3-Hydroxy-3-methylglutaryl-coenzyme A reductase in normal and dystrophic hamsters

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The activity and diurnal variation of 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34; HMG-CoA reductase), the rate-limiting enzyme in the cholesterol-biosynthetic pathway, of normal and dystrophic hamsters was determined. Liver enzyme activity showed a diurnal pattern in the normal male, but not in the dystrophic male. Enzyme values in normal males at the midpoint of the 12 h dark period were 10 times those in dystrophic males. No evidence for diurnal variation in the HMG-CoA reductase of the brain was observed, and similar activities were found for normal and dystrophic animals. The apparent K_m for HMG-CoA reductase from the liver of normal or dystrophic hamsters was approx. $9\mu\text{M}$, and the V_{max} was 5.9 and 21.7 pmol/min per mg of protein for dystrophic and normal hamsters respectively.

Cholesterol is required by all tissues of the body; the liver, intestine, kidney and immature nervous system are the major sites of synthesis (Dietschy & Wilson, 1970; Hughes-Wiley *et al.*, 1977). The rate-limiting enzyme in the biosynthetic pathway for cholesterol is HMG-CoA reductase (EC 1.1.1.34) (Rodwell *et al.*, 1976). In rat liver and intestine, this enzyme follows a diurnal pattern, with the highest activity found at midnight (Shefer *et al.*, 1972; Rodwell *et al.*, 1976). An increase in the dietary intake of cholesterol, a decrease in energy intake, or the presence of bile acids, all act to decrease cholesterol synthesis. Each of these physiological variables has been shown to exert an effect on HMG-CoA reductase (Bochenek & Rodgers, 1979; Sabine & James, 1976; Arebalo *et al.*, 1981). Because an increase in cholesterol content of skeletal-muscle subcellular fractions from patients and animals with muscular dystrophy has been reported (Slack *et al.*, 1980; Hughes, 1972; Owens *et al.*, 1972; Owens & Hughes, 1970), we decided to investigate the activity of this enzyme in the normal Syrian and the dystrophic hamster. A genetically dystrophic strain of hamster (UMX 7.1) with marked cardiomyopathy was chosen for our study.

Materials and methods

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: D-glucose 6-phosphate (monosodium salt), NADP⁺

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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(monosodium salt), glucose 6-phosphate dehydrogenase and HMG-CoA (sodium salt). DL-3-Hydroxy-3-[³H]methylglutaryl-CoA (sp. radioactivity 11.7 Ci/mmol) and DL-[2-¹⁴C]mevalonic acid (sp. radioactivity 47.0 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A.

The scintillation cocktail was prepared as described by Anderson & McClure (1973).

The radiochemical purity of [³H]HMG-CoA and [¹⁴C]mevalonic acid was verified by using silica-gel-G-coated glass plates with butan-1-ol/acetic acid/water (5:2:3, by vol.) or propan-1-ol/conc.NH₃ (sp.gr. 0.880) (7:3, v/v) as the solvent system respectively.

Normal Syrian hamsters were purchased from Charles River, St. Constant, Quebec, Canada. Dystrophic hamsters (UM-X7.1) were obtained from Dr. G. Jasmin of the University of Montreal. The animals (130–140 days old) were maintained on Purina Rodent Laboratory Chow no. 5001 *ad libitum*, and exposed to light from 06:00 h to 18:00 h, followed by a 12 h dark period.

Hamsters were weighed, decapitated, and the livers and brains immediately excised and placed in 20–25 ml of ice-cold 0.9% NaCl. The livers and brains were homogenized in 3.5 vol. (v/w) of 0.1 M-sucrose/50 mM-KCl/40 mM-KH₂PO₄, pH 7.2 (buffer I), with a Potter-type homogenizer (clearance 0.5 mm; ten strokes for liver, six strokes for brain). The brain homogenate was centrifuged for 15 min at 12000 g then resuspended in 30 mM-EDTA/70 mM-NaCl/10 mM-mercaptoethanol, pH 6.8 (buffer II). The liver homogenate was centrifuged twice at

12000 g for 15 min and the supernatant at 100000 g for 1 h to obtain a microsomal pellet. The pellet was resuspended in buffer I and re-centrifuged at 100000 g for 45 min. The washed liver pellet was suspended in buffer II (one-fifth of the volume used for homogenization) and assayed for enzyme activity.

HMG-CoA reductase activity was measured by a modification of the procedure used by Cayen & Dvornik (1979); 5–8 mg of liver microsomal or brain homogenate protein was added to 1 ml of buffer II, which also contained 30 μ mol of glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, 3 μ mol of NADP⁺ and 4 nmol of [³H]HMG-CoA (3.2×10^5 d.p.m.). Incubations were performed in 1.5 cm $\times$ 15 cm tubes for 30 min at 37°C in a Dubnoff metabolic shaker. Enzymic activity was terminated and lactonization was enhanced by the addition of 0.2 ml of 10 M-HCl. To each sample [¹⁴C]mevalonic acid (1.75×10^4 d.p.m.) was added as an internal standard. The samples were shaken at 37°C for 30 min to allow complete lactonization of the mevalonic acid. Anhydrous Na₂SO₄ (1 g) was added to each tube and the mixture extracted with 3 $\times$ 8 ml of anhydrous diethyl ether. The extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μ l of acetone and 50 μ l was applied to a silica-gel-G-coated glass thin-layer plate and developed in benzene/acetone (1:1, v/v). The silica was scraped from the plate into counting vials which, after addition of a xylene/Triton cocktail (Anderson & McClure, 1973), were counted for radioactivity in a liquid-scintillation spectrometer. The enzyme activity was corrected by subtracting the activity of a blank in which the microsomal protein was boiled for 15 min and to which 0.2 ml of 10 M-HCl was added before the substrate. The total recovery of ¹⁴C was used to calculate the conversion of [³H]HMG-CoA into [³H]mevalonic acid as follows:

$$\mu\text{mol of mevalonate formed} = \frac{\text{d.p.m. of } ^{14}\text{C-labelled internal standard added}}{\text{d.p.m. of } ^{14}\text{C-labelled internal standard recovered}} \times \frac{\text{d.p.m. of } [^3\text{H}]\text{mevalonate recovered}}{\text{radioactivity of HMG-CoA added}}$$

Protein was determined by the method of Lowry *et al.* (1951), and statistical analysis was by Student's *t* test. A preliminary report describing some of the results has been published (Greenough & Boegman, 1981).

Results

Recovery of the [¹⁴C]mevalonolactone internal standard from the silica-gel plates was 55–60%.

Table 1. *Liver microsomal HMG-CoA reductase activity in the normal and dystrophic hamster*

HMG-CoA reductase activity was determined over a 24 h period in liver microsomal fractions from normal and dystrophic hamsters. Results presented are means $\pm$ S.E.M. Values in parentheses indicate the numbers of experiments, with triplicate assays carried out in each experiment.

Time (h)	Specific activity (pmol/min per mg of protein)	
	Normal	Dystrophic
09:00	6.00 $\pm$ 1.20 (n = 3)	3.88 $\pm$ 0.31 (n = 3)
12:00	1.80 $\pm$ 0.40 (n = 6)	3.83 $\pm$ 0.30 (n = 3)
18:00	4.33 $\pm$ 1.20 (n = 6)	3.88 $\pm$ 0.38 (n = 3)
24:00	16.32 $\pm$ 4.44 (n = 7)	1.63 $\pm$ 0.14 (n = 3)

The HMG-CoA reductase in normal male hamster liver showed a diurnal pattern similar to that described in rat liver and intestine (Shefer *et al.*, 1972; Rodwell *et al.*, 1976) (Table 1). The enzyme activity at 24:00 h was approx. 10 times that at 12:00 h ($P < 0.01$). This 10-fold increase in activity was first noted in the rat by Kandutsch & Saucier (1969) and confirmed by Shapiro & Rodwell (1969) and Hamprecht *et al.* (1969).

No diurnal variation in liver HMG-CoA reductase was found in the male dystrophic hamster (Table 1). The enzyme activity at the midpoint of the 12 h dark period showed a significant decrease in the dystrophic hamster ($P < 0.05$), with normal males having a 10-fold higher activity. The difference between dystrophic and normal males was not significant at 12:00 and 18:00 h. Lineweaver–Burk analysis of HMG-CoA reductase indicated that the

V_{max} for the liver enzyme was lower in dystrophic than normal hamsters (5.92 and 21.68 pmol/min per mg of protein respectively), whereas the apparent K_m values were approximately the same, 9.12 and 9.50 μ M respectively (Fig. 1).

Brain HMG-CoA reductase activity in normal and dystrophic male hamsters showed no evidence of diurnal variation. The enzyme activity at different time intervals was therefore pooled to give a mean HMG-CoA reductase activity for each group of

Fig. 1. Lineweaver-Burk plots of hepatic microsomal HMG-CoA reductase activity from normal and dystrophic hamsters

Each point represents the mean of triplicate assays on pooled microsomal fractions from three normal or dystrophic hamsters.

animals. The HMG-CoA reductase activity for normal animals was 5.38 ± 0.56 ($n = 13$), and that for dystrophic hamsters was 5.65 ± 0.67 ($n = 8$) pmol/min per mg of protein. No significant difference in enzyme activity was noted between each group.

Discussion

Liver HMG-CoA reductase activity in normal Syrian hamsters follows a diurnal pattern similar to that reported for rat liver and intestine (Shefer *et al.*, 1972; Rodwell *et al.*, 1976). Dystrophic hamsters, however, showed no evidence of a diurnal pattern, and enzyme activity at the peak of the 12h dark period was one-tenth of that in normal hamsters. The absence of a diurnal pattern and the decreased activity of hepatic HMG-CoA reductase activity in the dystrophic hamster, which has been shown to have increased membrane cholesterol (Slack *et al.*, 1980), is therefore different from that found in some other disease states, e.g. cancer, where feedback control is lacking although diurnal rhythm is retained (Sabine, 1980).

The liver is the main source of plasma cholesterol in man and rat (Dietschy & Wilson, 1970), and the rate of cholesterol synthesis (i.e. HMG-CoA reductase activity) decreases as the amount of cholesterol absorbed from the gut increases (Siperstein, 1970; Sabine, 1975). Since there is evidence for increased concentrations of cholesterol in the dystrophic hamster (Slack *et al.*, 1980; Barakat *et al.*, 1976), the activity of the rate-limiting enzyme, i.e. HMG-CoA reductase, might be expected to be lower in this animal. In our experiments, a 10-fold decrease was observed.

Two hypotheses have been put forward to explain the molecular mechanism controlling the activity of

HMG-CoA reductase. In the first, a reversible phosphorylation-dephosphorylation has been proposed to modulate the activity (Ingebritsen *et al.*, 1978; Nordstrom *et al.*, 1977), whereas in the second, the enzyme is thought to be regulated by the fluidity of the surrounding membrane microenvironment (Sabine & James, 1976; Mitropoulos & Venkatesan, 1977). The increased content of cholesterol in subcellular membrane fractions isolated from the dystrophic hamster (Slack *et al.*, 1980) could alter the membrane microenvironment (Demel & DeKruyff, 1976) and may account for the absence of a diurnal pattern and the low enzyme activity in the dystrophic hamster.

Crude homogenates rather than microsomal fractions were used for brain enzyme assays, because it has been reported that much of the HMG-CoA reductase and the microsomal marker NADPH-cytochrome *c* reductase activity is removed from brain homogenates by low-speed centrifugation (Sudjic & Booth, 1976; Aragon *et al.*, 1978). It is unlikely that our results are due to hydrolysis of the substrate by HMG-CoA lyase, since Maltese & Volpe (1979) found negligible lyase activity in a similar preparation from rat brain, and in our experiments, 88% of the substrate remained unhydrolysed.

Our kinetic data suggest that the affinity of HMG-CoA for HMG-CoA reductase is the same in both the normal and dystrophic hamster, but the rate of enzyme catalysis is greater in the normal hamster. Sabine & James (1976) reported that K_m values for HMG-CoA reductase did not change during feedback inhibition of the enzyme by cholesterol, indicating that feedback by classical allosteric inhibition was unlikely in the regulation of this enzyme. More recently, Sinensky *et al.* (1979) demonstrated that feedback inhibition of the enzyme is due to decreased enzyme synthesis. The greater V_{max} observed in the normal hamster therefore appears to be due to an increase in the amount of enzyme.

In summary, hepatic HMG-CoA reductase activity in the dystrophic hamster is lower than that in the normal hamster and does not show a diurnal pattern. Although the V_{max} is different, the apparent K_m appears to be the same in the normal and dystrophic hamster.

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