

Bile Acid Synthesis in Man

In Vivo Activity of the 25-Hydroxylation Pathway

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Abstract

During biosynthesis of bile acid, carbons 25-26-27 are removed from the cholesterol side-chain. Side-chain oxidation begins either with hydroxylation at the 26-position, in which case the three-carbon fragment is released as propionic acid, or with hydroxylation at the 25-position, in which case the three-carbon fragment is released as acetone. We have previously shown in the rat that the contribution of the 25-hydroxylation pathway can be quantitated in vivo by measuring production of [^{14}C]acetone from [^{14}C]26-cholesterol. In the present study, we adapted this method to human subjects. 4 d after oral administration of 100 μCi of [^{14}C]26-cholesterol and 1 d after beginning a constant infusion of 16.6 $\mu\text{mol}/\text{min}$ unlabeled acetone, three men and two women underwent breath collections. Expired acetone was trapped and purified as the 2,4 dinitrophenylhydrazine derivative. $^{14}\text{CO}_2$ was trapped quantitatively using phenethylamine. Specific activity of breath acetone was multiplied by the acetone infusion rate to calculate production of [^{14}C]acetone. [^{14}C]acetone production averaged 4.9% of total release of ^{14}C from [^{14}C]26-cholesterol, estimated by $^{14}\text{CO}_2$ output. The method was validated by showing that [^{14}C]acetone production from [^{14}C]isopropanol averaged 86.9% of the [^{14}C]isopropanol infusion rate. We conclude that in man, as in the rat, the 25-hydroxylation pathway accounts for < 5% of bile acid synthesis.

Introduction

Bile acids are synthesized from cholesterol by a series of ring modifications followed by oxidation and shortening of the side-chain. Oxidation of the side-chain can occur by either of two pathways (Fig. 1). The first begins with hydroxylation of carbon-26 followed by oxidation to a carboxylic acid and cleavage of the three-carbon fragment as propionic acid (1, 2). The second begins with hydroxylation of carbon-25 followed by oxidation to a ketone and cleavage of the three-carbon fragment as acetone (3, 4). Enzyme systems necessary for both pathways have been identified in rat and human liver (3, 5, 6). Moreover, intermediates exclusive to each pathway have been identified in bile of normal human subjects (7, 8).

An issue that has been difficult to resolve is the relative contribution of each of these two pathways to total bile acid synthesis in normal man. Administration to human subjects of

radiolabeled intermediates exclusive to either pathway has led to conflicting conclusions. In one study, < 20% of radiolabeled 5 β -cholestane-3 α ,7 α ,12 α ,25 tetrol was converted to bile acid (9). Since normally only trace amounts of this tetrol can be detected in human bile (8), this finding suggested that 25-hydroxylation was not normally very active in man. In another study, however, administration of radiolabeled trihydroxycoprostanoic acid (THCA),¹ an intermediate exclusive to the 26-hydroxylation pathway, resulted in production of cholic acid having a specific activity of only 20% of the THCA specific activity (10). This finding suggested that 80% of bile acid synthesis bypassed THCA, presumably via the 25-hydroxylation pathway.

The relative activity of these two pathways is especially important for understanding the rare sterol storage disease, cerebrotendinous xanthomatosis (CTX). Patients with this disease produce and excrete large amounts of 25-hydroxylated bile alcohols (11). There are two different hypotheses to explain this finding. The first proposes that the enzymatic defect in CTX is impairment of 26-hydroxylase, forcing side-chain oxidation through an otherwise little used 25-hydroxylation pathway (12). The second proposes that the 25-hydroxylation pathway is normally dominant, and that the enzymatic defect in CTX is deficient 24-hydroxylation, blocking further oxidation of normally produced 25-hydroxylated intermediates (13). Quantitating the relative contribution of the 25-hydroxylation pathway to normal bile acid synthesis could distinguish between these two alternative hypotheses.

We have recently developed and validated a method for quantitating use of the 25-hydroxylation pathway in the intact rat (14). Rather than studying the disposition of intermediates exclusive to one or the other pathway, this method assesses generation of a byproduct exclusive to the 25-hydroxylation pathway. Body cholesterol pools are first labeled with [^{14}C]26-cholesterol. Synthesis of bile acid from this labeled cholesterol by either pathway ultimately releases $^{14}\text{CO}_2$ (14, 15). However, synthesis via the 25-hydroxylation pathway results in intermediate production of [^{14}C]acetone (4). One can therefore quantitate use of the 25-hydroxylation pathway by relating production of [^{14}C]acetone to total $^{14}\text{CO}_2$ excretion. Using this method, we have shown that the normal rat synthesizes < 2% of its bile acid via the 25-hydroxylation pathway (14). In the present study, we describe adaptation and application of this technique to human subjects.

Methods

Five human volunteers ranging in age from 35 to 68 were studied. Three were male and two were female. All were in apparent good health according to previously published criteria (16). All procedures

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1. Abbreviations used in this paper: CTX, cerebrotendinous xanthomatosis; THCA, trihydroxycoprostanoic acid.

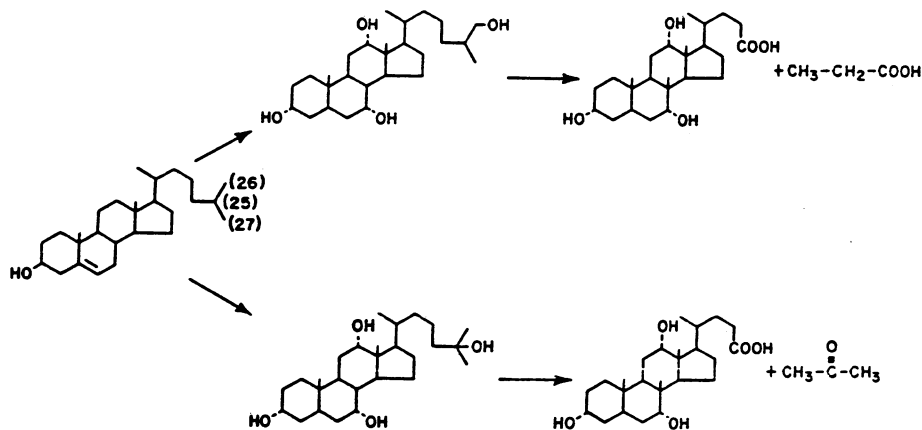


Figure 1. Abridged scheme showing the fate of the 25-26-27 three-carbon fragment during oxidation of cholesterol to cholic acid. The pathway beginning with 25-hydroxylation releases these three carbons as acetone, while the pathway beginning with 26-hydroxylation releases them as propionic acid.

were approved in advance by committees overseeing human experimentation at the Minneapolis Veterans Administration Medical Center and the University of Minnesota. Informed consent was obtained from each volunteer before study.

All solvents and chemicals were reagent grade except solvents used for HPLC, which were chromatography grade. All were used without additional purification. The scintillant used for trapping breath CO_2 was made from phenethylamine, methanol, and Liquifluor (New England Nuclear, Boston, MA) as previously described (14).

$[^{14}\text{C}]26$ -Cholesterol and $[^{14}\text{C}]2$ -acetone were purchased from New England Nuclear and checked for radiochemical purity as described previously (14). Neither material contained > 3% radiochemical impurities. $[^{14}\text{C}]2$ -Isopropanol was synthesized from $[^{14}\text{C}]2$ -acetone by catalytic hydrogenation and purified by distillation as previously described (14).

At least 5 d after oral administration of 100 μCi $[^{14}\text{C}]26$ -cholesterol, each subject was admitted to the Special Diagnostic and Treatment Unit of the Minneapolis Veterans Administration Medical Center. An 18-gauge catheter was placed in a forearm vein and a constant infusion of unlabeled acetone at 16.6 $\mu\text{mol}/\text{min}$ was begun. After 18–24 h of this infusion and with the infusion continuing, breath samples were obtained using the apparatus previously described (15). Briefly, each subject was placed under a plexiglass hood from which air was drawn through a series of traps containing the scintillation fluid described above to quantitatively collect breath CO_2 . After duplicate 10-min collections of breath CO_2 , the traps containing scintillant were replaced with traps containing 4.0 ml of an aqueous solution of 2.5 mg/ml 2,4-dinitrophenylhydrazine in 2.0 N HCl, 60.0 ml water, and 30 ml toluene. Air was then drawn from the plexiglass box through these traps for two separate 30-min collection periods. For validation studies, a known amount of $[^{14}\text{C}]$ isopropanol was included in the acetone infusion solution.

Isolation of the acetone dinitrophenylhydrazone from breath collections was accomplished by a series of thin-layer chromatographic and high performance chromatographic steps as previously described (14). Part of each isolate was analyzed for acetone mass by gas-liquid chromatography (14). Another part of each isolate was combusted on a biological oxidizer (Tri-Carb 306; Packard Instrument Co., Downers Grove, IL) for subsequent liquid scintillation counting as previously described (14). Because so little $[^{14}\text{C}]$ acetone was produced in the $[^{14}\text{C}]26$ -cholesterol experiments (Table II), raw radioactivity counts in these experiments were low, ranging from 4 to 42 cpm above a background of ~ 25 cpm. To obtain adequately precise counting statistics, we therefore counted each vial (including a background vial) for five 20-min periods, alternating the sample vials with a background vial. Assuming a background of exactly 25 cpm, standard methods for calculating and combining counting errors yield a 95% confidence interval of 2.5–5.5 for a count of 4.0 above background (17, 18). For a count of 42 above background, the corresponding 95% confidence limit is 40.1–43.9.

The final value for radioactivity was divided by acetone mass to calculate specific activity of breath acetone. This permitted calculation of the production rate of $[^{14}\text{C}]$ acetone by the equation $P = (I) (SA)$, where P is the $[^{14}\text{C}]$ acetone production rate; I , the infusion rate of unlabeled acetone; and SA , the specific activity of breath acetone.

Results

Table I provides values for $[^{14}\text{C}]$ acetone production in three subjects during constant intravenous infusion of $[^{14}\text{C}]$ -isopropanol. For these individuals, measured $[^{14}\text{C}]$ acetone production was 79.5, 98.9, and 82.4% of the $[^{14}\text{C}]$ isopropanol infusion rate. Since 80–95% of isopropanol is converted to acetone (14, 19, 20), these data indicate that our technique measured $[^{14}\text{C}]$ acetone production in these subjects with reasonable accuracy.

Table II provides values for $[^{14}\text{C}]$ acetone production and $^{14}\text{CO}_2$ output in five subjects given $[^{14}\text{C}]26$ -cholesterol at least 5 d earlier. Mean production of $[^{14}\text{C}]$ acetone was 4.9% of simultaneously determined $^{14}\text{CO}_2$ output. Judging from these data, average contribution of the 25-hydroxylation pathway to total bile acid synthesis was < 5% in these five subjects.

Discussion

The methods of the present study have been extensively explained and discussed in a previous publication (14). To briefly summarize, the method is unusual because it quantitates activity of a metabolic pathway in vivo by radiolabeling the original substrate, cholesterol, and monitoring generation of a unique radiolabeled byproduct of that pathway, acetone. The usual way of studying metabolic pathways in vivo is to administer a

Table I. $[^{14}\text{C}]$ Acetone Production during $[^{14}\text{C}]$ Isopropanol Infusion

Subject	$[^{14}\text{C}]$ Isopropanol infusion rate	$[^{14}\text{C}]$ Acetone production rate	Fraction $[^{14}\text{C}]$ acetone recovered
<i>dpm/min</i>			
1	235,500	187,200	0.795
2	235,500	232,860	0.989
3	235,500	193,950	0.824
		Mean	0.869

Table II. [¹⁴C]Acetone Production from [¹⁴C]26-Cholesterol

Subject	¹⁴ CO ₂ output	[¹⁴ C]Acetone production	Percent synthesis via 25-OH intermediates
	<i>dpm/min</i>		
1	530	49.4	9.3
2	396	31.2	7.9
3	513	3.9	0.8
4	528	24.1	4.6
5	304	5.6	1.8
		Mean	4.9

radioactive hypothetical intermediate and monitor incorporation of radioactivity into the final product. That approach can give falsely positive results if the hypothetical intermediate is converted to product but is not actually formed *in vivo* during normal metabolism. Conversely, it can give falsely negative results if the administered intermediate fails to quantitatively reach the cellular location at which further conversion to product normally occurs. The monitoring of a unique metabolic byproduct, such as acetone, suffers from neither of these potential pitfalls. It is an approach that presumably would be used more often if not for the fact that very few metabolic reactions have byproducts that are both unique and measurable.

Production of [¹⁴C]acetone in the present study was calculated as the product of the infusion rate of unlabeled acetone and the breath acetone specific activity (see Methods). The prolonged constant infusion of unlabeled acetone serves two critical functions. First, in the fed state, endogenous acetone production is negligible compared with the infusion rate used in the present study (21). The infusion rate thus provides a reasonable estimate of total acetone input into the system. Second, this input of unlabeled acetone floods the liver, helping to assure that [¹⁴C]acetone produced in the liver is not rapidly metabolized before reaching the periphery, where it can be measured. Indeed, the major biological prerequisite for validity of this method is that [¹⁴C]acetone produced in the liver cell must thoroughly mix with the infused acetone so that the measured specific activity is representative. To show that this prerequisite was satisfied, we measured production of [¹⁴C]acetone during constant infusion of [¹⁴C]isopropanol, which is catabolized almost exclusively to acetone (14, 19, 20). In these studies (Table I), measured production of [¹⁴C]acetone was nearly identical to the infusion rate of [¹⁴C]isopropanol. These data provide strong evidence that the measured specific activity of breath acetone was representative of that in the liver, and that the method overall provides an accurate estimate of [¹⁴C]acetone production in man. We have also reported similarly close agreement between measured [¹⁴C]acetone production and input of [¹⁴C]isopropanol in the rat (14).

In five human subjects given [¹⁴C]26-cholesterol, we found that production of [¹⁴C]acetone averaged < 5% of total production of ¹⁴CO₂. This finding indicates very little use of the 25-hydroxylation pathway for bile acid synthesis in man and is fully consistent with nearly identical findings by the same method in the rat (14). Two earlier independent observations *in vivo* also suggested that the 25-hydroxylation pathway

played at most a minor role in normal bile acid synthesis. First was the incomplete (< 20%) conversion of 5 β -cholestane-3 α ,7 α ,12 α ,25 tetrol to cholic acid in man (9). Second was the absence of 25-hydroxylated intermediates, in spite of the presence of THCA, in patients with Zellweger's syndrome, a disease in which side-chain cleavage is defective (22). Neither observation, alone or in combination, absolutely excluded a major role for the 25-hydroxylation pathway. The 25-tetrol thus might have failed to reach the cellular location required for further conversion to bile acid. In addition, children with Zellweger's syndrome are extremely abnormal and terminally ill, so the implications of their bile acid pattern for normal biochemistry was uncertain. Nevertheless, these two previous independent observations further strengthen the case against a major role for the 25-hydroxylation pathway in normal human bile acid synthesis.

A weakness of the present study is that we were forced to rely on a negative finding, namely the lack of production of [¹⁴C]acetone. The study would have been considerably strengthened had we been able to document that ¹⁴C released from [¹⁴C]26-cholesterol was quantitatively incorporated into propionic acid. However, our efforts to measure incorporation of radioactivity into propionic acid in a previous study were unsuccessful because propionic acid levels in serum and breath were so low that adequate amounts of this metabolite could not be collected for analysis (14). Unfortunately, therefore, this desirable confirmatory data proved impossible to obtain.

The only *in vivo* evidence for a major role of the 25-hydroxylation pathway in normal man is a study by Salen et al. in which radiolabeled THCA was administered continuously over a period of 14 d to one normal volunteer (10). For the last 8 d of the experiment, specific activity of biliary THCA and cholic acid were both constant, indicating the existence of a steady state. During this time, specific activity of cholic acid was only 20% of the specific activity of THCA. Because THCA is not an intermediate of the 25-hydroxylation pathway, this difference in specific activities suggested that 80% of bile acid synthesis occurred via the 25-hydroxylation pathway. The authors correctly pointed out, however, that there was an alternative explanation, namely that the labeled THCA did not reach the appropriate cellular location for conversion to cholic acid before being conjugated and/or excreted. In light of the results of the present study and the other evidence against extensive use of the 25-hydroxylation pathway in man, this alternative explanation now seems the more likely of the two.

The rare sterol storage disease, CTX, is characterized in part by production and excretion of large amounts of 25-hydroxylated bile alcohols (11). Two separate hypotheses have been advanced to explain this finding. The first is that CTX patients have impaired 26-hydroxylase activity, which results in accumulation of 5 β -cholestane-3 α ,7 α ,12 α triol and shunting of this intermediate through an otherwise inactive 25-hydroxylase pathway (12). The second supposes that the 25-hydroxylation pathway is normally dominant and that impaired 24-hydroxylation of 25-hydroxylated intermediates results in their excretion rather than their further transformation to bile acid (13). *In vitro* studies have failed to distinguish between these two possibilities because activities of both the 24- and the 26-hydroxylase have been reported to be low in CTX patients (12, 13). The present study, by demonstrating normal dominance of the 26-hydroxylation pathway, eliminates reduced 24-hydroxylation as an explanation for the production of large

amounts of 25-hydroxylated bile alcohols in CTX. This is not to say that 24-hydroxylation is not impaired in CTX patients. Indeed, impaired activity of this enzyme remains one possible reason that so little of the 25-hydroxy bile alcohols produced in CTX is further oxidized to bile acid.

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