THE MECHANISM OF THE DIURNAL VARIATION OF HEPATIC HMG-CoA REDUCTASE ACTIVITY IN THE RAT

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SUMMARY. Direct measurements of the incorporation of ^{3}H-leucine into highly purified β-hydroxy-β-methylglutaryl CoA reductase (E.C. 1.1.1.34) have shown that the diurnal variation in activity observed in crude fractions is due to synthesis of new enzyme protein for approximately six hours, followed by complete cessation of synthesis for a duration of approximately 15 hours. The rate of degradation does not appear to be significantly altered.

The molecular mechanisms of the control of isoprenoid synthesis in mammals have been the subject of investigation by several laboratories (for a recent review see Ref. 1). The hepatic synthesis of cholesterol is regulated to a major extent by the activity of β-hydroxy-β-methylglutaryl CoA reductase which catalyzes the NADPH dependent reduction of HMG-CoA to MVA. The activity of this enzyme, as measured in microsomal fractions, is drastically reduced after cholesterol feeding (e.g., 2), and is increased by various treatments such as Triton WR 1339 (3), noradrenaline (4), or biliary drainage (5). Back, Hamprecht and Lynen (6), Hamprecht, Nüssler and Lynen (7), Kandutsch and Saucier (3), Shapiro and Rodwell (8), Dugan et al. (9), and others have demonstrated large diurnal variations in the activity of the enzyme and cholesterol synthesis in mouse and rat liver preparations; it has been suggested that this phenomenon is due to changes in the rate of synthesis and/or degradation of the enzyme while inhibition by cholesterol

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Abbreviations: MVA, mevalonic acid; HMG-CoA, β-hydroxy-β-methylglutaryl-CoA.

feeding may be due to the action of an as yet undiscovered inhibitor (e.g., 10).

In the aforementioned work data was obtained via measurement of HMG-CoA reductase activity in crude microsomal preparations. A more direct approach to studying the regulation of enzyme levels is to determine the rate of synthesis and degradation of the isolated enzyme protein. We report here studies on the incorporation of ^{3}H-leucine into the highly purified preparation of HMG-CoA reductase isolated by Kawachi and Rudney (11).

Methods

Assay of HMG-CoA Reductase. Incubation conditions were as described by Kawachi and Rudney (11) but using 1 ml or 2 ml volumes. After termination of the reaction with NaOH, addition of an accurately known quantity of MVA-lactone, acidification with H_2SO_4 and saturation of the solution with Na_2SO_4, MVA-lactone was extracted with diethyl ether (5 x 1 vol). The ether extract, dried by anhydrous Na_2SO_4, was divided into two aliquots. One fraction, after evaporation to a small volume by a stream of nitrogen, was quantitatively applied to a thin layer chromatographic plate of silica gel G (0.3 mm wet thickness), which was developed with benzene:acetone (1:1 v/v). MVA-lactone markers were visualized by spraying successively with 2 M NH_2OH (pH 7.0) and a ferric chloride solution. The area corresponding to MVA-lactone was scraped from the plate directly into a scintillation counter vial. The amount of MVA-lactone in the other fraction was determined by the hydroxamate method of Lynen and Grassl (12), or by the gas chromatographic method of Hinse and Lupien (13).

Animals and Isolation of Enzyme. Female rats, approx 200 g, were obtained from Sprague-Dawley, Inc., Madison, Wisconsin, and were allowed free access to Rockland rat chow and water. At the specified times the radioactive amino acid dissolved in 0.1 ml isotonic saline was injected intraperitoneally. After an appropriate time interval the rats were killed

by decapitation, or injected with 25 mg unlabelled leucine dissolved in isotonic saline.

HMG-CoA reductase was isolated according to the method described by Kawachi and Rudney (11) through chromatography on Sephadex G-200. This material was contaminated by a minor component (as shown by disc gel electrophoresis).

Results and Discussion

Table 1 displays the data from an HMG-CoA reductase preparation purified from the livers of rats injected with ^{3}H-leucine. The correspondence in specific radioactivity and specific enzyme activity in the final purification steps is evidence that the radioactivity observed is indeed constitutive of the enzyme molecule.

Table 1. Purification of ^{3}H-HMG-CoA-Reductase from Rat Liver

	Enzyme Activity	mg Protein	% Recovery of Activity	^{3}H dpm	dpm ^{3}H/mg Protein
Microsomal fraction	1.9	890	100	1.35×10^{6}	1,521
Deoxycholate supernatant	5.7	425	140	6.05×10^{5}	1,400
Ammonium sulfate fraction	10.1	169	109	2.48×10^{5}	1,470
DEAE cellulose	78.0	18.1	82	3.5×10^{4}	1,950
Hydroxyapatite	277.0	3.1	51	7.65×10^{3}	2,470
G-200 Sephadex	301	2.7	49	7.0×10^{3}	2,630
Disc-gel electrophoresis*	-	0.5	-	1.35×10^{3}	2,700

Twenty rats were injected intraperitoneally with 50 μCi each of ^{3}H-leucine (390 mCi/mmole) in 0.1 ml isotonic saline. Protein in column fractions was precipitated with trichloroacetic acid after addition of 1 mg bovine albumen to each tube and filtered on Whatman 3GA discs. After digestion with Beckman Soluene-100 radioactivity was determined directly using a toluene based scintillant. Protein was determined by the Lowry-Folin method. Enzyme activities expressed as nmoles MVA/mg protein/hr.

* 0.5 mg protein was subjected to polyacrylamide disc gel electrophoresis in 12 tubes. Gels were sliced, dissolved in piperidine and counted (15).

Figure 1 shows data obtained by isolation of the enzyme 35 min after the injection with ^{3}H-leucine. The microsomal enzymic activity was measured at each time point and the total radioactivity of the purified enzyme, from the same rats, was subsequently determined.

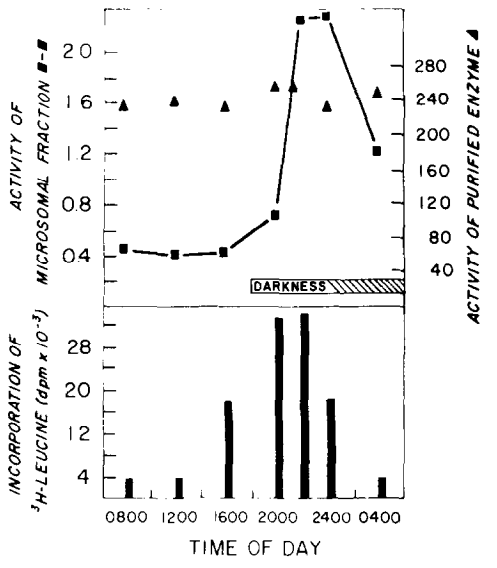

Figure 1. Variation of Microsomal HMG-CoA Reductase Activity and Incorporation of ^{3}H-leucine. Groups of 8 rats were injected with 40 µCi of ^{3}H-leucine (390 mCi/mmole) at the times shown. Livers were removed 35 min later and enzyme purification commenced. Assays are as described in the text and legend to Table 1.

The specific activity of the purified enzymes does not change during the diurnal variation period. The variation in incorporation of leucine reflects the rise and fall of the enzyme activity in crude fractions during the diurnal period; however, the total radioactivity in microsomal fractions remains constant as expected. It is interesting to note that the increase in incorporation seems to precede the rise in microsomal enzymic activity. This may be merely an artifact of the system, reflecting the time required for increased synthesis of protein to be reflected as enzyme activity, or it could indicate a more complex control where the enzyme is inhibited at that time.

Assuming that the incorporation of amino acid reflects enzymic syn-

thesis (and not a large decrease in amino acid pool size - unlikely here since the animals begin to feed at the same time), it was reasonable to suggest that the diurnal variation in enzyme is at least partly due to a change in rate of synthesis. To gain some insight into the role played by degradation of the protein, groups of rats were injected with ^{3}H-leucine, then 35 min later with 25 mg unlabeled leucine. The total radioactivity and specific radioactivity of purified HMG-CoA reductase were determined at time intervals as shown in Figure 2. The contrast between the results obtained from injections at the time of maximum incorporation and the injection just before the rate of incorporation starts to increase is striking. In the former case, the total isolatable radioactivity and protein declines rapidly, while the specific radioactivity remains essentially constant. Conversely, while the enzyme activity and the amount of isolatable protein were rising, the specific radioactivity of the "pulse" labeled protein decreased rapidly. Our explanation of these results is that synthesis of new enzyme protein essentially stops after approximately six hours. Protein labeled at this time is not diluted by newly synthesized enzyme.

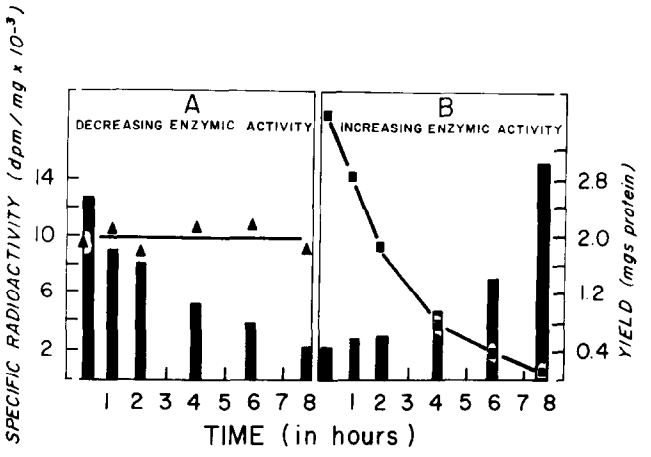

Figure 2. The Variation of Specific Radioactivity after a Single Injection of ^{3}H-leucine. Groups of 36 rats were injected with 25 μCi ^{3}H-leucine each (440 μCi/mmole) at 2200 hr (A) or 1630 hr (B) and with 25 mg unlabeled leucine 35 min later. Livers of 6 rats were excised at the times shown and the enzyme isolated. Dotted lines represent specific radioactivity, histogram the total radioactivity.

The isolatable radioactivity at intervals after a single pulse label is plotted on semilogarithmic scales (Fig. 3). The decay in radioactivity, both while the enzyme activity in the microsomal fraction is rising and while it is falling, follows approximately first order kinetics. From this data the half life of the protein is approximately 3 hr. The slight differences in slope of the two lines can be explained by reutilization of the radioactive amino acid.

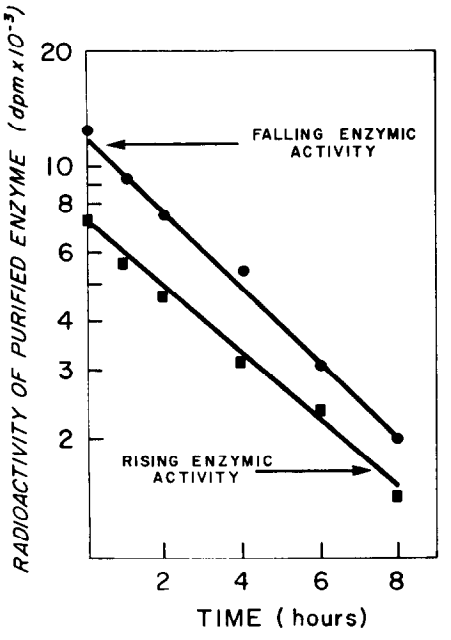

Figure 3. Decay of Radioactivity Associated with HMG-CoA Reductase. Experimental conditions are as described for Figure 2.

If the assumption is made that the enzyme behaves identically in the purification procedures at high and low levels, and in the light of mixing experiments (which will be reported elsewhere) which failed to show an inhibitor of the enzyme during the low part of the cycle, this data unequivocally demonstrates that the diurnal variation in activity is due to an increase in the rate of synthesis of the enzyme during the rising portion of the cycle and an apparent complete cessation of enzyme synthesis during the falling phase. Present experimental conditions make it difficult to pin point the exact initiation of these phases. Changes in the rate of

degradation do not seem to be significantly different during both phases of the cycle.

The conclusion that it is a rapidly changing rate of enzyme synthesis, rather than a marked change in the rate of degradation, is consistent with data obtained by others using antibiotic inhibitors of protein synthesis. Kandutsch and Saucier (3) were able to prevent the cyclic rise in activity using puromycin, while cycloheximide had the same effect in the experiments of Shapiro and Rodwell (14). Our results do not throw any light on the prevention of the decrease in activity brought about by cycloheximide (14) unless, of course, the degradation of HMG-CoA reductase is effected by a protein also with a very short half life.

The demonstration of this work on complete repression of enzyme synthesis playing a role in a diurnal variation is worthy of note. The nature of the signals for the repression and subsequent derepression of the synthesis of this key enzyme in isoprenoid biosynthesis remains to be investigated.

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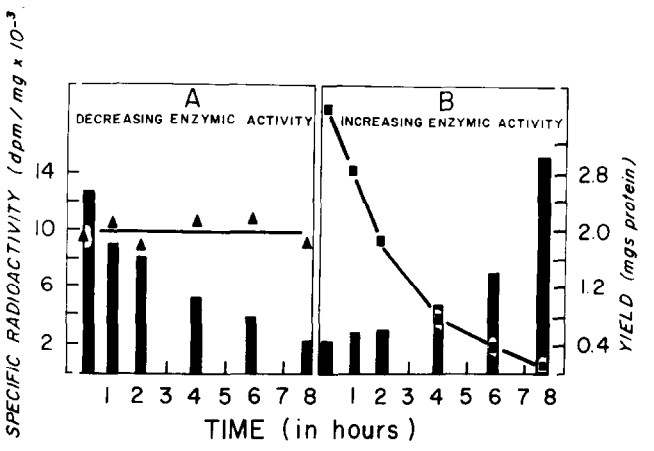

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