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Effects of Bile Acid Administration on Bile Acid Synthesis and Its Circadian Rhythm in Man

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In man bile acid synthesis has a distinct circadian rhythm but the relationship of this rhythm to feedback inhibition by bile acid is unknown. We measured bile acid synthesis as release of $^{14}CO_2$ from [26-^{14}C]cholesterol every 2 hr in three normal volunteers during five separate 24-hr periods. Data were fitted by computer to a cosine curve to estimate amplitude and acrophase of the circadian rhythm. In an additional six volunteers, we measured synthesis every 2 hr from 8:00 a.m. to 4:00 p.m. only. During the control period, amplitude (expressed as percentage of mean synthesis) averaged 52% and acrophase averaged 6:49 a.m. During administration of ursodeoxycholic acid (15 mg per kg per day), synthesis averaged 126% of baseline ($p < 0.1$), amplitude averaged 43% and acrophase averaged 6:20 a.m. During administration of chenodeoxycholic acid (15 mg per kg per day), synthesis averaged 43% of baseline ($p < 0.001$), amplitude averaged 53% and acrophase averaged 9:04 a.m. Addition of prednisone to this regimen of chenodeoxycholic acid to eliminate release of $^{14}CO_2$ from corticosteroid hormone synthesis resulted in a mean amplitude of 62% and a mean acrophase of 6:50 a.m., values very similar to those in the baseline period. Administration of prednisone alone also did not significantly alter the baseline amplitude (40%) or acrophase (6:28 a.m.). We conclude that neither chenodeoxycholic acid nor ursodeoxycholic acid significantly alters the circadian rhythm of bile acid synthesis in man. Moreover, because this method of measuring synthesis does not require subtraction of large amounts of exogenous bile acid, these data also demonstrate more clearly than ever before that chenodeoxycholic acid causes inhibition of total bile acid synthesis while ursodeoxycholic acid causes either no change or a slight increase in synthesis.

Hepatic production of bile acids is a major catabolic route for cholesterol elimination (1) and also provides detergent for solubilization of biliary cholesterol (2). However, regulation of bile acid synthesis remains incompletely defined, especially in man. Negative feedback inhibition by bile acids undergoing enterohepatic circulation is one important modulator of bile acid synthesis (3). In addition, there is a remarkable circadian rhythm of bile acid synthesis (4), which in animal models appears to be independent of feedback inhibition (5).

Chenodeoxycholic acid and/or ursodeoxycholic acid are being used with increasing frequency to treat cholesterol gallstones (6, 7). Although structurally very similar, these two bile acids differ strikingly in their physiologic effects. Chenodeoxycholic acid appears to inhibit bile acid synthesis, and this inhibition may be important in determining both efficacy and potential toxicity of this bile acid in gallstone dissolution therapy (3, 8). Ursodeoxycholic acid, on the other hand, appears to inhibit bile acid synthesis little, if at all, and may actually stimulate synthesis (7, 8).

In the present study, we have attempted to define the effects in man of both chenodeoxycholic acid and ursodeoxycholic acid on total bile acid synthesis measured as output of $^{14}CO_2$ from [26-^{14}C]cholesterol corrected for cholesterol specific activity (4). This method allows measurement of bile acid synthesis with an intact enterohepatic circulation (EHC) at time intervals short enough to define circadian variation. It also permits direct measurement of synthesis during bile acid administration, without requiring subtraction of administered bile acid. This is especially advantageous during suppression of synthesis, when most bile acid "output" measured by radioisotope dilution or fecal acid sterol balance actually represents ingested exogenous bile acid.

SUBJECTS AND METHODS

Nine normal volunteers (six males and three females) were studied. They ranged in age from 34 to 68 years. All were without medical problems as determined by previously published criteria (9).

[1-^{14}C]Propionic acid and [3-^{14}C]propionic acid were purchased (New England Nuclear, Boston, Mass.), and both were found to be >97% pure by high-performance liquid chromatography as previously described (4). [26-^{14}C]Cholesterol was purchased (New England Nuclear) and assayed for isotopic purity by thin layer chromatography as previously described (4). No labeled cholesterol containing more than 3% radiochemical impurity was used.

Collections of breath $^{14}CO_2$ were performed and analyzed as previously described (4). Briefly, the subject placed his head in a plexiglass hood fitted with a flexible collar to provide an air-

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tight seal at the neck. Air was pulled through this hood at 10 liters per min. After an initial equilibration period, air flow from the hood was diverted through a series of three traps for 8 min. Each trap contained 40 to 60 ml of an alkaline scintillation fluid capable of trapping 1.4 mmoles of CO_2 per ml. The trapping solution consisted of phenethylamine, methanol, toluene and Liquifluor (New England Nuclear) in the proportions 27:27:41:5. At the end of collection, volume of scintillation fluid in each of the three traps was measured, and duplicate 10-ml aliquots were counted on a Packard Tricarb 4640 liquid scintillation counter (Packard Instrument United Technologies, Downers Grove, Ill.). Quenching correction was made by internal standardization.

Each volunteer was given a total of 90 to 100 μCi of [26-^{14}C] cholesterol orally. In some cases, this was given as a single dose and in others it was given as two doses of 45 to 50 μCi each. In either case, an interval of at least 10 days was interposed between administration of isotopic cholesterol and breath sampling. On the day of each breath sampling, a 10-ml sample of serum was obtained for analysis of cholesterol specific activity by the ferric chloride method (10). Bile acid synthetic rate was calculated by the equation:

$$\text{Synthesis} = (^{14}CO_2 \text{ output})/(\text{cholesterol specific activity})$$

The circadian rhythm of bile acid synthesis was determined by measuring synthesis at 2-hr intervals throughout a 24-hr period. Three healthy adult male volunteers were studied over 24-hr periods using the following interventions: (i) none (baseline); (ii) after 14 days of taking chenodeoxycholic acid (15 mg per kg per day); (iii) after 14 days of taking ursodeoxycholic acid (15 mg per kg per day); (iv) after 14 days of taking chenodeoxycholic acid (15 mg per kg per day) plus prednisone (15 mg per day) for 5 days, and (v) after 5 days of prednisone (15 mg per day). The purpose of prednisone during the last two periods was to assess the contribution of corticosteroid hormone synthesis to total $^{14}CO_2$ output. For clarity, it should be emphasized that there was an interval of at least 14 days between all studies except for addition of prednisone to chenodeoxycholic acid, in which case the interval was 5 days. Thus, whenever a medication given in one study period was not included in the following study period, a minimum interval of 14 days was interposed between measurements for those two periods.

These three subjects also underwent separate studies during infusion of [1-^{14}C]propionate and [3-^{14}C]propionate to correct $^{14}CO_2$ output from [26-^{14}C]cholesterol for delay in transit through propionate pools as previously described (4). Briefly, 5 μCi of the appropriate isotope were diluted in 100 ml of normal saline and infused into a forearm vein at a constant rate. Output of $^{14}CO_2$ was determined every 90 min following the start of infusion. (Any residual breath $^{14}CO_2$ from [26-^{14}C]cholesterol was measured before starting the propionate infusion and subtracted from each determination during infusion. These residual values were always <10% of the $^{14}CO_2$ output during infusion and usually much lower.) To calculate first-order turnover constants of ^{14}C in both the 1- and 3-positions of propionic acid, the $^{14}CO_2$-time relation was transformed to a linear function (see "Appendix"). Propionate turnover constants were used to correct amplitude and acrophase of the $^{14}CO_2$-time relation obtained in the studies of [26-^{14}C]cholesterol for delay in transit of ^{14}C through propionic acid pools. These calculations are described in more detail in the "Appendix" and a previous publication (4).

Corrected values for bile acid synthesis as a function of time of day were fitted to a cosine curve for statistical analysis and calculation of F-values as described by Nelson et al. (11).

An additional six volunteers (three males and three females) were studied over 8-hr periods (8:00 a.m. to 4:00 p.m.) using only the first three interventions listed above. Bile acid synthesis was measured at each 2-hr interval between 8:00 a.m. and 4:00 p.m. These values were averaged to obtain the synthetic rates shown later in Table 2. Identical averaging, limited to this 8-hr period, was also done for the three subjects who underwent sampling for 24 hr (*vide supra*). These data were statistically analyzed by paired t test.

Informed consent was obtained from each subject before initiation of experiments. The study protocol was approved by committees on the use of human subjects from both the University of Minnesota and the Minneapolis Veterans Administration Medical Center. Radiation dosimetry calculations yielded a total body dose of 0.3, 0.000023 and 0.000073 rem for 100 μCi of [26-^{14}C]cholesterol, 5 μCi of [1-^{14}C]propionate and 5 μCi of [3-^{14}C]propionate, respectively. Because of its high concentration of biliary cholesterol, the gallbladder was regarded as the critical organ for isotopic cholesterol. Radiation dose to the gallbladder for 100 μCi of [26-^{14}C]cholesterol was calculated to be 10 rem. For isotopic propionate, the liver was regarded as the critical organ. For 5 μCi of [1-^{14}C]propionate and 5 μCi of [3-^{14}C]propionate, critical organ dose was calculated to be 0.0007 and 0.0022 rem, respectively. All these calculations were reviewed by the Department of Environmental Health and Safety of the University of Minnesota.

RESULTS

For the three subjects undergoing circadian rhythm studies, the first-order rate constants for ^{14}C turnover in the 1-position of propionic acid were 0.56, 0.37 and 0.43 per day and for ^{14}C turnover in the 3-position of propionic acid were 0.14, 0.14, and 0.37 per day. These values are similar to those we reported previously (4). They resulted in amplitude corrections of 11 to 29% and acrophase corrections of 133 to 180 min.

As shown in Figure 1, amplitude of the baseline circadian rhythm averaged 52% around mean synthesis, while acrophase averaged 6:49 a.m. On ursodeoxycholic acid (Figure 2), amplitude of the circadian rhythm averaged 43% around mean synthesis and acrophase averaged 6:20 a.m. These data were not significantly different from baseline ($F = 1.13$).

On chenodeoxycholic acid, amplitude averaged 53% around mean synthesis and acrophase averaged 9:04 a.m. This change was not quite statistically significant compared to baseline ($F = 3.28$). Because during inhibition of bile acid synthesis by chenodeoxycholic acid (see below) corticosteroid hormone synthesis makes a greater relative contribution to $^{14}CO_2$ output, we also studied all three subjects on chenodeoxycholic acid together with prednisone (15 mg per day) to inhibit corticosteroid hormone synthesis. On this combination, the circadian rhythm had an average amplitude of 62% around mean synthesis and an acrophase of 6:50 a.m., which is not significantly different from baseline ($F = 0.68$).

Finally, administration of prednisone alone resulted in no change in the circadian rhythm of $^{14}CO_2$ output compared to baseline (amplitude 40% and acrophase 6:28 a.m., $F = 0.28$). All of the above data are summarized in Table 1 and depicted in Figures 3 and 4.

Mean bile acid synthesis rates for the period 8:00 a.m. to 4:00 p.m. in all nine subjects are presented in Table 2. These include measurements during the control period, during ingestion of chenodeoxycholic acid and during ingestion of ursodeoxycholic acid. As graphically depicted in Figure 5, chenodeoxycholic acid, 15 mg per kg

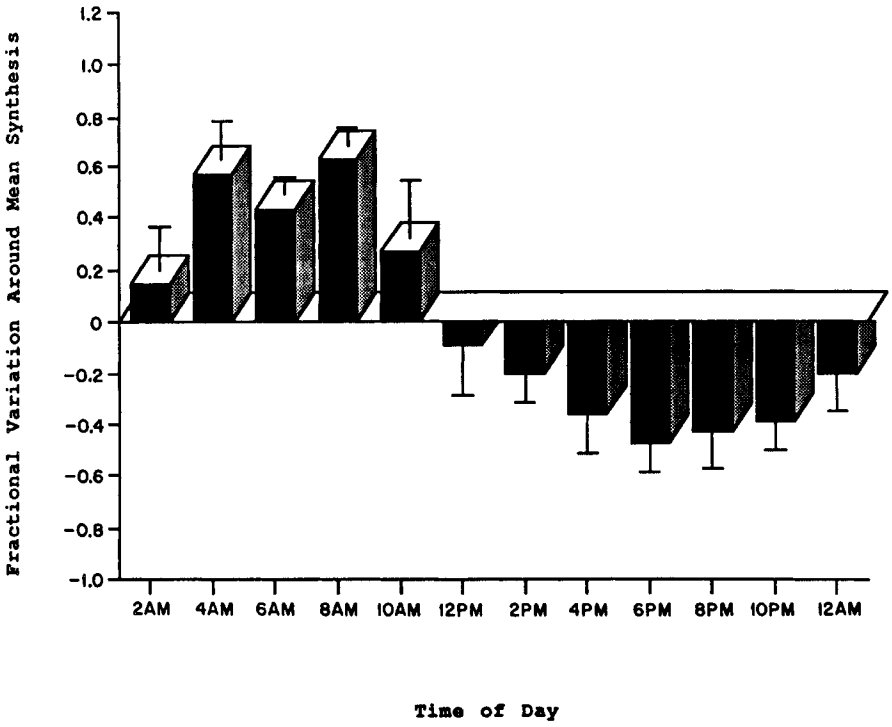

FIG. 1. Baseline circadian rhythm of bile acid synthesis in three normal volunteers. Values are expressed as fraction of mean daily synthesis. Each *bar* represents mean ± S.E. for the indicated time of day.

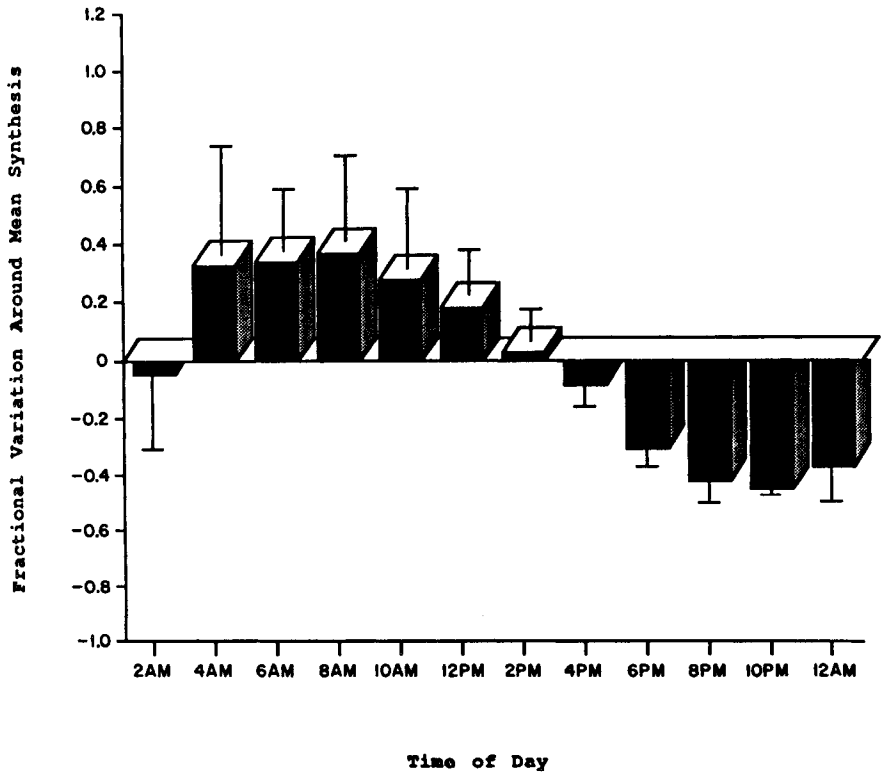

FIG. 2. Circadian rhythm of bile acid synthesis after 2 weeks on ursodeoxycholic acid. Subjects and calculations are the same as in Fig. 1.

per day, inhibited synthesis to a mean of 43% of baseline ($p < 0.001$). During ingestion of ursodeoxycholic acid, however, synthesis averaged 126% of baseline, a change which was at the borderline of statistical significance ($p < 0.1$).

DISCUSSION

We have previously shown in man that bile acid synthesis has a distinct circadian rhythm, varying about 50% around mean synthesis (4). We have also shown in

the rat that the circadian rhythm of bile acid synthesis continues despite complete interruption of the EHC, suggesting independence of feedback inhibition and the circadian rhythm (5). In man, however, there is no information on the relationship of feedback inhibition and the circadian rhythm of synthesis. In the present study, therefore, we have examined the effects of chenodeoxycholic acid and ursodeoxycholic acid on total bile acid synthesis and its circadian rhythm in human subjects.

To accomplish this, we measured bile acid synthesis as output of $^{14}CO_2$ from [26-^{14}C]cholesterol. This method, unlike all other available means for measuring bile acid synthesis with the EHC intact, permits measurements over sufficiently short time periods to define circadian variation. Even so, to accurately define acrophase and amplitude of the circadian rhythm of bile acid synthesis, one must account for the delay between cleavage of propionic acid from the cholesterol side-chain and conversion of the labeled propionic acid to $^{14}CO_2$. This delay tends to dampen the amplitude and delay the acrophase of $^{14}CO_2$ release. We accounted for this delay by measuring output of $^{14}CO_2$ as a function of time during infusions of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid. By mathematically fitting these data to first-order kinetics and the circadian rhythm to a cosine curve, we could make appropriate corrections in acrophase and amplitude of $^{14}CO_2$ output. Details and equations for these procedures have been published previously (4) and are presented in the "Appendix."

This $^{14}CO_2$ method is also uniquely suited to studies of effects of exogenous bile acids on bile acid synthesis apart from circadian rhythm studies. Determination of total bile acid synthesis by either fecal acidic sterol output or isotope dilution requires subtraction of exogenous bile acid from total measured "production." The more synthesis is inhibited, the closer to identical these two numbers become and the greater the potential error. This difficulty can be circumvented in part by measuring, for example, cholic acid synthesis by isotope dilution during administration of chenodeoxycholic acid, but this provides a value for only part of total synthesis.

There are several potential difficulties with the $^{14}CO_2$ technique. First, to the extent that newly synthesized cholesterol is used preferentially for bile acid synthesis, this method will underestimate synthesis. There can be extensive (20 to 50%) preferential use of newly synthesized cholesterol for bile acid synthesis when bile acid and cholesterol synthesis are stimulated by interrupting the EHC (12). However, when the EHC is intact, both in animal models and in man there is very little (5 to 15%) preferential use of newly synthesized cholesterol for bile acid synthesis (13, 14). Accordingly, in a careful study of this method in baboons, Redinger et al. (15) found virtually perfect correspondence between bile acid synthesis measured by fecal acidic sterol output and the $^{14}CO_2$ method when the EHC was intact. It should be noted, however, that even if the $^{14}CO_2$ method does underestimate synthesis, as long as it does so consistently

TABLE 1. **Amplitude and acrophase of circadian rhythms of bile acid synthesis**

	Absolute amplitude (μmoles/min)	Normalized amplitude (% of mean synthesis)	Acrophase (time of day)
Baseline	0.22 ± 0.05	52 ± 12	6:49 ± 0.30 a.m.
Ursodeoxycholic acid	0.18 ± 0.04	43 ± 7	6:20 ± 1:40 a.m.
Chenodeoxycholic acid	0.11 ± 0.07	53 ± 18	9:04 ± 0:27 a.m.
Chenodeoxycholic + prednisone	0.12 ± 0.05	62 ± 22	6:50 ± 2:05 a.m.
Prednisone	0.17 ± 0.06	40 ± 9	6:28 ± 1:58 a.m.

Values are mean ± S.E. for three subjects.

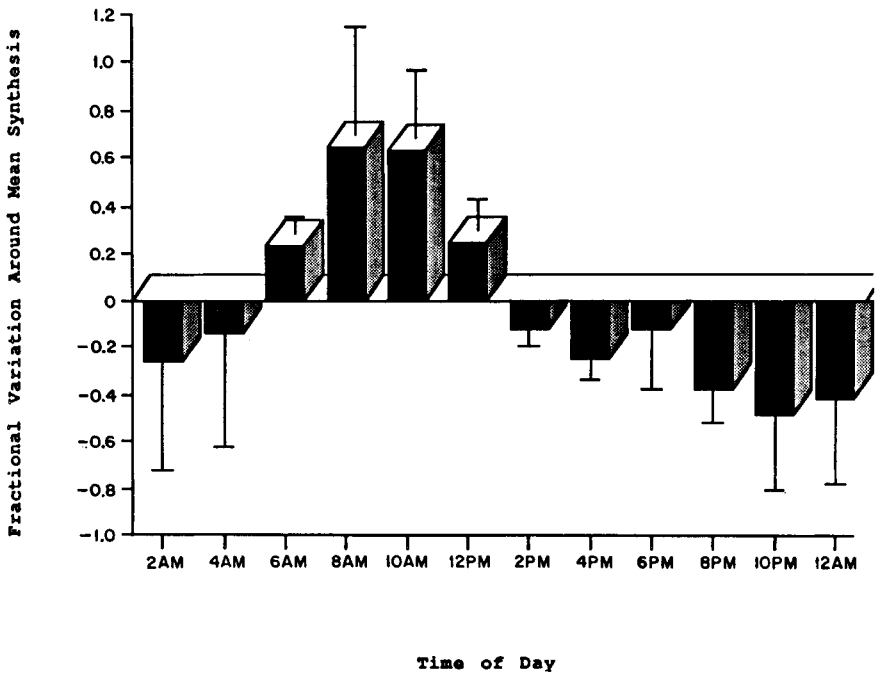

FIG. 3. Circadian rhythm of bile acid synthesis after 2 weeks on chenodeoxycholic acid. Subjects and calculations are the same as in Fig. 1.

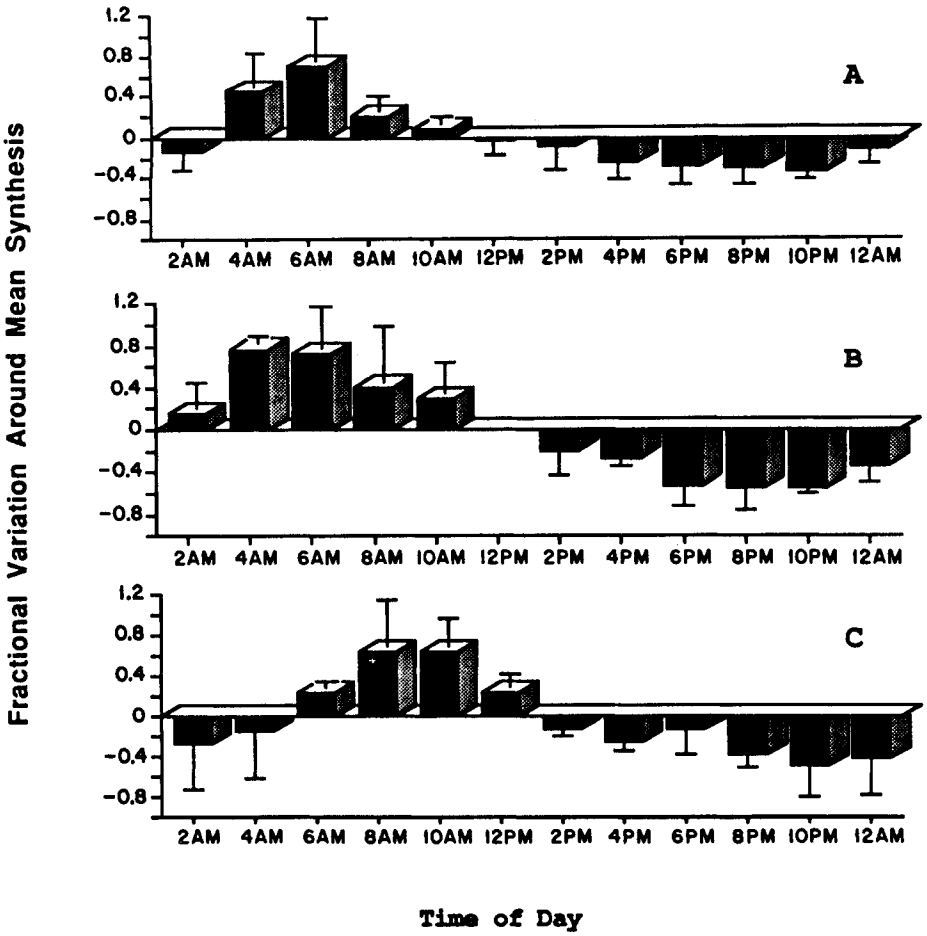

FIG. 4. Circadian rhythms of bile acid synthesis after 5 days on prednisone alone (A), after 2 weeks on chenodeoxycholic acid with the last 5 days on prednisone (B) and after 2 weeks on chenodeoxycholic acid only (C). Without inhibition of bile acid synthesis, prednisone alone did not appreciably alter the circadian rhythm compared to baseline (Fig. 1). Chenodeoxycholic acid alone appeared to delay slightly the acrophase of synthesis, although the result was not statistically significant (see text). However, the combination of chenodeoxycholic acid and prednisone resulted in return of the acrophase to baseline values. Thus, the shift in acrophase with chenodeoxycholic acid alone may have resulted from a greater relative contribution of corticosteroid hormone synthesis to output of $^{14}CO_2$ during inhibition of bile acid synthesis.

TABLE 2. Bile acid synthesis rates

Subject	Synthesis (μmoles/min)		
	Baseline	Chenodeoxycholic	Ursodeoxycholic
1	0.35	0.11	0.28
2	0.49	0.36	0.67
3	0.43	0.13	0.38
4	0.44	0.19	0.64
5	0.37	0.14	0.34
6	0.28	0.12	0.47
7	0.46	0.09	0.78
8	0.29	0.23	0.37
9	0.28	0.09	0.27

throughout the day, we will obtain a valid description of the circadian rhythm.

A second potential source of error with the $^{14}CO_2$ method is that synthesis of steroid hormones also releases $^{14}CO_2$ from [26-^{14}C]cholesterol. However, the magnitude of this synthesis is small (35 to 45 mg) compared to uninhibited bile acid synthesis (200 to 500 mg). Nevertheless, in the present study we directly assessed the importance of this factor by administering prednisone to inhibit synthesis of glucocorticoid hormones. [Glucocorticoids account for about 75% of steroid hormone synthesis, and synthesis of other steroid hormones apparently does not vary diurnally in adult subjects (4).] On prednisone, the circadian rhythm of $^{14}CO_2$ output was

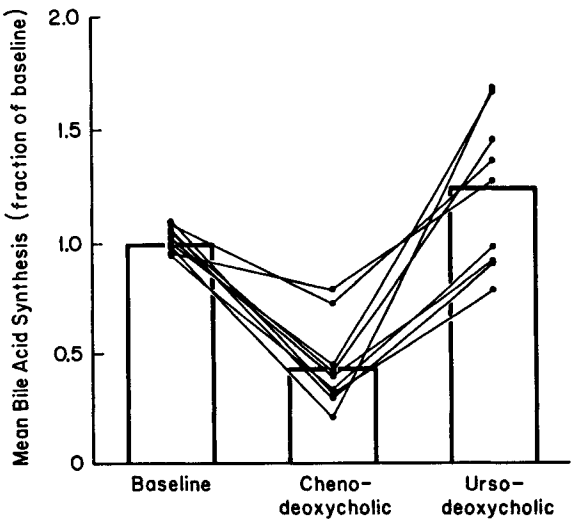

FIG. 5. Mean bile acid synthesis during the hours from 8:00 a.m. to 4:00 p.m. in nine normal volunteers. Values are expressed as fraction of baseline. By paired analysis, chenodeoxycholic acid significantly lowered mean synthesis to 43% of control ($p < 0.001$). Ursodeoxycholic acid resulted in an increase in mean synthesis to 126% of control, but the change was of borderline statistical significance ($p < 0.1$).

unchanged with respect to acrophase and amplitude (Figure 4). Thus, when bile acid synthesis is not inhibited, neglecting steroid hormone synthesis as a source of $^{14}CO_2$ appears to be a reasonable approximation.

As shown in Figure 5, chenodeoxycholic acid inhibited total bile acid synthesis in all nine subjects studied. Synthesis during chenodeoxycholic acid administration ranged from 22 to 80% of baseline with a mean of 43% of baseline. This finding is consistent with reports of chenodeoxycholic acid decreasing cholic acid synthesis by isotope dilution and decreasing total synthesis by fecal acidic sterol output (3, 8, 16). Ursodeoxycholic acid, on the other hand, did not inhibit bile acid synthesis by $^{14}CO_2$ output (Figure 5). In fact, mean synthesis during ursodeoxycholic acid administration was actually higher than baseline, although the change was of borderline statistical significance. Previous measurements of bile acid synthesis during administration of ursodeoxycholic acid have been somewhat inconsistent. Some report inhibition of synthesis, some report no change and some report increases in synthesis (7, 17).

In view of the minimal effects of ursodeoxycholic acid on total bile acid synthesis, it is not surprising that there was no change in acrophase or amplitude of the circadian rhythm of synthesis during ursodeoxycholic acid administration (Figure 2). During marked inhibition of bile acid synthesis with chenodeoxycholic acid, however, there was a proportional reduction in mean absolute amplitude of the circadian rhythm (Table 1), although this was not a statistically significant change. Amplitude expressed as fraction of mean synthesis remained unchanged on chenodeoxycholic acid compared to baseline (Figure 3). In addition, there was a small shift in acrophase from 6:49 a.m. to 9:04 a.m. Although this change was not quite statistically significant, there is a possible reason for such a phase shift to occur. During inhibition of bile acid synthesis, the contribution of steroid hormone synthesis to $^{14}CO_2$ output may cease to be negligible and its circadian rhythm might make an appreciable contribution to the overall rhythm of $^{14}CO_2$ output. To eliminate this as a confounding variable, we suppressed corticosteroid hormone synthesis by administering prednisone concurrently with chenodeoxycholic acid. In these studies, acrophase and amplitude (as fraction of mean synthesis) were indistinguishable from baseline values (Figure 4). Thus, chenodeoxycholic acid, even at a dose which markedly suppresses bile acid synthesis, appears to have no effect on the circadian rhythm except perhaps to reduce absolute amplitude in proportion to the reduction in total synthesis.

These findings provide the first direct evidence in man that the circadian rhythm of bile acid synthesis is independent of feedback inhibition. In a preliminary study of one volunteer, we observed no change in the circadian rhythm of synthesis during a 48-hr fast (18). Since fasting eliminates the normal daily variation in hepatic flux of bile acid, this observation lends indirect support to independence of the circadian rhythm from feedback inhibition. This conclusion is also consistent with our studies in the rat which showed that the circadian rhythm persisted despite fasting and despite prolonged interruption of the EHC (5).

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APPENDIX

Commercially available isotopic cholesterol, designated as [26-^{14}C]cholesterol, is actually randomly labeled at carbons 26 and 27 because these two carbons are chemically indistinguishable. Therefore, during oxidation to bile acid, 50% of the propionic acid released will be labeled in the 1-position and 50% in the 3-position. For analysis of [1-^{14}C]propionic acid kinetics, we define:

$P_1 \equiv$ amount of ^{14}C product of [1-^{14}C]propionic acid in whatever pool just precedes the rate-limiting step in conversion of propionic acid to CO_2

$k_1 \equiv$ the rate constant of removal from the pool

$A(t) \equiv$ the rate of formation or input of [1-^{14}C]propionic acid.

then:

$$\frac{dP_1}{dt} = A(t) - k_1P_1 \quad (1)$$

If A(t) is constant at A_o (as in our [1-^{14}C]propionic acid infusion studies), then the solution of this equation yields:

$$P_1(t) = P_s\,[1 - \exp(-k_1t)] \quad (2)$$

where P_s is the steady-state amount of [1-^{14}C]propionic acid.

Rearranging and taking the natural logarithm:

$$\ln\left[1 - \frac{k_1P_1(t)}{k_1P_s}\right] = -k_1t \quad (3)$$

Since we know k_1P_s (rate of $^{14}CO_2$ output in the steady state) and k_1P_1 (rate of output of $^{14}CO_2$ at various times), we are able to determine k_1 as the slope of the line defined by plotting the left-hand side of Equation (3) vs. t.

However, release of [1-^{14}C]propionic acid from [26-^{14}C] cholesterol may follow a circadian rhythm rather than being constant. If we approximate the circadian variation of bile acid synthesis as a cosine curve with a period, ω ($= \frac{2\P}{24}$ hr^{-1} where the period is 24 hr and the angles are in radians), and a phase angle, θ:

$$A(t) = A_o\cos(\omega t + \theta) \quad (4)$$

Substituting Equation (4) into Equation (1) yields a differential equation which can be solved as previously described (4). This yields the following expression for $^{14}CO_2$ output:

$$k_1P_1(t) = A_o[1 + (\omega/k_1)^2]^{-1/2}\cos(\omega t + \theta + \phi_1) \quad (5)$$

where:

$$\phi_1 = -\tan^{-1}(\omega/k_1) \quad (6)$$

Equation (5) indicates that the measured amplitude of $^{14}CO_2$ output will differ from the amplitude of [1-^{14}C] propionic acid input by a factor of $[(\omega/k_1)^2 + 1]^{-1/2}$ and the phase of $^{14}CO_2$ output will differ from the phase of [1-^{14}C]propionic acid input by the angle, ϕ_1, which is defined by Equation (6).

Identical analysis for [3-^{14}C]propionic acid kinetics yields:

$$k_3P_3(t) = A_o[(\omega/k_3)^2 + 1]^{-1/2} \cos(\omega t + \theta + \phi_3) \quad (7)$$

where:

$$\phi_3 = -\tan^{-1}(\omega/k_3) \quad (8)$$

Then, total $^{14}CO_2$ output is:

$$k_1P_1(t) + k_3P_3(t) = A_o[(\omega/k_1)^2 + 1]^{-1/2} \cos(\omega t + \theta + \phi_1) + A_o[(\omega/k_3) + 1]^{-1/2} \cos(\omega t + \theta + \phi_3) \quad (9)$$

For any given k_1 and k_3 the phase shift (ϕ) and change in amplitude (A) can be calculated using the general relation:

$$A_1\cos(\omega t + \phi_1) + A_2\cos(\omega t + \phi_2) = A\cos(\omega t + \phi) \quad (10)$$

where:

$$A = [A_1^2 + A_2^2 + 2A_1A_2\cos(\phi_1 - \phi_2)] \quad (11)$$

$$\phi = \tan^{-1}\left[\frac{A_1\sin\phi_1 + A_2\sin\phi_2}{A_1\cos\phi_1 + A_2\cos\phi_2}\right] \quad (12)$$

This relation is derived by expanding the cosine terms in Equation (10) using standard trigonometric identities.

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