# Diurnal rhythm of rat liver cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase

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Rat liver cytosolic 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase exhibits a diurnal rhythm of enzyme activity which coincides with the diurnal rhythm of HMG-CoA synthase protein. The peaks of activity and protein (determined by SDS/PAGE and immunoblotting) both occur at D10 (the tenth hour of the daily 12 h dark cycle). The peak of mRNA levels (measured by slot-blot hybridization of liver RNA) is slightly advanced with respect to that of protein, by about 4 h, and shows a maximum at D6. Cytosolic HMG-CoA synthase activity and protein in rats fed on a normal diet were approx. 2-fold higher during the peak at D10 than in the nadir at D2. HMG-CoA synthase mRNA levels were approx. 4-fold higher during the peak at D6 than in the nadir at D2. These results point to a transcriptional and translational regulation of the cytosolic HMG-CoA synthase.

## INTRODUCTION

The formation of mevalonate from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) catalysed by HMG-CoA reductase is believed to be the rate-limiting step in cholesterol biosynthesis [1,2]. Mevalonate also serves as a precursor for non-sterol products such as ubiquinone, dolichol and isopentenyl t-RNA [3]. However, the synthesis in cytosol of HMG-CoA, catalysed by HMG-CoA synthase, that precedes the reaction catalysed by HMG-CoA reductase is also a regulatory step in the cholesterol pathway [4]. The operation of this pathway is subject to feedback suppression by cholesterol and other end-products exerting their main influence on these two regulatory steps [4–6].

HMG-CoA reductase has been extensively studied in the past, and it is now well documented that, in addition to feedback repression by cholesterol, it is influenced by protein phosphorylation [7,8], by diet [1] and by drugs [9], and also exhibits a diurnal rhythm of activity [9,10]. On the other hand, the influence of hormones and nutritional states on the activity of HMG-CoA synthase has been less studied. In liver two distinct forms of HMG-CoA synthase are present, a mitochondrial and a cytosolic form [11]. Most of this enzyme is located in the mitochondria, and the product of its activity, HMG-CoA, is directed towards ketone bodies, which are exported by liver cells under conditions of rapid fatty acid oxidation [12,13].

To investigate further the regulation of cytosolic HMG-CoA synthase as a point of control of the cholesterol pathway, we have examined whether or not there are changes in specific activity of the hepatic enzyme during the diurnal cycle. Quantification of hepatic cytosolic HMG-CoA synthase protein was also performed to determine whether potential variations in enzyme activity could be due to changes in enzyme mass or to activation of the enzyme. Furthermore, the levels of mRNA for cytosolic HMG-CoA synthase were determined. We found that there is a daily rise and fall in cytosolic HMG-CoA synthase activity which is correlated with the mass of the enzyme and with the level of mRNA for cytosolic HMG-CoA synthase. These changes in the levels of cytosolic HMG-CoA synthase mRNA appear to play a major role in producing the diurnal behaviour.

## MATERIALS AND METHODS

### Materials

$^{32}$P-labelled nucleotides were purchased from New England Nuclear and Amersham. [1-$^{14}$C]Acetyl-CoA (59.3 mCi/mmol) came from New England Nuclear and $^{125}$I-labelled Protein A from Amersham. Acetoacetyl-CoA and acetyl-CoA were purchased from Sigma. DEAE-cellulose DE-32 was obtained from Whatman; BA 83 nitrocellulose paper was from Schleicher & Schuell; and blotting nylon membranes were from Bio-Rad.

### Buffers

Buffer A: 40 mM-potassium phosphate, 100 mM-sucrose, 30 mM-EDTA, 50 mM-KCl and 10 mM-β-mercaptoethanol, pH 7.2. Buffer B: 60 mM-potassium phosphate, 0.1 mM-EDTA and 10 mM-β-mercaptoethanol, pH 7.0. Buffer C: 150 mM-potassium phosphate, 0.1 mM-EDTA and 10 mM-β-mercaptoethanol, pH 7.0.

### Animals

Male Wistar rats (125–150 g) were kept under a 12 h-light/12 h-dark cycle for 15 days before use and fed *ad libitum*. Hepatic cytosol was prepared at 4 °C by homogenizing the livers in 3 vol. of buffer A in a Potter–Elvehjem homogenizer with 10 strokes of a Teflon pestle. The homogenate was centrifuged twice at 30000 *g* for 20 min. The last supernatant was centrifuged at 100000 *g* for 60 min and frozen at −80 °C in 1 ml portions.

### Partial purification of cytosolic protein

Cytosolic HMG-CoA synthase activity cannot be measured directly in the cytosolic fraction because of the presence of other activities that interfere with the assay method [4,12]. We have carried out a procedure to measure accurately cytosolic HMG-CoA synthase activity by partial purification of the protein by batch fractionation in DEAE-cellulose. Cytosol (0.5 ml) was loaded into an Eppendorf tube containing 0.5 ml of DEAE-

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cellulose, and the mixture was shaken for 15 min. The samples were then centrifuged at 15000 rev./min for 2 min in a microfuge and the supernatant fraction was discarded. The DEAE-cellulose pellet was washed successively with buffers A and B, and the supernatants were discarded; the cytosolic HMG-CoA synthase activity was eluted with 0.5 ml of buffer C.

## HMG-CoA synthase assay

A modification of the radiochemical method of Clinkenbeard *et al.* [12] was used to measure the synthesis of $[^{14}C]$HMG-CoA from $[^{14}C]$acetyl-CoA. The standard reaction contained the following components, in a final volume of 0.2 ml: 0.1 M-Tris/HCl; 20 $\mu$M-acetoacetyl-CoA; 0.1 mM-EDTA; 20 mM-MgCl$_2$; 20 $\mu$g of cytosolic protein partially purified by DEAE-cellulose fractionation; and 0.2 mM-$[1-^{14}C]$acetyl-CoA (4000 c.p.m./nmol), pH 8.0. The reaction mixture was preincubated for 2 min at 30 °C, and the reaction was started by adding $[1-^{14}C]$acetyl-CoA. After 10 min of incubation at 30 °C, the whole reaction mixture was removed and added to a scintillation-counting vial that contained 0.3 ml of 6 M-HCl. Each counting vial was then heated in an oven at 95 °C for 90 min, and non-volatile radioactivity was counted to determine the amount of $[^{14}C]$HMG-CoA formed. Enzyme activity was linear with time and protein concentration. Protein was determined by the Bradford method [14].

## Antibodies against synthetic peptides

Peptides corresponding to amino acids 303–317 (DVKLEDTYFDRDVEK) (peptide 1) and 506–520 (TAA-ESESAVISNGEH) (peptide 2) of hamster cytosolic HMG-CoA synthase [15] were synthesized by using the solid-phase methods developed by Marglin & Merrifield [16]. In both peptides, a cysteine residue was added to the N-terminal end. The compositions of the peptides were confirmed by amino acid analysis. The peptides were coupled to keyhole-limpet haemocyanin with maleimidobenzoyl-N-hydroxysuccinimide ester [17]. New Zealand White rabbits were injected sub-cutaneously on days 0, 14 and 28 with a mixture of the two peptides (0.1 mg of each) coupled to haemocyanin. The synthetic peptide–haemocyanin conjugates were emulsified with Freund's complete adjuvant (day 0) or incomplete adjuvant (days 14 and 28) in a total of 1 ml. Rabbits were bled on day 35 and also 7 days after each booster injection. Antibodies were purified by $(NH_4)_2SO_4$ fractionation [18]. Immunoblotting of cytosolic HMG-CoA synthase was carried out as described by Beisiegel *et al.* [19], with minor modifications.

## RNA analysis

Total RNA from rat liver was isolated as described [20] with minor modifications. Slot-blotting was performed by following the manufacturer's recommendations (Zeta-Probe Blotting Membranes; Bio-Rad). For measurement of cytosolic HMG-CoA synthase mRNA, 0.6 $\mu$g of RNA was loaded in each well and then transferred to a nylon membrane and probed with the $^{32}$P-labelled 1.1 kb fragment (*Apa*I–*Sac*I) of the rat liver cytosolic HMG-CoA synthase cDNA [21]. In order to confirm the specificity of the probe used, 1 $\mu$g of poly(A)$^+$ RNA, purified by oligo(dT)–cellulose chromatography [22], was fractionated on 1% agarose/formaldehyde gel, transferred and u.v.-cross-linked to a nylon membrane. The nylon membrane was then hybridized either with the 1.1 kb *Apa*I–*Sac*I probe or with the 0.5 kb *Sac*I probe which corresponds to the mitochondrial HMG-CoA synthase cDNA [11], and washed according to the manufacturer's recommendations.

## RESULTS AND DISCUSSION

Our first goal was to determine cytosolic HMG-CoA synthase activity in liver with accuracy, avoiding the interference by the mitochondrial activity present in the samples. The mitochondrial/cytosolic HMG-CoA synthase ratio is 8:1 [13], and therefore any residual mitochondrial HMG-CoA synthase activity present in the samples would mask the activity of the cytosolic HMG-CoA synthase. The DEAE-cellulose batch treatment removed this interference, as no change was detected in HMG-CoA synthase activity when the treated samples were measured in either the presence or the absence of 20 mM-MgCl$_2$. It is well documented that a concentration of 20 mM-Mg$^{2+}$ inhibits the activity of mitochondrial HMG-CoA synthase by about 65% without any significant change in the cytosolic enzymic activity [23].

The second aim was to develop specific antibodies against cytosolic HMG-CoA synthase that could not recognize the mitochondrial enzyme. These were obtained after injecting
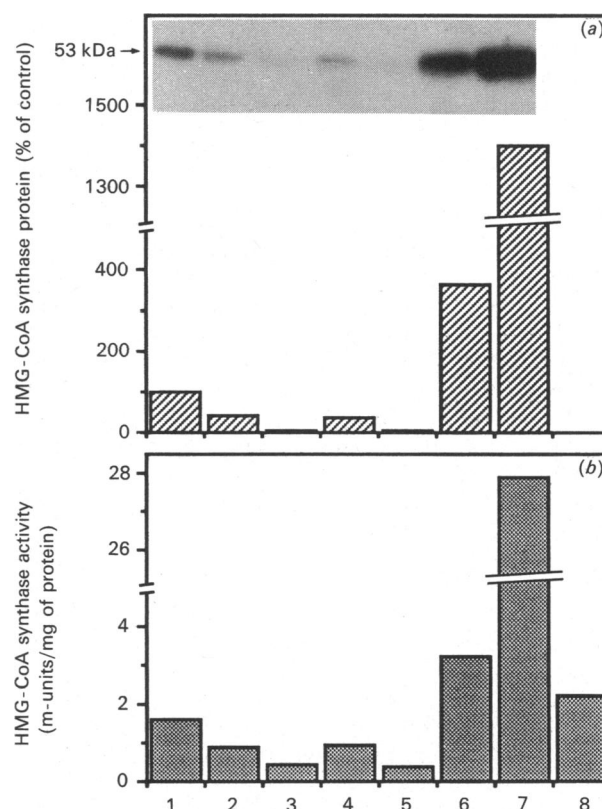


**Fig. 1. Quantification of cytosolic HMG-CoA synthase protein by immunoblot**

(*a*) Rat liver cytosol was isolated from rats killed at the seventh hour of the dark period after feeding on a normal diet (control, lane 1), a diet supplemented with 3% cholesterol for 10 days (lane 5), or 5% cholestyramine for 5 days (lane 6), or 5% cholestyramine plus 0.1% mevinolin for 5 days (lane 7), or fed on a normal diet and then given mevalonolactone by stomach intubation 3 h before cytosolic iso-lation (1 mg/g body wt.) (lane 4), or from rats fasted for 24 h (lane 2) or 48 h (lane 3). Lanes 1–6 contained 100 $\mu$g of cytosolic protein and lane 7 contained 50 $\mu$g. Lane 8 contained 100 $\mu$g of mitochondrial protein from a 24 h-fasted rat. HMG-CoA synthase protein values are expressed relative to those of control rats, defined as 100%. The inset shows the Western blot corresponding to a representative experiment from which the densitometric scan was obtained. Arrow indicates the molecular mass (kDa) of cytosolic HMG-CoA synthase. (*b*) Enzymic activities of the samples corresponding to the lanes shown above. The mitochondrial HMG-CoA synthase activity was determined in the absence of Mg$^{2+}$.
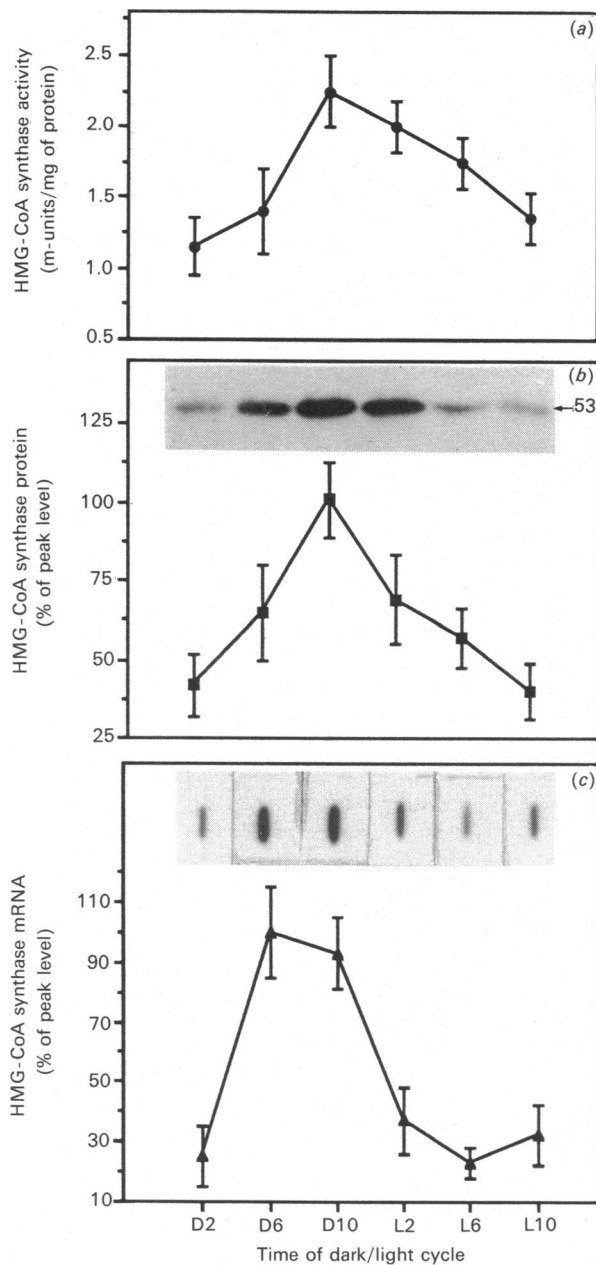
**Fig. 2. Quantitative comparison of cytosolic HMG-CoA synthase specific activity and levels of cytosolic HMG-CoA synthase protein and mRNA over the course of the diurnal cycle**

Liver cytosol and total RNA were prepared at designated times from rats fed on a normal diet. Cytosolic HMG-CoA synthase specific activity (●) was determined as described in the Materials and methods section. Levels of cytosolic HMG-CoA synthase protein (■) were quantified by densitometric scanning of Western-blot analysis; the area of the densitometric scan at D10 (the peak level) was set at 100% for the purpose of comparison. Levels of cytosolic HMG-CoA synthase mRNA (▲) were quantified by densitometric scanning; the area of the densitometric scan at D6 (the peak level) was set at 100% for the purpose of comparison. Data of specific activity, protein levels and mRNA levels are means ± S.D. for three experiments for samples obtained from different rats receiving the same diet and killed at the same hour. The inset in (b) shows a representative Western-blot analysis in which the same quantity of protein (100 μg) was applied to each lane. Arrow indicates the molecular mass (kDa) of cytosolic HMG-CoA synthase. The inset in (c) shows a representative slot-blot analysis in which 0.6 μg of total RNA was loaded in each well and then transferred to a nylon membrane and probed with the $^{32}$P-labelled 1.1 kb ApaI–SacI fragment of the cytosolic HMG-CoA synthase cDNA.

rabbits with synthetic peptides deduced from the nucleotide sequence corresponding to a cDNA clone of hamster cytosolic HMG-CoA synthase [15]. In addition, we have recently cloned and sequenced a cDNA for rat cytosolic HMG-CoA synthase [21]. The comparison of the deduced amino acid sequence with that of the hamster enzyme [15] shows that peptide 1 is 100% identical with that of the rat enzyme and that peptide 2 has three conservative substitutions with respect to the rat enzyme: Ala-2 and Ala-3 to Ser and Gly respectively, and Ser-5 is changed to Pro in the rat enzyme. The antibodies obtained did not recognize mitochondrial HMG-CoA synthase (53 kDa) (Fig. 1, lane 8), but they were able to reflect the expected variations in the mass of the cytosolic synthase (53 kDa) after nutritional or pharmacological treatment of the rats. Fig. 1 shows the results of a representative experiment reflecting the changes in cytosolic HMG-CoA synthase protein observed in rats fasted for 1 or 2 days, rats given mevalonolactone, rats fed with cholesterol, or rats fed with cholestyramine or cholestyramine plus mevinolin. This experiment was carried out five times, and the results obtained were essentially the same (results not shown). The enzymic activities paralleled the mass of protein observed in the blots.

Because of the known circadian rhythm in rat liver HMG-CoA reductase, we set out to determine whether a diurnal variation could be demonstrated in rat liver cytosolic HMG-CoA synthase, and if so, whether or not this rhythm would correspond with the rhythm of protein mass and mRNA for cytosolic HMG-CoA synthase. To this end, rats were exposed to a repetitive light/dark cycle for 2 weeks, and then groups of animals were killed at 4 h intervals throughout a 24 h cycle. Fig. 2 shows the results obtained for cytosolic HMG-CoA synthase activity (a), for mass of protein (b) or for mRNA levels (c). There is a diurnal rhythm in enzymic activity with a maximum at D10 (tenth hour of the 12 h dark cycle), which coincides with the maximum of cytosolic HMG-CoA synthase protein. The activity at D10 represents a 2-fold increase in relation to the basal activity seen at D2. A similar increase of approx. 2-fold is observed in cytosolic HMG-CoA synthase protein, from which it is concluded that no changes in cytosolic HMG-CoA synthase specific activity were produced throughout the diurnal cycle. In addition, mRNA for cytosolic HMG-CoA synthase presents, analogously, a diurnal rhythm, with the maximum located in the middle of the dark cycle, which means that the peak of mRNA is slightly advanced in relation to both enzymic activity and protein. There is an increase of about 4-fold in the level of cytosolic HMG-CoA synthase mRNA between the minimum at D2 and maxima at D6. Fig. 3 shows that the cDNA probe used was able to recognize only the mRNA for the cytosolic HMG-CoA synthase, but not that corresponding to the mitochondrial HMG-CoA synthase. These results suggest that the circadian rhythm of cytosolic HMG-CoA synthase from rat liver is a direct consequence of the cyclic variation of mature mRNA levels.

Clinkenbeard et al. [12] did not find this diurnal rhythm in rats maintained on a 12 h-light/12 h-dark cycle. We believe that a small mitochondrial contamination could mask the final result. Given that the percentage of mitochondrial HMG-CoA synthase activity is near 90% [13], a small contamination of the mitochondrial enzyme in the assay would be enough to abolish the effect of the cycle. To avoid interference by the mitochondrial HMG-CoA synthase, Balasubramaniam et al. [24] studied the diurnal cycle of the cytosolic HMG-CoA synthase activity in the adrenal gland, in which no significant synthesis of ketone bodies is observed. They found a circadian rhythm with an activity maximum located between D7 and D10, which agrees fairly well with our results.

It is also of value to compare our results with those of the HMG-CoA reductase diurnal cycle. Apparently, the synthase
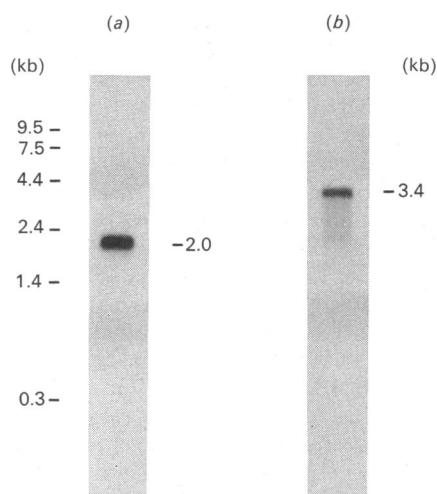
**Fig. 3. Northern-blot analysis of rat liver mRNA**

Poly(A)$^+$ RNA was isolated from rats fed on a normal diet and killed at the seventh hour of the dark period. A 1 μg portion was electrophoresed and hybridized with either (a) the 0.5 kb SacI fragment of the rat mitochondrial HMG-CoA synthase [11] or (b) the 1.1 kb ApaI–SacI fragment of the rat cytosolic HMG-CoA synthase cDNA [21], as described in the Materials and methods section. The positions of molecular-size markers are shown.

synthesis of the HMG-CoA) is low (left side of its curve), HMG-CoA reductase activity is maximal. Conversely, when reductase activity is minimal (right side of its curve), HMG-CoA synthesis is at the highest level. This reciprocal effect between HMG-CoA reductase and HMG-CoA synthase in the middle of the dark period may represent a fine co-ordinate regulation for mevalonate synthesis and also for the isoprenoid pathway.

peak was delayed by 4 h in comparison with the reductase peak. This is also observed in the adrenal gland [24]. A possible interpretation for this delay could be formulated in terms of transcriptional control caused by the product of the reaction, HMG-CoA. When the maximum of HMG-CoA reductase activity is reached, there is a decrease in HMG-CoA concentration as it is transformed into mevalonate. Then this decrease could produce a stimulus to increase the transcription of the cytosolic HMG-CoA synthase gene. An alternative explanation for this delay could be based on the fact that the diurnal peak of insulin, which is produced upon refeeding, could cause a lower transcriptional influence on the cytosolic HMG-CoA synthase gene than on the reductase gene. In any case, this delay in the cytosolic HMG-CoA synthase cycle would maintain the mevalonate production even when the levels of HMG-CoA reductase were decreasing after the maximum of activity, by increasing the substrate availability.

Gibbons et al. [25], comparing the HMG-CoA reductase activity and sterol synthesis in rat hepatocytes treated with insulin and glucagon, reached the conclusion that the amount of the substrate, HMG-CoA, present is the main factor responsible for the increase in HMG-CoA reductase activity, and that this was an inverse correlation. When the availability of HMG-CoA was decreased, HMG-CoA reductase activity increased. These results seem to be consistent with our results. Owing to the 4 h delay in the diurnal peak of HMG-CoA synthase compared with HMG-CoA reductase, when the synthase activity (and thus the

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