases severalfold during this time. The different effects of cholesterol feeding and fasting suggest that they may regulate HMG-CoA reductase through different mechanisms.

Cholesterol synthesis (Back *et al.*, 1969) and 3-hydroxy-3-methylglutaric acid (HMG)-CoA reductase activity[1] (Shapiro and Rodwell 1971) undergo cyclic variations with peak activity at midnight. Cycloheximide (Shapiro and Rodwell, 1971) and puromycin (Kandutsch and Saucier, 1969) prevent the cyclic rhythm. Although several hepatic enzymes, including tyrosine transaminase (Wurtman and Axelrod, 1967) and tryptophan pyrrolase (Rapoport *et al.*, 1966), undergo cyclic variations, molecular explanations of cyclic rhythms remain generally obscure. Wurtman (1969) reported that fasting extinguishes the tyrosine transaminase cyclic rhythm and proposed that cyclic variations in dietary tryptophan intake were the primary cause of cyclic variations in tyrosine transaminase levels.

Investigation of the HMG-CoA reductase cyclic rhythm at short time intervals has revealed distinct, protein synthesis dependent activity peaks at about midnight and 1:45 a.m. The double-peaked rhythm persists in fasted rats.

Materials and Methods

Animals. Female Wistar rats from our colony weighing 75–125 g were used in most experiments. In one experiment female Sprague-Dawley rats (Laboratory Supply Co., Indianapolis, Ind.) weighing 100–120 g were used. Rats were kept in a windowless room with a controlled light–dark cycle (light 6 a.m.–6 p.m.; dark 6 p.m. –6 a.m., EST) for at least 10 days and were housed two to a cage for at least 3 days prior to experimental use. Control rats were allowed access to food and water ad libitum. Where indicated, stock diet (Inkpen, 1968) containing 5% cholesterol was used. Red light, not visible to rats, was used when rats were removed from the room just prior to sacrifice.

Assay of HMG-CoA Reductase. The preparation of the substrate ([3-^{14}C]HMG-CoA) and the procedures for isolation, quick freezing, and assay of the microsomal fraction of rat liver have been described previously (Shapiro *et al.*, 1969; Shapiro and Rodwell, 1969, 1971). Unless otherwise indicated, two rats were killed at each time and their livers pooled, minced, and homogenized; microsomal fraction was isolated and quick frozen for assay the following day. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. For assay of HMG-CoA reductase activity, standard incubations were for 15 min at 37° and contained, in 1.0 ml of 30 mM EDTA, 70 mM NaCl, 10 mM β-mercaptoethanol (Linn, 1967), at pH 6.8: 30 μmoles of glucose 6-phosphate, 2.0 enzyme units of glucose-6-phosphate dehydrogenase, 3.0 μmoles of $NADP^+$, 300 nmoles of DL-[3-^{14}C]-HMG-CoA (specific activity 400 cpm/nmole) and the quantities of microsomal protein indicated in figure and table legends. The protein concentrations used (0.6–4.5 mg) are within the range where mevalonate formation is proportional to protein concentration (Shapiro and Rodwell, 1971). Mevalonate was isolated by thin-layer chromatography and quantitated as described previously (Shapiro *et al.*, 1969; Shapiro and Rodwell, 1971). HMG-CoA reductase activity is expressed as nanomoles of mevalonate synthesized per minute per milligram of microsomal protein (nanomoles per minute per milligram). Data in tables and figures are mean values for triplicate incubations.

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[1] The use of the term *HMG-CoA* (3-hydroxy-3-methylglutaric acid-coenzyme A) *reductase activity* does not imply a specific mechanism for the changes we observe *in vitro* in the rate of conversion of HMG-CoA to mevalonic acid. These may be due to changes in the activity of existing enzyme molecules or, as seems more likely, to changes in the number of enzyme molecules.

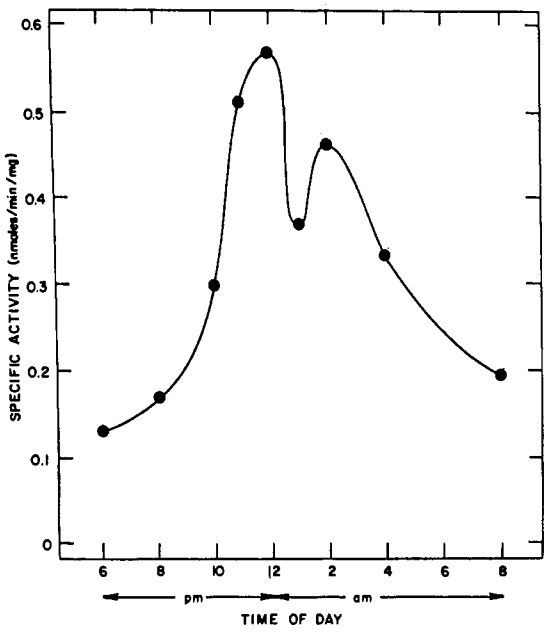

FIGURE 1: Cyclic variations in HMG-CoA reductase activity. The data are for reductase activity of microsomes from rats killed at the indicated times and incubated and assayed under standard conditions (see Methods). The standard error of the mean for triplicate incubations containing 2.8–4.5 mg of microsomal protein was less than 7% in all cases.

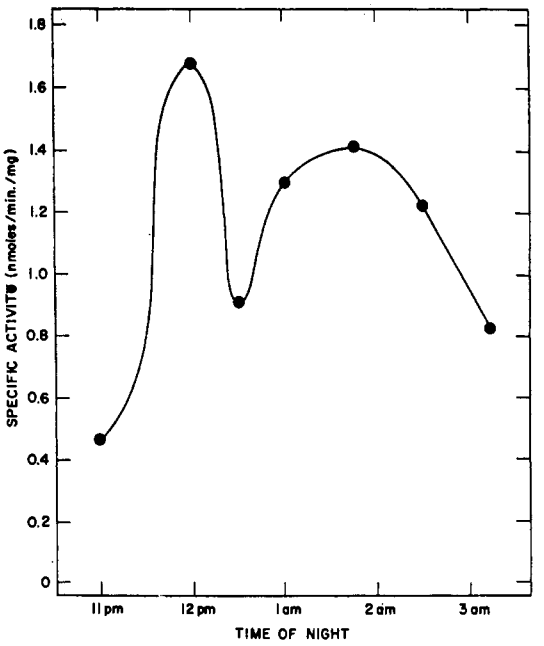

FIGURE 2: Cyclic variations in HMG-CoA reductase activity in rats sacrificed on different days. The single time point studied each night represents the only room entry. The experiment was conducted on successive nights, proceeding from the latest time (3:20 a.m.) taken the first night to the earliest (11 p.m.) taken on the seventh. Triplicate incubations containing 1.6–2.9 mg of microsomal protein were performed under standard conditions as described in Methods. The standard error of the mean was less than 6% in all cases.

Results

Fine Structure of the Cyclic Rhythm of HMG-CoA Reductase. Investigation of the HMG-CoA reductase cyclic rhythm at short time intervals suggested the existence of two activity peaks (Figure 1). More detailed studies at the times in question (11 p.m.–3 a.m.) demonstrated the presence of distinct activity peaks at about midnight and 1:45 a.m. (Figures 2 and 3).

To determine whether the occurrence of a double peak in reductase activity is a general phenomen, we investigated the cyclic rhythm in a second strain of rats (Sprague-Dawley) fed a different diet (Wayne Lab-Blox) and housed in a different building. Although the first peak occurred at 11 p.m. rather than at midnight, a similar cyclic rhythm was observed in these animals (Figure 4). Since environmental stress may influence HMG-CoA reductase activity (D. J. Shapiro and V. W. Rodwell, unpublished observations), repeated room entry, even in red light, could conceivably affect the cyclic rhythm. To minimize possible stress effects, an experiment was performed with only one room entry and one time point studied each night. Two peaks in HMG-CoA reductase activity were also observed under these conditions (Figure 2).

Both the first (6:00 p.m.–12:00 p.m.) (Shapiro and Rodwell, 1969, 1971) and the second (12:30 a.m.–1:45 a.m.) rise in HMG-CoA reductase activity are prevented by cycloheximide injection and thus appear to involve processes dependent on protein synthesis (Table I).

Effects of Dietary Cholesterol and Fasting on the Cyclic Rhythm. The cyclic rise in HMG-CoA reductase activity is depressed by short-duration (10-hr) cholesterol feeding (Shapiro and Rodwell, 1971). Fasting, which reduces reductase activity to low levels (Regen *et al.*, 1966; White and Rudney, 1970), does not suppress the cyclic rise (Hamprecht *et al.*, 1969). The effect of fasting on the double cyclic peaks in HMG-CoA reductase activity was therefore investigated.

We observed a 20- to 25-fold decrease in HMG-CoA reductase activity in rats fasted 36 hr. The cyclic rhythm with both peaks nevertheless persists and is comparable to that seen in control rats (Figure 3). This suggests that cyclic variations in dietary amino acid intake, which may affect the activity of HMG-CoA reductase, do not trigger the cyclic rhythm. Fasting beyond 18–24 hr is reported to stimulate glucocorticoid secretion and actually raises tyrosine transminase levels (Wurtman, 1969). Reductase levels, however, are extremely low in rats fasted 36 hr (Figure 3).

The effects of short-term fasting and cholesterol feeding on the cyclic rhythm were compared. Reductase activity decreases in rats fasted for 10 hr although the cyclic rise persists (Table II). Reductase activity at 6 p.m. is similar in cholesterol-fed and fasted rats. At midnight and 1:45 a.m. reductase activity is lower than the 6-p.m. level in cholesterol-fed rats and severalfold higher in fasted rats. The differential effects of dietary cholesterol and of fasting on the HMG-CoA reductase cyclic rhythm are apparent within 18 hr. This suggests that cholesterol feeding and fasting may regulate HMG-CoA reductase, and thereby control the rate of cholesterol synthesis, *via* different mechanisms.

Discussion

The rate of hepatic reduction of HMG-CoA to mevalonate rises sharply from 6 p.m. to midnight and then declines by 30–50% from about 12:00 to 12:30 a.m. A second rise in reductase activity occurs from about 12:30 to 1:45 a.m. and is followed by a decline in the early morning hours. Both the initial and the second rise are prevented by cycloheximide (Table I) and therefore appear to require synthesis of new protein. This suggests that changes in reductase levels rather

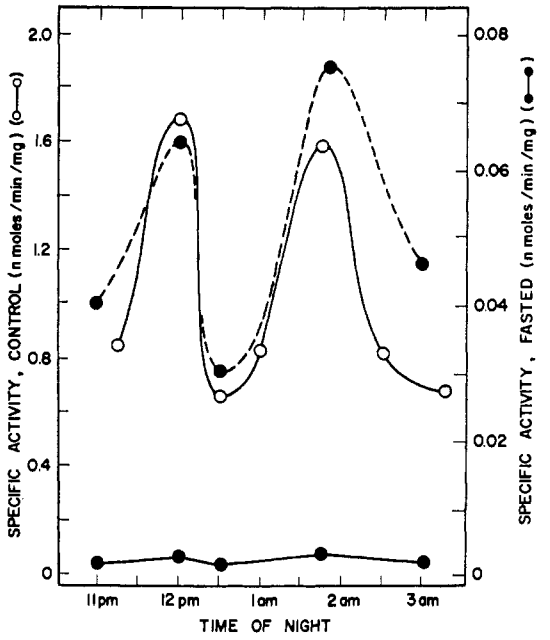

FIGURE 3: Cyclic variations in HMG-CoA reductase activity in control and fasted rats. Incubations contained 1.0–2.3 mg of microsomal protein and were under standard conditions (see Methods). The standard errors for triplicate incubations performed with microsomes from control rats (O—O) were less than 5% in all cases. The standard errors for triplicate incubations of microsomes from rats fasted 36 hours prior to 11 p.m. (●---●), ranged from 7 to 17%. Data from fasted rats is also plotted on the same axis as data from control rats (●—●).

TABLE I: Effect of Cycloheximide on the Cyclic Increases in HMG-CoA Reductase Activity. Specific Activity of HMG-CoA Reductase at Indicated Times.[a]

During the first cyclic increase	6 p.m.	8 p.m.	10 p.m.	12 p.m.
Control	0.107	0.353	0.522	1.12
Cycloheximide injected			0.104	0.103
During the second cyclic increase	**12:30 a.m.**	**1:15 a.m.**	**1:45 a.m.**	
Expt 1.				
Control	1.12	1.27	1.83	
Saline injected	1.28	1.87	2.57	
Cycloheximide injected	1.07		1.15	
Expt 2.				
Control	0.684		1.15	
Saline injected	0.627		1.32	
Cycloheximide injected			0.716, 0.720[b]	

[a] Cycloheximide (0.5 mg/100 g) was injected intraperitoneally in 0.5 ml of 0.9% saline. Injection was at 6 p.m. for studies of the first cyclic peak and at 12:25 a.m. for studies of the second peak. Saline injection at 6 p.m. is without effect at times beyond 8 p.m. (D. J. Shapiro and V. W. Rodwell, unpublished observations). The standard error of the mean for triplicate incubations performed as described under Methods was less than 10% in all cases. [b] Two pairs of rats were sacrificed and assayed separately as described under Methods.

than activation of preexisting enzyme may be responsible for both cyclic increases in HMG-CoA reductase activity.

To our knowledge, dual peaks have not been reported in cyclic rhythms for other liver enzymes. The absence of data taken at short time intervals in other systems makes it difficult to determine whether this phenomenon is unique to HMG-CoA reductase or is of more general occurrence. Possible explanations of the dual-peak phenomenon include the following.

The Transient Appearance of a Labile, HMG-CoA Reductase Specific Degradative System. Such a system might be responsible for the rapid decline in HMG-CoA reductase activity observed between midnight and 12:30 a.m. Following its disappearance, continued rapid synthesis of reductase would result in the second peak of activity. Boctor and Grossman (1970) have reported a 3- to 4-fold increase in the rate of tyrosine transaminase degradation following glucocorticoid induction. They suggest that glucocorticoids act at two loci to produce changes in the rate of transaminase synthesis and degradation.

Isozymes. The double peak could conceivably represent asynchronous production of two isozymes of HMG-CoA reductase. Differential temporal regulation might result in one isozyme attaining peak levels near midnight and the other near 1:45 a.m. A related possibility is that dual peaks represent synthesis of HMG-CoA reductase in different membrane fractions.

Periodic Oscillations. HMG-CoA reductase may undergo periodic oscillations which are detectable only at the peak of the cyclic rhythm. Maintenance of steady-state levels of labile regulatory enzymes may involve control elements which sense the level of enzyme or of metabolites. When enzyme levels rise above the preprogrammed value, cellular controls which lower the enzyme level by altering the rate of enzyme synthesis or degradation are activated. These changes may take the form of damped oscillations similar to the "overshoot" observed for several enzymes in developing systems (Moog, 1971) and following refeeding (Freedland and Szep-

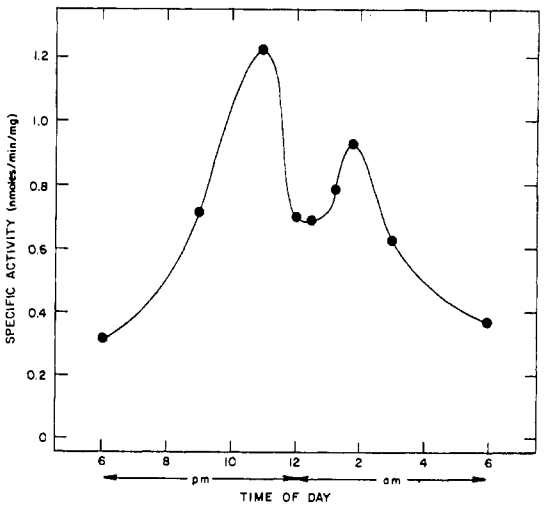

FIGURE 4: Cyclic variations in HMG-CoA reductase activity in Sprague-Dawley rats. Rats were housed in a different building from those used for other experiments. Four 100- to 120-g female rats were killed at each time. Each data point represents the mean value for two preparations of microsomes incubated and assayed under standard conditions (see Methods) using 0.6–2.4 mg of microsomal protein/assay.

TABLE II: Effect of Dietary Cholesterol and of Fasting on the Cyclic Rise in HMG-CoA Reductase Activity.[a]

	HMG-CoA Reductase Activity in Microsomes from Livers of Rats Fed					
	Control Diet		Control Diet + 5% Cholesterol		Fasted	
Time (EST)	Sp Act. (nmoles/min per mg)	Fraction of 6 p.m. Act. (%)	Sp Act. (nmoles/min per mg)	Fraction of 6 p.m. Act. (%)	Sp Act. (nmoles/min per mg)	Fraction of 6 p.m. Act. (%)
			Experiment 1			
6:00 p.m.	0.169 ± 0.007[b]	(100)	0.121 ± 0.002	(100)	0.093 ± 0.009	(100)
10:00 p.m.	0.211 ± 0.008	125	0.049 ± 0.003	40	0.185 ± 0.013	199
12:00 p.m.	0.589 ± 0.026	349	0.073 ± 0.003	60	0.231 ± 0.011	248
1:45 a.m.	0.365 ± 0.014	216	0.075 ± 0.008	62	0.238 ± 0.021	356
			Experiment 2			
6:00 p.m.	0.188 ± 0.01	(100)	0.094 ± 0.002	(100)	0.093 ± 0.003	(100)
12:00 p.m.	0.452 ± 0.009	240	0.062 ± 0.006	66	0.276 ± 0.004	300
2:00 a.m.	0.579 ± 0.001	310	0.069 ± 0.008	74	0.308 ± 0.012	330

[a] Feeding of 5% cholesterol diet and fasting commenced at 8 a.m. and continued throughout the experiment. Incubations containing from 1.22- to 2.21-mg microsomal protein were performed as described under Methods. [b] Data are expressed as ± the standard error of the mean for triplicate assays.

esi, 1971). The second cyclic peak could represent such a damped oscillation. Alternatively, it could represent a periodic oscillation as the enzyme level rises to, and then declines from, a limiting value. At the low basal levels of HMG-CoA reductase, oscillations might be obscured by differences between animals.

This model suggests that the decline in HMG-CoA reductase activity following the cyclic peak is due to activation of cellular controls when the level of reductase, or of its end product, cholesterol, reaches critical values. The persistence of the cyclic rhythm in fasted rats favors the alternative hypothesis that the entire cyclic rhythm is internally programmed and is independent of the level of enzyme or end product. Since HMG-CoA reductase activity in rats fasted 36 hr is only about 5% of control activity, neither a high level of enzyme nor an elevated rate of cholesterol synthesis is required to initiate the cyclic decline in reductase levels.

Our ability to validate these or other possible causes of the dual cyclic peaks is hampered by the obscurity of the factors responsible for cyclic rhythms. Cyclic variations in food intake, with resulting variations in intracellular tryptophan levels, have been cited by Wurtman (1969) as triggering cyclic variations in hepatic enzymes. The persistence of the cyclic rhythm in rats fasted 36 hr suggests, however, that nutritional factors, which can profoundly affect reductase levels, do not in fact trigger the cyclic rhythm in HMG-CoA reductase.

Suppression of the cyclic rhythm by cholesterol and persistence of the rhythm in fasted rats suggest that fasting and cholesterol feeding may regulate HMG-CoA reductase by different mechanisms. Fasting may act to affect the levels of numerous enzymes and cellular processes (Freedland and Szepesi, 1971) while cholesterol affects HMG-CoA reductase and cholesterol synthesis in a highly specific way (Siperstein and Guest, 1960).

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