Regulation of Bile Acid Synthesis in Man

PRESENCE OF A DIURNAL RHYTHM

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ABSTRACT Regulation of bile acid synthesis in man is incompletely understood, in part because of difficulty in making measurements over short time periods when the enterohepatic circulation is intact. We investigated the possibility of a diurnal rhythm of bile acid synthesis in three human subjects given [26-^{14}C]cholesterol. When this isotope of cholesterol, which is randomly labeled in the 26 and 27 positions, is converted to bile acid, the ^{14}C is released as propionic acid randomly labeled in the 1 and 3 positions. The labeled propionic acid is then oxidized to $^{14}CO_2$, output of which is a function of bile acid synthesis. However, delays in transit of the ^{14}C through propionic acid and CO_2-HCO_3^- pools would shift the phase and dampen the amplitude of $^{14}CO_2$ output relative to an existing diurnal rhythm of bile acid synthesis. Therefore, using constant infusion methods, we determined the turnover constants for conversion to $^{14}CO_2$ of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid to be 0.36–0.59 h^{-1} and 0.14–0.16 h^{-1}, respectively. Using these constants and modeling the diurnal rhythm as a cosine function, we determined that amplitude of $^{14}CO_2$ output from [26-^{14}C]cholesterol was reduced 35% and acrophase was delayed 2.4–3.0 h relative to the diurnal rhythm of bile acid synthesis. None of the diurnal rhythm in $^{14}CO_2$ output from [26-^{14}C]cholesterol resulted from diurnal variation in propionic acid or CO_2-HCO_3^- metabolism since constant infusion of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid for 30 h revealed no diurnal variation in output of $^{14}CO_2$. These studies demonstrate for the first time that humans with an intact enterohepatic circulation have a diurnal rhythm of bile acid synthesis with an amplitude of ±35–55% around mean synthesis, and an acrophase at about 9 a.m.

INTRODUCTION

Hepatic production of bile acids is a major pathway of cholesterol catabolism (1), and at the same time provides a micellar species for solubilization of biliary cholesterol (2). Abnormalities of bile acid synthesis may be important in the pathogenesis of cholesterol gallstone disease and certain hyperlipidemias (3–6); however, in man regulation of bile acid synthesis is incompletely understood.

In both animal models and man bile acid production is under negative feedback control by bile acid returning to the liver and suppressing activity of the rate-limiting enzyme, 7α-hydroxylase (7–9). Two observations suggest that at least one other regulatory mechanism also exists. First, fasting, which is associated with a decreased flux of bile acid through the liver, reduces bile acid synthesis and 7α-hydroxylase activity (10, 11). Second, bile acid synthesis and 7α-hydroxylase activity undergo a diurnal rhythm with peak activity during the waking and feeding hours (11, 12) when hepatic flux of bile acid is also highest (13). We have demonstrated effects of fasting on synthesis in man as well as in animal models (10, 12). However, the diurnal rhythm of bile acid synthesis has never been studied in humans with an intact enterohepatic circulation because of technical difficulties in making many measurements of synthesis over a 24-h period.

A technique for measuring bile acid synthesis that could potentially overcome this difficulty is production of $^{14}CO_2$ from [26-^{14}C]cholesterol. As shown in Fig. 1, during oxidation of cholesterol to bile acid, the terminal three-carbon fragment of the cholesterol side-chain is cleaved, probably as propionic acid (14). The propionic acid is then converted to succinic acid, which is oxidized

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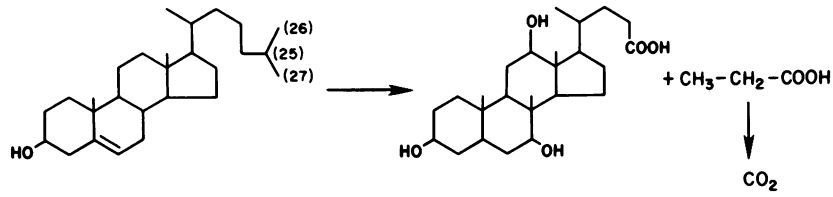

FIGURE 1 Conversion of cholesterol to bile acid and propionic acid. Commercially available isotopic cholesterol designated as [26-^{14}C]cholesterol is randomly labeled at the 26 and 27 positions. Propionic acid derived from this cholesterol is randomly labeled at the 1 and 3 positions.

to CO_2. This technique has been used successfully to demonstrate a diurnal rhythm of bile acid synthesis in the rat (15). One major potential difficulty in such an application is that delays in converting propionic acid to CO_2 would result in both a shift of phase and a dampening of amplitude of $^{14}CO_2$ output relative to the diurnal rhythm of bile acid synthesis. Because the 26 and 27 carbons on the cholesterol side-chain are chemically indistinguishable, commercially designated [26-^{14}C]cholesterol is randomly labeled on these two terminal carbons. As a result, 50% of the propionic acid generated during bile acid synthesis will be labeled in the 1 position and 50% in the 3 position. Moreover, as shown in Fig. 2, the ^{14}C from the 1 position may not be released as $^{14}CO_2$ at the same rate as ^{14}C from the 3 position. In the present study therefore, we determined the kinetics of $^{14}CO_2$ output from both [1-^{14}C]propionic acid and [3-^{14}C]propionic acid. We modeled the diurnal rhythm as a cosine function and used these kinetic parameters to correct phase and amplitude of $^{14}CO_2$ output from [26-^{14}C]cholesterol. These studies demonstrate for the first time that bile acid synthesis in humans with an intact enterohepatic circulation undergoes a definite diurnal rhythm.

METHODS

Three adult male volunteers participated in the study. All were without medical problems as determined by previously published criteria (10, 16) in addition to ultrasonic examination of the liver and biliary tree. Informed consent was obtained from each subject before initiation of experiments.

For determination of kinetics of $^{14}CO_2$ released from labeled propionic acid, [1-^{14}C]propionic acid (1–5 mCi/mmol) and [3-^{14}C]propionic acid (2–10 mCi/mmol) were purchased (New England Nuclear, Boston, MA) and found to be >98% isotopically pure by high-performance liquid chromatography of the phenacyl ester derivatives. This analysis was performed on a Hewlett-Packard 1084B chromatograph (Hewlett-Packard Co., Palo Alto, CA) using a C-18 column and a moving phase of acetonitrile/water, 50:50. [26-^{14}C]Cholesterol (50–60 mCi/mmol) was purchased (New England Nuclear) and assayed for isotopic purity by thin-layer chromatography on silica gel G using a moving phase of ethyl ether/heptane, 55:45 and by high performance liquid chromatography using a C-8 column and a moving phase of acetonitrile/tetrahydrofuran/water, 65:35:3. No labeled cholesterol containing more than 3% isotopic impurity was used. Other chemicals

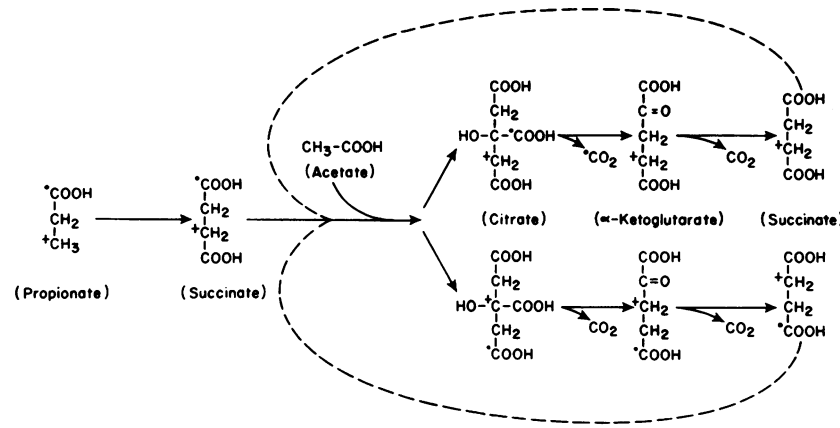

FIGURE 2 Metabolic pathway of propionic acid catabolism. Dots and crosses trace the fate of a carbon atom at the 1 and 3 positions of propionic acid, respectively. Carbon in the 1 position is readily converted to CO_2 by citric acid cycle enzymes. Carbon in the 3 position can be converted to CO_2 only by catabolism of citric acid cycle intermediates.

and solvents were used as purchased without further purification.

The apparatus for quantitative collection of breath CO_2 is shown in Fig. 3. The subject was placed in a plexiglass hood fitted with a flexible rubber collar to provide an air-tight seal at the neck. Air from this hood was pulled through a series of traps and collection flasks by a pump (Gast Manufacturing Corp., Benton Harbor, MI) at a rate of 10 liters/min. From the hood, air passed through a cold trap (methanol-dry ice) to remove water vapor and then through a flowmeter that was used to monitor and adjust air flow. From the flowmeter, air passed through a solution of methanol-HCl to saturate the atmosphere with methanol and reduce evaporation from the scintillation fluid. Air then passed through either an open bypass line or, if the bypass line was closed, through a series of three traps containing 40–60 ml of an alkaline scintillation fluid capable of trapping 1.4 mmol of CO_2 per ml (Oxifluor-CO_2, New England Nuclear). From there, air passed through a trap containing 40 ml of 0.05 M hyamine hydroxide and a small amount of thymolphthalein as an indicator. This trap provided constant visual assurance that no substantial amount of CO_2 escaped trapping in the three flasks of Oxifluor-CO_2. Finally air was passed through another cold trap (methanol-dry ice) to minimize exposure of the pump to organic solvent fumes.

To obtain a timed quantitative collection of breath CO_2, the subject was placed in the hood and air was pulled through the system at 10 liter/min with the bypass line open for 8 min. This equilibration period was provided to reach a steady-state in which flux of CO_2 through all parts of the system equaled output of CO_2 from the subject. After equilibration, the bypass line was closed and CO_2 was trapped for exactly 8 min. At the termination of collection, volume of scintillation fluid in each of the three traps was carefully measured and duplicate 10-ml aliquots were taken for counting in a Packard Tricarb 4640 scintillation counter (Packard Instrument United Technologies, Downers Grove, IL). Correction for quenching was made using internal standardization.

For determination of $^{14}CO_2$ output from [1-^{14}C]propionic acid or [3-^{14}C]propionic acid, ~5 μCi of the appropriate isotope was diluted in isotonic saline and infused into a forearm vein at a constant rate with a Harvard infusion pump (Harvard Apparatus Co., Inc., South Natick, MA). To calculate first-order turnover constants of ^{14}C in both these positions of propionic acid, the $^{14}CO_2$ output-time relation was transformed to a linear function as described in the Appendix. In these calculations the steady-state output of $^{14}CO_2$ was assumed to be 80% of the infusion rate. This approximation was based on the fact that during infusions of $H^{14}CO_3^-$, 80% of the ^{14}C is recovered on the breath as $^{14}CO_2$, most of the remainder being channeled into bone and undefined large molecules (17). Output of $^{14}CO_2$ during infusion of [1-^{14}C]propionic acid seemed to reach a plateau level within 5–6 h of infusion. This plateau also was ~80% of the ^{14}C infusion rate. In early studies we observed that allowing the subject to eat during this plateau resulted in a transient rise in output of $^{14}CO_2$ which returned to the original plateau level within ~60 min. We assumed this to be a result of disequilibration of CO_2-HCO_3^- pools. Because of this effect, no breath samples were taken within 60–90 min of eating.

For measurement of bile acid synthetic rates, each subject was injected intravenously with 30 μCi of [26-^{14}C]cholesterol at least 10 d before breath sampling. On the day of each breath sample, a 5–10-ml sample of serum was obtained. This was analyzed for cholesterol by ferric chloride method (18) and for carbon-14 by liquid scintillation counting in order to calculate cholesterol specific activity. Bile acid synthetic rate was then calculated by dividing the output of $^{14}CO_2$ (dpm/min) by serum cholesterol specific activity (dpm/mol). The circadian rhythm of $^{14}CO_2$ output was determined by performing measurements at 90-min intervals throughout the 24-h period in two or three separate 8–12-h sittings. During this time subjects were permitted to eat three regular meals at about 9 a.m., 1 p.m., and 7 p.m., as well as a light snack at about 1 a.m. When measurements were being made between about 11:00 p.m. and 6:00 a.m., subjects were encouraged to nap between sampling periods. Correction of the amplitude and phase of this diurnal rhythm for turnover of ^{14}C in propionic acid pools was performed as described in the Appendix.

Total daily bile acid synthetic rate by the $^{14}CO_2$ output technique was calculated by multiplying the bile acid synthetic rate (micromoles per minute) for each 90-min interval by 90 and summing. The resulting value was divided by 0.8 to correct for incomplete recovery of $^{14}CO_2$ as described above.

RESULTS

Fig. 4 shows breath $^{14}CO_2$ output as a function of time during constant intravenous infusion of both [1-^{14}C]propionic acid and [3-^{14}C]propionic acid in separate experiments for a single subject. Output of $^{14}CO_2$ increased more slowly during infusion of [3-^{14}C]propionic acid than during infusion of [1-^{14}C]propionic acid in all three subjects. First-order turnover constants for ^{14}C in both positions (Table I) were calculated as indicated in the Appendix using measurements made well before the plateau in $^{14}CO_2$ output, i.e., $k_1P_1(t) < (0.9)k_1P_s$

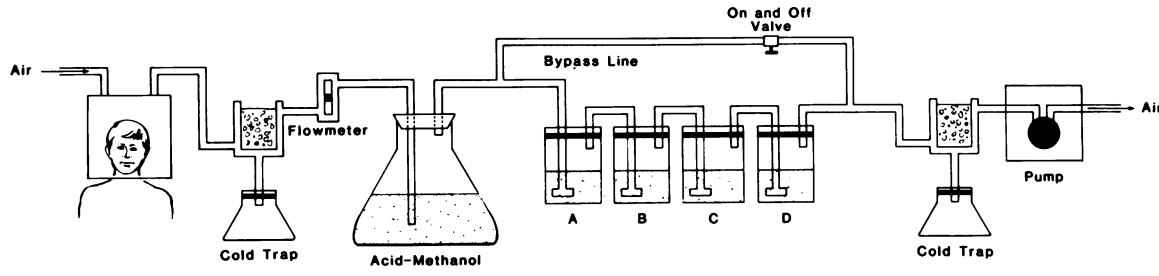

FIGURE 3 Apparatus for measurement of $^{14}CO_2$ output on the breath as described in the text.

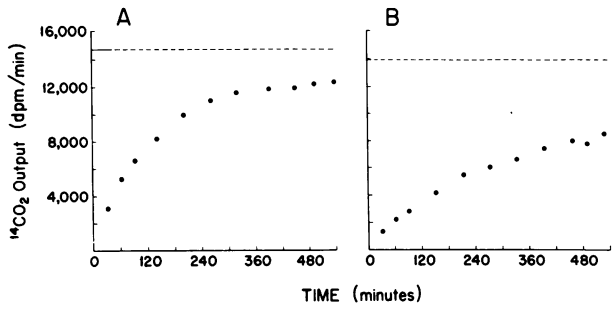

FIGURE 4 Output of $^{14}CO_2$ on the breath during constant intravenous infusion of [1-^{14}C]propionic acid (*A*) and [3-^{14}C]propionic acid (*B*). Infusions were started at time zero.

(Eq. 3, Appendix). These plots were linear by visual inspection and all had correlation coefficients of 0.99.

To better define turnover of ^{14}C in the 1 and 3 positions of propionic acid, we also performed a 30-h constant intravenous infusion of a 50-50 mixture of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid. Breath $^{14}CO_2$ output during this infusion (Fig. 5) slowly increased until at 30 h ~75% of the ^{14}C being infused was being excreted on the breath as CO_2. There was no evidence of a circadian rhythm in $^{14}CO_2$ output during this infusion.

In all three subjects, output of $^{14}CO_2$ after administration of [26-^{14}C]cholesterol varied diurnally with a peak in the mid-morning hours. Correction of these $^{14}CO_2$ output data for turnover of ^{14}C in propionic acid pools resulted in an increase in amplitude of this diurnal rhythm of ~35% and a shift in phase of 2.4–3.0 h (Table I). After this correction, data for all subjects were averaged, yielding the circadian curve presented in Fig. 6. Uncorrected overall daily bile acid synthesis rates in subjects 1, 2, and 3, respectively were 1,020, 1,110, and 1,100 μmol/d. Correcting for incomplete recovery of $^{14}CO_2$ yielded values of 1,275, 1,390, and 1,375 μmol/d, respectively.

TABLE I
Kinetic Constants for [1-^{14}C]Propionic Acid (k_1) and [3-^{14}C]Propionic Acid (k_3) Used to Correct Amplitude and Phase of $^{14}CO_2$ Output

Subject	$-k_1$	$-k_3$	Amplitude correction*	Phase shift
	h^{-1}			h
1	0.588	0.140	0.66	2.4
2	0.452	0.138	0.64	2.7
3	0.356	0.158	0.65	3.0

* Amplitude of $^{14}CO_2$ output as fraction of the amplitude of [^{14}C]-propionic acid input.

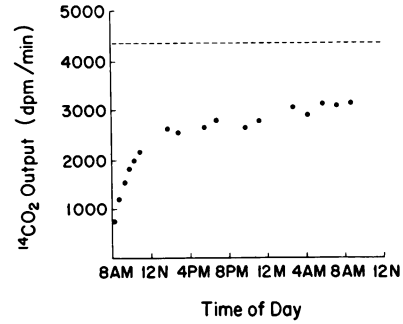

FIGURE 5 Output of $^{14}CO_2$ on the breath during a prolonged constant intravenous infusion of an equal mixture of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid. There was no diurnal variation in output of $^{14}CO_2$ during this infusion. After 30 h, 70–75% of the infused ^{14}C was being recovered as $^{14}CO_2$.

DISCUSSION

Diurnal rhythm studies require many measurements over a period of 24 h. Of the methods currently available for measuring bile acid synthesis with the enterohepatic circulation intact, only $^{14}CO_2$ production from [26-^{14}C]cholesterol would enable determinations over the necessary short time periods. To make valid use of this method in defining a diurnal rhythm, it is necessary to account for delays in metabolism of the three-carbon fragment removed from cholesterol during its conver-

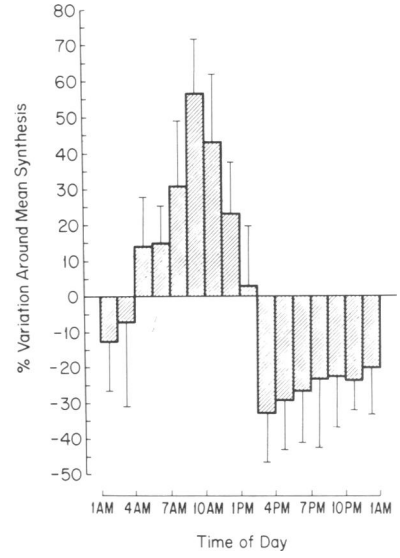

FIGURE 6 Mean±SEM of bile acid synthesis during 90-min periods throughout the 24-h cycle. Values and times have been corrected for delays in conversion of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid to $^{14}CO_2$ as described in the text. The data describe a diurnal rhythm of bile acid synthesis with an amplitude of ±35–55% around mean synthesis and an acrophase at about 9 a.m.

sion to bile acid. Because the rate-limiting step in bile acid synthesis occurs before cleavage of this three-carbon fragment (9), delays in conversion of the rest of the molecule to bile acid can be neglected.

According to the prevailing theory of bile acid intermediary metabolism, side-chain oxidation occurs predominantly via hydroxylation at carbon 27 with subsequent oxidation of this hydroxyl group to a carboxylic acid (14, 19, 20). The 25-26-27 carbon fragment is then removed as propionic acid (Fig. 1). Therefore to account for delays in conversion of the three-carbon fragment to CO_2, we determined the kinetics of $^{14}CO_2$ release from both [1-^{14}C]propionic acid and [3-^{14}C]propionic acid, which are generated in equal amounts from [26-^{14}C]cholesterol. Metabolism of [1-^{14}C]propionic acid was rapid, with a turnover rate constant of $\sim$0.5 h^{-1}. This is consistent with data obtained by administering a bolus of [1-^{14}C]propionic acid (21). Moreover, since the turnover rate constant of the CO_2-HCO_3^- pool is also $\sim$0.5 h^{-1} (17), these data suggest that the actual release of ^{14}C from this propionic acid isotope was rapid relative to transit of the ^{14}C through the CO_2-HCO_3^- pool. Interestingly, turnover of [3-^{14}C]propionic acid to $^{14}CO_2$ was prolonged compared with that of [1-^{14}C]propionic acid. The explanation for this difference can be quickly appreciated by inspection of Fig. 2, which shows that ^{14}C in the 1 position is released by metabolic activity of citric acid cycle enzymes while ^{14}C in the 3 position can be released only when citric acid cycle intermediates are metabolized through other pathways. Even with this prolonged conversion of [3-^{14}C]propionic acid to $^{14}CO_2$, the correction of phase and amplitude in $^{14}CO_2$ output was relatively small (Table I and Appendix).

Note that some fraction of bile acid synthesis may take place via an alternate pathway in which side-chain oxidation begins with hydroxylation at carbon 25 (14, 22). In that case, after oxidation of the hydroxyl group, the three-carbon fragment would be removed as acetone rather than propionic acid. However, available evidence suggests that 25-hydroxylation is at most a minor pathway for synthesis of bile acid in man (14, 19, 20, 22). Even if some fraction of the radioactivity from [26-^{14}C]cholesterol had been released as acetone, any resulting errors in correction of phase and amplitude (Table I) would have been small because the turnover constant of acetone is intermediate between our observed turnover constants for 1- and 3-labeled propionic acid (23). However, if this method were to be used in circumstances where the 25-hydroxylation path predominates, e.g., cerebrotendonous xanthomatosis, it would be wise to precisely define the rate of release of $^{14}CO_2$ from labeled acetone.

In using the $^{14}CO_2$ output method, we assume that specific activity of the immediate precursor pool of cholesterol is the same as the specific activity of the serum cholesterol. In animal models with an interrupted enterohepatic circulation given [4-^{14}C]cholesterol, this is not the case, since synthesized bile acid has a lower specific activity than serum cholesterol (24). However, in humans with an intact enterohepatic circulation, specific activity of bile acid has been virtually identical to specific activity of serum [4-^{14}C]cholesterol (25), indicating little or no preferential use of newly synthesized cholesterol for bile acid synthesis.

Not quite all catabolized cholesterol is converted to bile acid. In males $\sim$45 mg/d of cholesterol is converted to hormonal steroids (26, 27), presumably liberating $^{14}CO_2$ from [26-^{14}C]cholesterol. About 10 mg/d of this steroid hormone production is represented by testicular synthesis of testosterone, which, in sexually mature men, undergoes little if any diurnal variation (28). The remaining 35 mg/d represents adrernal steroid hormone production, at least part of which (cortisol production) undergoes a diurnal rhythm (26). However, bile acid production rate in our subjects represents oxidation of 400–500 mg/d of cholesterol or 11- to 14-fold more than would be used for adrenal hormone production. Therefore, even if all adrenal steroid hormone production stopped at night and resumed in the morning, the resulting fluctuation in $^{14}CO_2$ output would account for no more than $\pm$7–9% of the amplitude of bile acid synthesis rate shown in Fig. 6.

For purposes of the present study, we are not forced to assume that ^{14}C released from [26-^{14}C]cholesterol is quantitatively recovered as $^{14}CO_2$ on the breath. We can obtain valid estimates of the diurnal rhythm of synthesis as long as a constant fraction of this ^{14}C is recovered as $^{14}CO_2$. Fig. 5 shows $^{14}CO_2$ output during a 30-h infusion of an equal mixture of [1-^{14}C]propionic acid and [3-^{14}C]propionic acid. There was no diurnal variation of $^{14}CO_2$ output during this prolonged infusion, indicating that none of the diurnal rhythm of $^{14}CO_2$ output from [26-^{14}C]cholesterol was attributable to variation in conversion of labeled propionic acid to $^{14}CO_2$. At the end of 30 h of infusion, 70–75% of the infused [^{14}C]propionic acid was being excreted as $^{14}CO_2$ on the breath. Interestingly, even when $H^{14}CO_3^-$ is administered to human subjects, only $\sim$80% is recovered as $^{14}CO_2$ on the breath (17). A very small amount of the remaining 20% is excreted in the urine, presumably as $H^{14}CO_3^-$. Fate of the missing ^{14}C is not known. Winchell et al. (17) speculate it is incorporated into bone and large molecules.

It was not the purpose of the present study to evaluate the accuracy of bile acid synthesis as measured by the $^{14}CO_2$ output technique. We found overall daily synthesis rates by this technique to be $\sim$1,050 μmol/d without correction for possible incomplete recovery of ^{14}C as $^{14}CO_2$ and $\sim$1,300 μmol/d when such a correc-

tion was applied. We cannot say whether or not such a correction is appropriate. The ^{14}C from labeled propionic acid or bicarbonate that does not appear as $^{14}CO_2$ over a period of hours might be eliminated as something other than $^{14}CO_2$. On the other hand, over periods of days or weeks, as slowly miscible pools of CO_2-HCO_3^- come into equilibrium, recovery as $^{14}CO_2$ might approach 100%. Calculated either way, our values for bile acid synthesis by this technique are similar to synthetic rates determined by isotope dilution or fecal acidic sterol balance in man (29, 30). This similarity is consistent with the work of Redinger et al. (31), who found that bile acid synthesis by $^{14}CO_2$ output in subhuman primates with an intact enterohepatic circulation almost exactly equaled synthesis by fecal acidic sterol balance.

In the rat, diurnal rhythm of bile acid production and activity of 7α-hydroxylase have an acrophase at about 10 p.m. (11, 12). Because the rat is a nocturnal animal, acrophases of most of its diurnal rhythms occur in the evening hours. In contrast, humans have diurnal rhythms that generally peak between 6 a.m. and 12 noon (32). It is not surprising, therefore, that our observed diurnal rhythm in bile acid synthesis peaked at about 9 a.m. Amplitude of the diurnal rhythm in our subjects was ±35–55%, which is similar to the amplitude of the rhythm of bile acid production in the rat (12, 15).

The reason for the diurnal rhythm in bile acid synthesis is not provided by our studies. Indeed, the basis for most diurnal rhythms has been difficult to clearly define. Diurnal rhythms usually persist for at least some length of time when rhythmic environmental clues, e.g., eating and light cycles, are removed (12, 33). Such clues, however, may serve to entrain the rhythm to a given period and acrophase. In the present study, we simply attempted to simulate usual and typical daily patterns of eating to avoid altering the rhythm. Much more extensive and difficult studies will be required to define the role of food intake, feedback suppression by bile acids returning to the liver, and other cyclic variables in producing the diurnal rhythm of bile acid production in man.

APPENDIX

Commercially available isotopic cholesterol designated as [26-^{14}C]cholesterol is 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 as the 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 as the rate constant of removal from the pool, and $A(t)$ as the rate of formation or input of [1-^{14}C]propionic acid. Thus:

$$dP_1/dt = A(t) - k_1P_1. \quad (1)$$

If $A(t)$ is constant at A_0 (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 can determine k_1 as the slope of the line defined by plotting the left-hand side of Eq. 3 vs. t.

However, release of [1-^{14}C]propionic acid from [26-^{14}C]cholesterol may follow a circadian rhythm rather than be constant. The cosine function is customarily used to model circadian periodicity (32). If we approximate the circadian variation of bile acid synthesis as a cosine curve with a period, ω (=[$2\pi/24$] h^{-1}, where the period is 24 h and the angles are in radians) and a phase angle, θ:

$$A(t) = A_0 \cos(\omega t + \theta). \quad (4)$$

Substituting Eq. 4 into Eq. 1 yields a differential equation for which an asymptotic (steady-state) solution can be obtained from standard tables of differential equations using the change of variable $u = \omega t + \theta$. It is appropriate to employ the asymptotic solution because the turnover of cholesterol is much longer than 24 h and because the delay between administering labeled cholesterol and sampling is long compared with the turnover of propionic acid (k_1 and k_3). Using this solution, the expression for $^{14}CO_2$ output is:

$$k_1P_1(t) = A_0[1 + (\omega/k_1)^2]^{-1/2} \cos(\omega t + \theta + \phi_1) \quad (5)$$

where:

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

Eq. 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 Eq. 6.

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

$$k_3P_3(t) = A_0[(\omega/k_3)^2 + 1]^{-1/2} \cos(\omega t + \theta + \phi_3) \quad (7)$$

where:

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

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

$$k_1P_1(t) + k_3P_3(t) = A_0[(\omega/k_1)^2 + 1]^{-1/2} \cos(\omega t + \theta + \phi_1) + A_0[(\omega/k_3) + 1]^{-1/2} \cos(\omega t + \theta + \phi_3). \quad (9)$$

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:

$$A = [A_1^2 + A_2^2 + 2A_1A_2 \cos(\phi_1 - \phi_2)]^{1/2} \quad (11)$$

$$\phi = \tan^{-1}(A_1 \sin\phi_1 + A_2 \sin\phi_2/A_1 \cos\phi_1 + A_2 \cos\phi_2). \quad (12)$$

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

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