In vivo demonstration of the circadian rhythm of cholesterol biosynthesis in the liver and intestine of the rat

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Abstract The existence of a circadian rhythm in the rate of hepatic cholesterol synthesis in the rat has been demonstrated in vivo by measuring the conversion of both [1-^{14}C]acetate and ^3H_2O to cholesterol. By both methods there was observed a similar increase in the rate of hepatic cholesterol synthesis between the nadir at noon and the peak at midnight. Circadian changes in the rate of hepatic cholesterol synthesis measured in vivo with [1-^{14}C]acetate were very similar to changes in the activity of hepatic microsomal HMG CoA reductase. Cholesterol synthesis in the jejunum and in the distal ileum was also shown to exhibit the same circadian rhythm in vivo but with smaller amplitude (1.6- and 1.3-fold, respectively). Rats trained to eat during a 4-hr period (9 AM–1 PM) while housed under normal illumination showed changes in the timing of the circadian rhythm of cholesterol synthesis; in the liver the maximum rate of cholesterol synthesis occurred at 6 PM, 9 hr after the presentation of food, while the two sections of the intestine investigated exhibited a maximum synthetic response between noon and 6 PM. Results obtained in this study support the hypothesis that the major portion of the rise in HMG CoA reductase activity and the increase in overall rate of cholesterol synthesis in liver and intestine during the circadian rhythm are due to the ingestion of food. Under the limited feeding schedule (food access 9 AM–1 PM), the rates of hepatic and intestinal synthesis of fatty acids from the injected acetate also showed a circadian rhythm with a peak at about 3 hr after presentation of food.

Supplementary key words small intestine · ^3H_2O · [1-^{14}C]acetate · HMG CoA reductase

THE CIRCADIAN RHYTHM of hepatic cholesterol synthesis was first reported by Kandutsch and Saucier (1), who used mouse liver slices. Back, Hamprecht, and Lynen (2) used similar techniques and confirmed the phenomenon in rats. Hamprecht, Nüssler, and Lynen (3) and Shapiro and Rodwell (4) have reported a circadian rhythm in the activity of HMG CoA reductase (EC 1.1.1.34), the primary rate-limiting step in the synthesis of cholesterol from acetate under most experimental conditions (5–7).

Published data on the hepatic circadian rhythm of cholesterol synthesis have been obtained only by in vitro methods (1–4, 8). In the large body of literature on factors affecting cholesterol synthesis in rat liver, marked differences have frequently been observed between the results obtained by in vitro and in vivo methods. Fasting depresses liver sterol synthesis to extremely low levels as measured in vitro in liver slices (9) or homogenates (10). On the other hand, as measured in vivo, fasting results in a reduction of sterol synthesis of about 50% as measured with [^{14}C]acetate (11, 12) and 40% with ^3H_2O (12). It therefore seemed pertinent to ascertain whether a rhythm in hepatic cholesterol synthesis occurred in vivo, and if so, whether the characteristics of the in vivo and in vitro rhythms were similar.

In rats, the liver and intestine synthesize most of the total body cholesterol; on low cholesterol diets these two tissues synthesize roughly equal amounts (13, 14). However, changes in the rate of sterol biosynthesis in liver and intestine frequently do not parallel one another. For example, cholesterol feeding depresses hepatic sterol biosynthesis but has little effect on intestinal synthesis (14–16). Since in this case different control mechanisms apparently regulate the rates of sterol synthesis in the liver and intestine, we have investigated the question of whether or not there is a common circadian rhythm in cholesterol synthesis in these two tissues.

Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaric acid coenzyme A.

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We have previously reported that reversing the lighting schedule reverses the timing of the rhythm of hepatic HMG CoA reductase activity (8); however, this could be due to an altered feeding pattern brought about by changing the lighting schedule. Hence, we have investigated the effects of both reversed illumination conditions and an artificial feeding schedule (food available 9 AM–1 PM under normal illumination conditions) on the timing and amplitude of the in vivo rhythm in both liver and intestine.

To obtain information on the specificity of the changes in rate of cholesterol synthesis, we also measured the in vivo rates of synthesis of total fatty acids in liver and intestine from [1-^{14}C]acetate.

MATERIALS AND METHODS

Materials were obtained as follows: sodium [1-^{14}C]-acetate, New England Nuclear, Berkeley, Calif.; glucose-6-phosphate dehydrogenase, Calbiochem, La Jolla, Calif.; glucose-6-phosphate (disodium salt) and NADP$^+$, Sigma, St. Louis, Mo.; digitonin, Merck, Rahway, N.J.; and mylar-backed silica gel chromatography sheets, Eastman, Rochester, N.Y.

Preparation of [3-^{14}C]HMG CoA and assay of liver HMG CoA reductase activity in vitro have been described previously (8).

Animals

Male Sprague-Dawley rats (200–250 g) were housed individually in windowless rooms with free access to food (Purina laboratory rat chow) and water, unless otherwise noted. Rats were acclimatized to strict lighting conditions for periods longer than 3 wk before they were killed by exsanguination. Normal illumination is defined as a 6 AM–6 PM light period and a 6 PM–6 AM dark period.

In vivo incorporation of [1-^{14}C]acetate into nonsaponifiable lipids, cholesterol, and fatty acids

Rats were lightly anesthetized with ether, an external jugular vein was exposed, and sodium [1-^{14}C]acetate in 0.3 ml of isotonic saline was given by iv injection over a 5-sec period. The wound was closed by clips. Exactly 35 min later the rat was killed by cardiac exsanguination under ether anesthesia. The small intestine and liver were immediately removed and placed in ice-cold saline. The intestinal contents were removed by a stream of cold saline prior to being cut longitudinally and blotted dry. The 25-cm portion of the ileum proximal to the ileocecal junction and a portion of the jejunum (the 25 cm proximal to the midpoint of the small intestine) were removed.

The samples of ileum, jejunum, and liver were treated with alcoholic sodium hydroxide. The nonsaponifiable lipid and fatty acid fractions were extracted separately, and cholesterol was isolated as its digitonide derivative by previously described methods (16). The dry sterol digitonide was dissolved in warm isopropanol, and the radioactivity was counted in Bray's scintillation fluid (17) in a Packard Tri-Carb liquid scintillation spectrophotometer; nonsaponifiable lipids and fatty acids were counted directly in Bray's solution as previously described (18).

In vivo incorporation of ^3H_2O into nonsaponifiable lipid and cholesterol

Rats were injected intraperitoneally with isotonic saline containing ^3H_2O (10 mCi/100 g body wt). After 90 min the rats were killed and the livers were removed and assayed for ^{3}H-labeled nonsaponifiable lipid and cholesterol as previously described (18).

RESULTS

Diurnal rhythm of cholesterol synthesis in the liver and intestine in vivo

Rats housed under conditions of normal illumination and fed ad lib. exhibited a circadian rhythm in the rate of hepatic biosynthesis of nonsaponifiable material (Fig. 1). Control experiments with both liver and intestine established that nearly 70% of the ^{14}C in the nonsaponifiable fraction was isolated as cholesterol digitonide; consequently, in subsequent experiments the assay of the non-

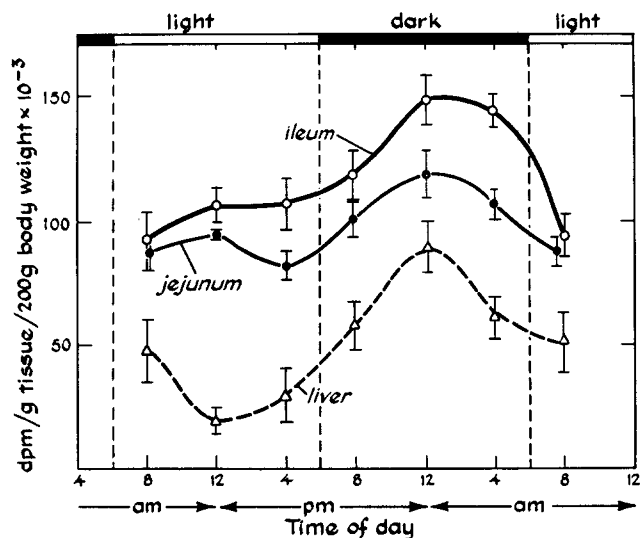

FIG. 1. Circadian rhythm of synthesis of nonsaponifiable lipid in liver and intestine in vivo. Rats were fed ad lib. [1-^{14}C]Acetate (100 μCi; SA, 50 μCi/μmole) in 0.3 ml of saline was injected into the jugular vein 35 min before the animals were killed. Each point represents the mean of determinations from five animals ± SD. P values by Student's t test between maximum and minimum of each curve are: Δ, liver, $P < 0.001$; ○, ileum, $P < 0.002$; ●, jejunum, $P < 0.05$.

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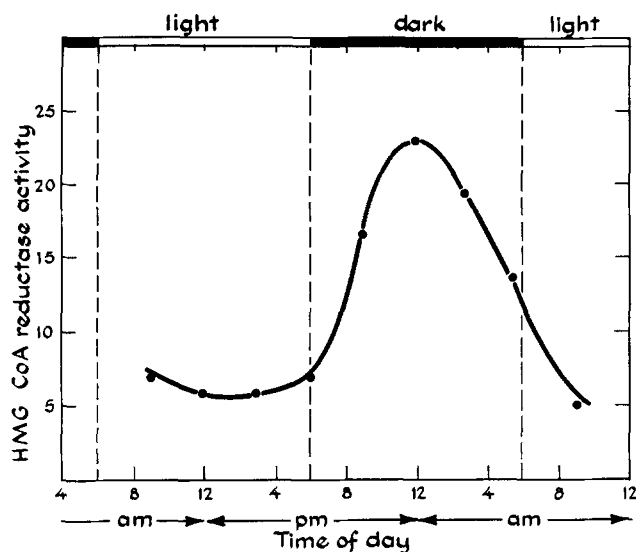

FIG. 2. Circadian rhythm of activity of hepatic microsomal HMG CoA reductase. Rats were housed under normal illumination and fed ad lib. Nine groups of two rats each were killed at various times over a 24-hr period and microsomes were prepared from each liver. The microsomes were assayed for HMG CoA reductase activity as described under Materials and Methods; the mean value for each pair is given in nmoles × 10^2 mevalonic acid synthesized/mg protein/min. The variation in reductase activity for the two rats killed at the same time was generally less than 10%.

saponifiable fraction was routinely employed as an index of cholesterol synthesis. The difference is due to squalene and to sterols other than cholesterol.

The minimal rate of synthesis at noon and the maximum at midnight correlate well with the timing of the minimum and maximum obtained from assay of hepatic microsomal HMG CoA reductase activity in vitro (Fig. 2). The amplitude of the rhythm, as defined by the ratio of the maximum to the minimum rates, was approximately 4.0, measured by either the in vitro ($P < 0.001$) or in vivo ($P < 0.001$) method. In order to eliminate the possibility that a changing acetate–acetyl CoA pool size accounted for the changing rate of incorporation of injected [1-^{14}C]acetate into cholesterol, ^3H_2O was injected intraperitoneally 45 min before noon (the nadir of the rhythm) or midnight (the peak of the rhythm), and the rats were killed 1.5 hr later. Incorporation of ^{3}H into the nonsaponifiable lipid fraction varied 2.6-fold between the two groups (Table 1) ($P < 0.001$). Assay of liver HMG CoA reductase from these same rats varied over the same range (Table 1).

Using the [1-^{14}C]acetate in vivo assay technique, the ileal and jejunal sections of the intestine also showed circadian rhythmic changes in the rate of synthesis of nonsaponifiable lipid (Fig. 1). Although the changes in the rate of cholesterogenesis were much less than for liver, the determinations showed a low degree of variability. The ileum showed a ratio of 1.6 ($P < 0.002$) and the jejunum a ratio of 1.3 ($P < 0.05$). The maximum rate of sterol biosynthesis in both ileum and jejunum occurred at midnight and the minimum between 8 AM and 4 PM.

TABLE 1. In vivo incorporation of ^3H_2O into hepatic nonsaponifiable fraction

Time of Killing Rats	Number of Animals	^{3}H Incorporated into Nonsaponifiable Lipid	Hepatic HMG CoA Reductase Activity
		dpm/g liver	*nmoles/min/mg protein*
Noon	4	4,872 ± 1,070	0.06 ± 0.005
Midnight	6	12,742 ± 1,130[a]	0.16 ± 0.008[a]
Ratio, midnight/noon		2.6	2.7

Rats housed under conditions of normal illumination were given an intraperitoneal injection of isotonic saline containing ^3H_2O (10 mCi/100 g body wt) 45 min before noon or midnight and were killed 1.5 hr later. Both the incorporation of tritium into the liver nonsaponifiable fraction and the activity of microsomal HMG CoA reductase were assayed for each animal.
[a] $P < 0.001$.

Effect of illumination and feeding habits on lipid synthesis in vivo

Reversed illumination periods. Rats housed under a reverse illumination cycle (lights on 6 PM–6 AM, off 6 AM–6 PM) showed a shift in the circadian rhythm when the ^{14}C-in vivo assay method was used. After being housed for 1 wk on the new light cycle, the rate of hepatic and intestinal sterol synthesis was greater in the animals killed at noon (the middle of the dark period) than in those killed at midnight. The hepatic and intestinal (ileum) values at noon were 1.7 and 1.4 times the values at midnight.

Restricted feeding with normal lighting. Rats consumed over 80% of their food during the daily 12-hr dark period and the 2.5 hr preceding the onset of darkness (Table 2). 180-g rats showed a dramatic increase in the rate of food ingestion during the 3-hr period preceding the onset of darkness (Table 2). The importance of feed-

TABLE 2. Feeding pattern of Sprague-Dawley rats housed under normal illumination

Time of Day	Food Consumed	
	g	*g/hr/rat*
9:30 AM–3 PM	0.84 ± 0.51	0.15
3 PM–5 PM	3.66 ± 0.28	1.83
5 PM–6 AM	13.5 ± 0.70	1.0
6 AM–9:30 AM	1.5 ± 0.42	0.43
Total per 24 hr	19.5	

The food intake of five rats (180 g body wt) housed for 3 wk on the normal light cycle (lights on 6 AM–6 PM) was measured by weighing food remaining in external feeders at the stated times. The pattern of feeding was identical with that exhibited by 350-g rats housed under the same conditions for 10 wk.

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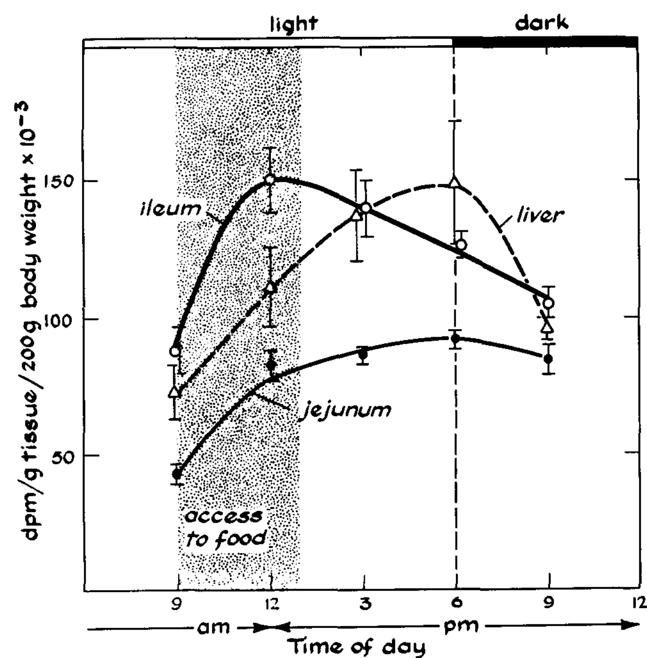

FIG. 3. Effect of daily restricted feeding on the rate of hepatic and intestinal synthesis of nonsaponifiable lipid in vivo. Rats were allowed access to food between 9 AM and 1 PM for 19 days. [1-^{14}C]-Acetate (30 μCi; SA, 2 μCi/μmole) was injected into the jugular vein 35 min prior to killing. Each point is the mean of determinations for five or six animals ± SEM. Student's t test was applied between the maximum value and the 9 AM value. For the liver (Δ), $P < 0.02$; the ileum (○), $P < 0.002$; and the jejunum (●), $P < 0.001$. For further details see text.

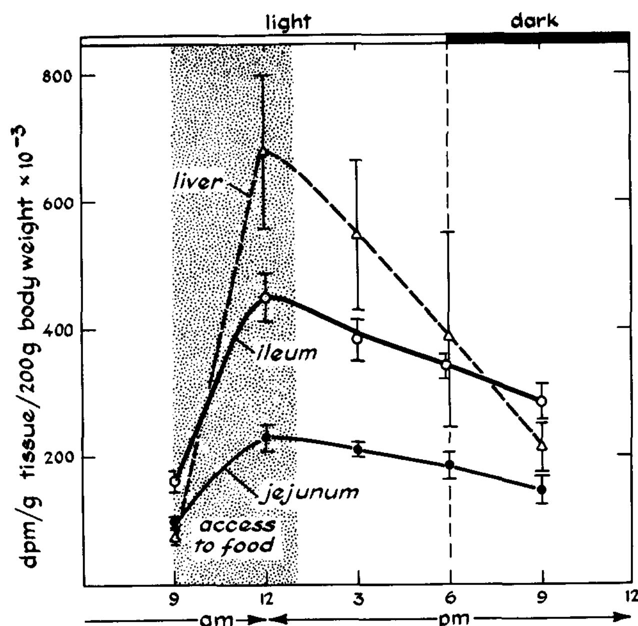

FIG. 4. Effect of restricted feeding on the rate of synthesis of fatty acids in the liver and intestine measured in vivo. For the liver (Δ), $P < 0.002$; the ileum (○), $P < 0.001$; and the jejunum (●), $P <$ 000.1. See Fig. 3 and text for further details.

ing habits rather than lighting per se in regulating sterol synthesis was demonstrated by rats housed under normal illumination conditions and offered food from 9 AM to 1 PM only (during the illuminated period) for 19 days prior to being killed. Their daily gain in body weight was in the same range as that of ad lib.-fed control rats (4–6 g/day), showing that they were acclimatized to the new feeding schedule. The maximal rate of hepatic sterol synthesis measured by the ^{14}C-in vivo technique occurred ca. 9 hr after food presentation (Fig. 3), but the maximal rate was only 2.1 times the minimal rate. Both ileum and jejunum showed significant increases in the rate of sterol synthesis after food presentation (Fig. 3); in the ileum the maximum to minimum ratio was 1.7 and the peak occurred at 3 hr, whereas in the jejunum the ratio was 2.0 and the peak was essentially constant from 3 to 12 hr.

There was a rapid rise in the rate of hepatic and intestinal fatty acid synthesis shortly after the presentation of food. The maximum rate of synthesis occurred during the 3-hr period after food presentation (Fig. 4).

DISCUSSION

Estimation of changes in the rate of hepatic cholesterol synthesis in vivo by determination of the conversion of [1-^{14}C]acetate to cholesterol in the liver of the intact rat necessarily assumes the absence of significant changes in the size and turnover rate of the acetate–acetyl CoA pool. ^{3}H-labeled water is theoretically a better precursor for in vivo determinations of cholesterol synthesis than [^{14}C]-acetate because it equilibrates rapidly with all labile hydrogens in all the intermediates in cholesterol biosynthesis. This method is consequently free from the uncertainty regarding variable precursor pool size and specific activity associated with the use of [^{14}C]acetate. In our previous studies in which both [^{14}C]acetate and [^{3}H]water were injected simultaneously into rats with widely different rates of hepatic cholesterol synthesis, it was found that both methods gave essentially the same results; in irradiated and control rats with hepatic synthetic rates varying over a 10-fold range, the ratio of ^{14}C to ^{3}H in cholesterol was 0.51–0.55 with a mean of 0.53, and in control rats fasted for 48 hr the ratio was 0.374 (12). Consequently, it is highly improbable that the size of the acetate–acetyl CoA pool in the liver of the ad lib.-fed normal rat could change sufficiently during a 12-hr period to account for the fourfold range in the rate of cholesterol synthesis when [1-^{14}C]acetate was used in vivo. The results presented in this paper can then be regarded as confirming the existence of the circadian rhythm of sterol synthesis in normal intact rats.

Using the in vitro or in vivo techniques, the amplitude of the circadian rhythm of sterol synthesis and HMG CoA reductase activity in our experiments was approximately fourfold. These results are in good agreement

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with the values reported by Shapiro and Rodwell (19) from assays of HMG CoA reductase (4.4-fold) and with those of Back et al. (2) (3.7-fold) and Horter, Hickman, and Sabine (20) (3.4–6.5-fold) from assays of the rate of conversion of acetate to cholesterol by liver slices. Hamprecht et al. (3) have reported that HMG CoA reductase activity varies eightfold over a 24-hr period. Generally, then, it appears that measurements of HMG CoA reductase activities are a good measure of in vivo cholesterogenesis.

The times at which the maximum and minimum values were observed in this in vivo study correlate well with the published data on the phasing of the rhythm of the conversion of acetate to cholesterol or HMG CoA reductase activity measured in vitro in rats housed under similar conditions (2–4). Our in vivo studies also confirmed our earlier in vitro finding that reversal of the lighting reversed the phasing of the circadian rhythm (8).

The ileum and jejunum were also shown to exhibit a circadian rhythm of sterol synthesis similar to, but with a smaller amplitude than, that of the liver. The time of minimal rate of synthesis in the intestine segments (8 AM–12 noon) was not as well defined as in the liver (12 noon). This, to our knowledge, is the first evidence for the presence of a circadian rhythm of cholesterol synthesis in the intestine.

Since certain experimental conditions (e.g., cholesterol feeding, X-irradiation) affect liver but not intestinal sterol synthesis, it is of interest to note that both tissues do exhibit a similar circadian rhythm and that both respond in a similar but not identical way to ingestion of a normal diet (Purina laboratory rat chow).

In these experiments the rate of sterol synthesis in both liver and intestine increased after food was presented to rats with a limited access to food (9 AM–1 PM) even though this occurred during the light part of the cycle. Goldfarb and Pitot (21) have reported results in agreement with ours on the effects of restricted feedings (8 AM–4 PM) on the rhythm of hepatic HMG CoA reductase. The peak reductase activity occurred at 2 PM, 6 hr after the presentation of food, and was 3.6 times the minimal activity (12 PM–8 AM).

Certain evidence presented above indicates that food intake is responsible for the major increase in cholesterol synthesis during the circadian cycle. However, there does not appear to be general agreement on this point. Hamprecht et al. (3) have reported that rats starved for 24 hr showed a much reduced but still detectable circadian rhythm in HMG CoA reductase activity; the maximum value reached in fasted rats was less than 3% of the maximum for fed rats and the variability of the results was large. Kandutsch and Saucier (1) reported an almost 10-fold increase in the conversion of acetate to cholesterol in liver slices of A/Hej mice between 8:30 AM and 8:30 PM even though food was removed at 8:00 AM. Mice and rats, however, appear to react very differently to fasting. After 24 hr without food, the rate of conversion of acetate to cholesterol by mouse liver slices was reported to be 90% the value of that of ad lib.-fed controls (1), while fasted rats are well known to have very low rates of cholesterol synthesis as measured in vitro (9, 10). Additionally, rats fail to show the normal diurnal rise in activity of HMG CoA reductase if food is removed during the nadir of the cycle (20).[1]

The evidence available at present is not conclusive, but it suggests that there may be a circadian rhythm independent of food intake although the major increase in activity appears to be due to a response to eating. Quantitatively, the response to feeding seems to be more important since in the fasted rat the highest level of HMG CoA reductase activity reported is only a very small fraction of that produced after ingestion of a normal diet.

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