Preparation of [24,25-^{3}H]cholesterol. Oxidation in man as a measure of bile acid formation

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Abstract In order to devise a convenient method for measuring the rate of formation of bile acids from cholesterol in man, [24,25-^{3}H]cholesterol was prepared by catalytic tritiation of desmosterol where the Δ^5-unsaturation was protected by conversion to the 3α,5α-cyclosterol-6-methyl ether. It was shown that tritium in the purified labeled sterol was located exclusively in the side chain. Measurements of ^3H_2O and the specific activity of plasma cholesterol at timed intervals up to 96 hours after administration of the tracer to three subjects afforded values for cholesterol oxidation (equals bile acid formation) in good agreement with accepted figures for bile acid formation determined by other techniques.

Supplementary key words cholesterol oxidation · cholesterol metabolism

While the conversion of cholesterol to bile acids in vivo is a multistep process involving sequential nuclear and then side chain hydroxylations (1), the actual formation of the bile acid occurs when the C_{24}–C_{25} bond of the C_{27} steroid is cleaved to form the C_{24} steroid and the C_3 fragment. The latter is rapidly metabolized to CO_2 and water. The overall reaction, i.e., side chain oxidation of cholesterol which, in the intact rat (2), has been shown to take place with the formation of CO_2, has been used to great advantage by Chevallier and Lutton (3) who reported that under steady state conditions during ingestion of [26-^{14}C]-cholesterol, the expired $^{14}CO_2$ was a measure of bile acid formation in this animal. In the same year, Myant and Lewis (4) determined bile acid formation in man from the measurement of $^{14}CO_2$ excretion after an intravenous dose of [24-^{14}C]cholesterol. Later, Davis, Showalter, and Kern (5) used the single-injection technique to study bile acid synthesis in rats. These procedures suffer from the drawback of cumbersome and difficult methodology in collecting a gaseous product, particularly if studies in man are contemplated.

In our laboratory, measurements of tritium in body water have afforded insight into the metabolism of [17-^{3}H]estradiol (6) and [17α-^{3}H]testosterone (7) as indicators of endogenous hormone behavior and have led us to conclude that cholesterol appropriately labeled with tritium could be used to yield important data with regard to the formation of bile acids. If tritium were incorporated only into that portion of the cholesterol side chain that is degraded during conversion to bile acids, the ^{3}H in body water would be a measure of bile acid production.

In our hands, efforts to prepare [25-^{3}H]cholesterol from 5-cholestene-3β,25-diol-3-acetate according to the procedure described by Joly, Sauer, and Bonner (8) were unsuccessful. Replacement of the tertiary hydroxyl group by bromine (8, 9) proceeded uneventfully to yield the desired intermediate. However, attempts to convert 25-bromocholesteryl-3-acetate to labeled cholesteryl-3-acetate by reductive replacement of the halogen with tritium by treatment with lithium aluminum tritide afforded 5,24-cholestadien-3β-ol (desmosterol) as the major product. Reacting 25-bromocholesteryl-3-acetate or 25-tosyloxycholesterol with sodium borohydride in dimethyl sulfoxide at room temperature according to Hutchins and coworkers (10) again led to the introduction of unsaturation at C-24,25.

We then turned to techniques for hydrogenation (tritiation) of desmosterol where the double bond in the B-ring was protected by conversion to the intermediate 3,5-cylosteroid (*i*-sterol). *i*-Desmosterol derivatives were prepared by the technique of Kosower and Winstein (11) or by the earlier method of Riegel and Kaye (12). Catalytic reduction of 3α,5α-cyclocholest-24-ene-6-ol-6-methyl ether (12) with tritium gas gave a product which was rearranged back to the Δ^5-3β-ol structure (13). [^{3}H]Cholesterol, obtained in good yield, was purified to radiochemical homogeneity where

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; desmosterol, 5,24-cholestadien-3β-ol; *i*-desmosterol, 3α,5α-cyclo-24-cholesten-6-ol.

virtually all of the ^3H was shown to be in the side chain, most probably at C_{24} and C_{25}.

In order to evaluate the feasibility of the procedure for the study of bile acid formation and to establish an appropriate experimental protocol, tracer amounts of [24,25-^{3}H]cholesterol were administered to three men in whom bile acid formation was calculated from labeled body water and circulating plasma cholesterol. The results are in accord with data obtained by other investigators employing different methods.

EXPERIMENTAL

Preparation and radiochemical purity of [24,25-^{3}H]cholesterol

Preparation of desmosterol-3-tosylate. To 400 mg of desmosterol (Steraloids, Inc., Wilton, NH) dissolved in 1.5 ml of pyridine was added 440 mg of toluene sulfonyl chloride. After standing at room temperature for 3 days, the solution was mixed with ice and extracted with diethyl ether. The ether solution was consecutively washed with 5% hydrochloric acid, 5% sodium hydroxide, and water until the washings were neutral; then they were dried over sodium sulfate. Concentration of the ether solution yielded 395 mg of crystalline material which was recrystallized from methanol, mp 121–123°C: analysis for sulfur ($C_{34}H_{50}O_3S$), calculated 5.95%; found 5.89%.

Preparation of i-desmosterol (11). A solution of 364 mg of desmosterol-3-tosylate in 6 ml of acetone was mixed with 1.5 ml of water followed by 289 mg of potassium acetate. The mixture was refluxed for 9 hr, then it was left overnight at room temperature and continued at reflux for 7 hr. The reaction mixture was concentrated on a steam bath in vacuo to remove the acetone and the residue was extracted with ether; after washing the ether solution with water and drying it over sodium sulfate, removal of the solvent gave 285 mg of an oil that resisted crystallization. Gas–liquid chromatography (GLC) on 3% OV-1 and 3% OV-225 (Supelco, Inc. Bellefonte, PA) at 256°C showed minor amounts of desmosterol, with the major peak showing a shorter retention time. A portion of the product was chromatographed on a silica gel plate with ethyl acetate–cyclohexane 3:7; the major product, with an R_f value of 0.45, migrated between cholestadiene (R_f 0.65) and desmosterol (R_f 0.29).

The major portion of the reaction product, 250 mg, was chromatographed on 40 g of deactivated aluminum oxide. The early, nonpolar material eluted with petroleum ether–benzene 8:2 was crystalline, weighed 5 mg, and by GLC was very probably cholesta-3,5,24-triene. The main product that was eluted in petroleum ether–benzene 6:4 could not be crystallized and was further purified by chromatography on several 20 × 20 cm plates coated with silica gel, using the same system as above; 122 mg of material was obtained from the bands, R_f 0.45. The most polar fraction, 18 mg, that was eluted from the column with petroleum ether–benzene 1:1 was desmosterol as judged by melting point (116–118°C) and by GLC and TLC characteristics which were identical to those of authentic material. Data from NMR of the main product (R_f 0.45) were as follows: δ0.76 (3H, C-18); δ1.03 (3H, C-19); δ1.58, δ1.67 (3H, C-26 and C-27); δ5.08 (1H, C-24).

i-Desmosterol-6β-acetate. The product from TLC was acetylated in 2 ml of acetic anhydride–pyridine 1:1 under reflux for 3 hr. Work-up as usual afforded 107 mg which was crystallized from acetone–methanol, mp 111–113°C: calculated ($C_{29}H_{46}O_2$), C 81.6%, H 10.9%; found C 82.2%, H 11.1%. NMR δ0.72 (3H, C-18); δ0.98 (3H, C-19); δ1.58; δ1.65 (3H, C-26 and C-27); δ2.00 (3H,-CO-CH_3); δ4.50 (1H, 6α-H) (14); δ5.08 (1H, C-24).

i-Desmosterol-6β-methyl ether. The substance was prepared from 311 mg of desmosterol-3-tosylate according to Riegel and Kaye (12). In contrast to the crystalline material (mp 64–66°C) synthesized by Dasgupta, Crump, and Gut (15) by a different route, our product could not be crystallized. Two hundred and twelve mg of the oil was chromatographed on 45 g of silica. The column was prepared in and developed with petroleum ether–diethyl ether 99:1. Preceding the main fraction of 145 mg was 5 mg of crystalline material, probably the 3,5,22-triene; the more polar material was not investigated.

The main fraction showed a single spot on silica gel TLC (R_f 0.5 in benzene). NMR was in accord with the *i*-methyl ether structural assignment: δ0.70 (3H, C-18); δ1.00 (3H, C-19); δ1.60, δ1.63 (3H, C-26 and C-27); δ3.32 (3H,-OCH_3) (16), δ5.08 (1H, C-24). This material was reduced catalytically with tritium.

Reduction of i-desmosterol-6β-methyl ether with tritium. Eighty two milligrams were reduced according to the procedure of Fernholz and Ruigh (13) by New England Nuclear Corporation, Boston, MA. The material was dissolved in 2 ml of ethyl acetate to which was added 50 mg of palladium black catalyst and tritium gas; the mixture was stirred overnight at room temperature. Labile tritium was removed by repeated dilution with methanol–methylene chloride and concentration in vacuo. The solution was filtered from the catalyst, again concentrated, and dissolved in 10 ml of benzene.

Conversion of tritiation product to [24,25-^{3}H]cholesterol. One-half of the product from the tritiation of 82 mg of *i*-desmosterol-6β-methyl ether was processed.

[24,25-^3H]Cholesterol acetate. After evaporation of the benzene under a stream of nitrogen, the tritiated reaction product was refluxed in 5 ml of glacial acetic acid to which 200 mg of zinc acetate was introduced. After 6 hr the solution was diluted with cold water and extracted with diethyl ether. The ether solution was washed with cold 5% sodium hydroxide until the washings were alkaline and then were washed with water. Fifteen milligrams of nonradioactive cholesterol acetate was added prior to concentration of the ether solution.

[24,25-^3H]Cholesterol. Labeled cholesterol acetate obtained from the preceding step was refluxed for 2 hr in 5% sodium hydroxide in 80% ethanol solution. The mixture was cooled and extracted with petroleum ether. The petroleum ether phase was washed with water and concentrated to dryness prior to chromatography. The crystalline residue was chromatographed on 15 g of deactivated aluminum oxide (17). The column was developed in petroleum ether–benzene 1:1, benzene, then benzene–diethyl ether 19:1. About 46% of the total radioactivity (17.85×10^{12} cpm) was eluted in the crystalline fractions associated with cholesterol. This was based on a pilot chromatogram run with nonradioactive cholesterol under the same conditions. An aliquot of each fraction was diluted with carrier cholesterol and crystallized from acetone and methanol to constant specific activity. The fractions eluted with benzene were about 55% radiochemically pure and were combined.

At this point, 300 mg of cholesterol was added to the combined fractions and the material was brominated in glacial acetic acid. The crystalline 5,6-dibromide was washed with small portions of ether–acetic acid 2:1 and, after drying under nitrogen at room temperature, was treated with zinc dust–glacial acetic acid to regenerate cholesterol. By reverse isotope dilution as before, an aliquot of this material was shown to be about 83% radiochemically pure. A parallel run, carried out with 300 mg of nonradioactive cholesterol under identical experimental conditions afforded 40 mg of cholesterol.

The radioactive cholesterol obtained from the debromination step was finally crystallized once from methanol and a few of the crystals so obtained were checked for radiochemical purity by reverse isotope dilution with carrier cholesterol; there was essentially no change in specific activity after several crystallizations from methanol, (900,924,914 cpm per mg). The final [^{3}H]cholesterol was considered to be radiochemically pure.

Location of ^3H in the side chain of cholesterol

An aliquot of [^{3}H]cholesterol was diluted with 3.5 g of nonradioactive cholesterol and acetylated with acetic anhydride–pyridine. Cholesteryl acetate, 3 g, obtained after extraction and washing in the usual fashion (sp act 158×10^4 cpm per mmol), was dissolved in 30 ml of methylene chloride–diethyl ether 1:3, buffered with 50 ml of 1.2% sodium acetate in 96% acetic acid, and brominated at 10°C by dropwise addition of 15 ml of bromine in glacial acetic acid (100 mg per ml). The 5,6-dibromocholesterol-3-acetate was filtered from the reaction mixture and washed with cold glacial acetic acid.

The dibromoacetate, moist with acetic acid, was dissolved in 40 ml of glacial acetic acid–methylene chloride 5.5:1; celite was added and the mixture was treated with chromic oxide–acetic acid–sulfuric acid mixture at 16°C as described by Schwenk, Werthessen, and Colton (18). After decomposing excess oxidizing agent and filtering the solution from the celite as described, the filtrate and washings were stirred with zinc dust for 2 hr. Removal of zinc was followed by the addition of water and the slurry was extracted with diethyl ether. The ether solution was washed repeatedly with potassium hydroxide solution to remove the acidic decomposition products, then with water. The ether was evaporated, affording a neutral fraction of 325 mg. Gas–liquid chromatography (3% OV-225, 243°C) showed a peak with a retention time identical to that of 3β-acetoxy-5-androsten-17-one. The 17-ketosteroid was obtained in the ketonic fraction after the Girard separation, 117 mg. This crystalline material was saponified to afford 3β-hydroxy-5-androsten-17-one which was chromatographed on a small aluminum oxide column from which was obtained the 17-ketosteroid, identical in every respect to authentic material; sp act 0.78×10^4 cpm per mmol. Therefore, no more than 0.5% of the total radioactivity in cholesterol was located in the ring system, i.e., 99.5% of the label was present in the side chain.

Patients and protocols

Approval for the studies was given by the Human Research Committee of Montefiore Hospital and Medical Center. The purpose and design of the studies, as well as any possible risks, were carefully explained to the patients and consent was given by each participant. [24,25-^{3}H]Cholesterol was diluted with 100 μg of nonradioactive sterol and dissolved in 200 μl of ethanol. This was mixed with 2 ml of propylene glycol under sterile conditions and the solution was administered by vein through a venoset infusion in 5% dextrose; the procedure took approximately one minute. In this manner, three men, 37, 56, and 72 years old, each received about 70 μCi of [24,25-^{3}H]-cholesterol. Blood (10–15 ml) was withdrawn into heparinized tubes at frequent intervals 6–12 times during the first 24 hr, commencing at 10 min after the tracer and continuing once or twice per day up to 4 days. The blood was immediately chilled and

centrifuged to remove the red cells. Plasma was stored at −20°C until processing. In one subject, samples of saliva were obtained between meals at various intervals throughout the studies.

Isolation of plasma cholesterol

The removal of plasma proteins and the partition of the deproteinized residue between petroleum ether and 70% ethanol have been described (19). The lipid residue from the petroleum ether fraction was chromatographed on a 20 × 20 cm silica gel-coated plate (ethyl acetate–cyclohexane 2:8) to obtain free cholesterol and cholesteryl esters; the appropriate bands, identified by pilot spots, were scraped from the plates and the material was extracted with methanol–chloroform 1:2. Cholesteryl esters were hydrolyzed by refluxing in 5% sodium hydroxide in 80% ethanol and the nonsaponifiable fraction was extracted from the alkaline solution with petroleum ether; TLC was carried out as before. Free cholesterol and cholesterol from the ester fraction were dissolved in benzene; cholestane was added as an internal standard and aliquots were removed for counting prior to quantitation by GLC (3% SP 2250 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA) at 260°C.

Body water

Samples of plasma red cells or saliva were lyophilized to obtain the sublimate for radioactive assay. (Body water volume was measured by dilution of a small amount of tritium water administered 2 weeks before sampling (20).) Alternatively, total body water was estimated from urinary creatinine excretion (21).

Counting

Body water samples (0.5 ml) were mixed with 18 ml of scintillant (ScintiVerse, Fisher Scientific Co., New York, NY) and cholesterol samples were dried and dissolved in the same volume of scintillant containing 0.5 ml of water. All counting was done by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

The percentages of the dose of administered [24,25-^{3}H]cholesterol that appeared in body water in three subjects are shown in **Fig. 1.** The shapes of the curves are very similar: at 24 hr after the tracer, from 1.5 to 3.0% of the dose was present and by 5 days, 4–5% was found. It can be seen that the tritium content of body water is independent of its source; tritium water from plasma, red cells, or saliva, as would be expected, falls on the same line since tritium water reaches a steady state throughout the body within 2 hr (20).

Fig. 2 shows the specific activity curves for plasma

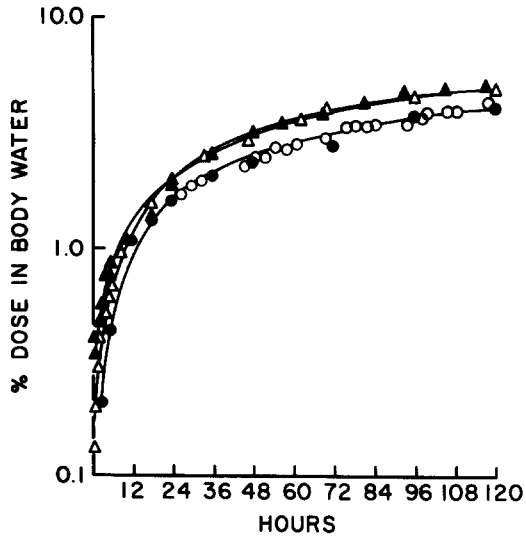

Fig. 1. Percent of [24,25-^{3}H]cholesterol in body water up to 96 hr after administration of the tracer to three subjects. ●, Water from red cells, patient 1; ○, water from saliva, patient 1; △, water from red cells, patient 2; ▲, water from red cells, patient 3.

free and esterified cholesterol from one of the patients (no. 2); they are similar to those that have been reported by us (22) and others (23–25) after the administration of [4-^{14}C]cholesterol or [1,2-^{3}H]cholesterol. The precipitous drop-off of cholesterol radioactivity in the plasma during the first 2 hr is a consequence of the rapid removal of nonlipoprotein-bound sterol by the liver (26). However, by 36 hr, both free and esterified cholesterol were distributed in the circulating pool and were declining with approximately the same specific activity. It is from this "inner pool" of cholesterol that bile acids are derived (27).

Since the only significant transformation of choles-

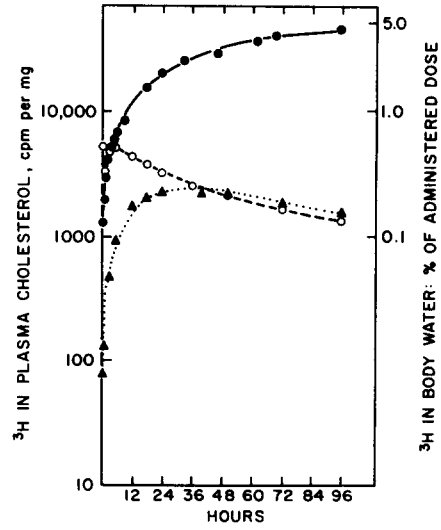

Fig. 2. Specific activity (cpm/mg) of plasma [^{3}H]cholesterol and percent of ^{3}H in body water of patient 2 up to 96 hours after administration of the tracer. ○ – – – ○, Free cholesterol; ▲ . . . ▲, ester cholesterol; ● —— ●, water.

TABLE 1. Cholesterol side chain oxidation calculated at increasing times after administration of [24,25-^{3}H]cholesterol

Subject and cpm Injected	Time after Tracer	Δ^3H_2O[a]	Cholesterol	Cholesterol Undergoing Side Chain Degradation	
				12 hr	24 hr
	hr	*%*	*cpm/mg*	*mg*	
1	36–48	0.38	2090	136	273
7.50×10^7	48–60	0.30	2000	113	226
	60–72	0.30	1900	118	237
	72–96	0.65	1790		272
2	36–48	0.45	2350	142	284
7.42×10^7	48–60	0.41	2150	141	283
	60–72	0.39	1950	148	297
3	36–48	0.55	2300	178	356
$7.44 \times 10_7$	48–60	0.45	2190	153	306
	60–72	0.40	2070	144	288
	72–96	0.70	1800		289

[a] Δ^3H_2O is the increment in % of dose in body water appearing between the times after the injection of the tracer indicated in column 2. Column 4 lists the specific activity of plasma cholesterol at the mid-point between the times indicated in column 2.

terol in man that involves loss of the side chain[1] is the conversion to bile acids, the tritium appearing in body water is a measure of bile acid formation. In Fig. 2, the percent dose as tritium in body water of patient 2 is plotted with the plasma cholesterol specific activity data. It can be seen that from 36 to 48 hr, the tritium in body water increased by 0.45% of the dose, from 2.65 to 3.10%. In this subject, who received 7.42×10^7 cpm of [24,25-^{3}H]cholesterol, it follows that 3.34×10^5 cpm of tritium were derived during this time from the conversion of cholesterol to bile acids. Between 36 and 48 hr, the specific activity of circulating cholesterol decreased from 2500 cpm per mg to 2200 cpm per mg and it is a reasonable assumption to consider the average specific activity between these points to be approximately 2350 cpm per mg. Therefore about 142 mg of circulating cholesterol (3.34×10^5 cpm/2.35×10^3 cpm per mg) underwent side chain degradation, i.e., conversion to bile acids during the 12 hr, or 284 mg per 24 hr. The calculations are essentially the same as those employed by us (28) and others (4, 29) in measuring bile acid excretion, using [4-^{14}C]cholesterol and the fecal acid fraction or [26-^{14}C]cholesterol and expired $^{14}CO_2$, respectively. **Table 1** shows data from the two other studies carried out in the same manner to evaluate the feasibility of using [24,25-^{3}H]cholesterol for determining bile acid synthesis in man from measurements taken between 36 and 48 hr; calculating the quantity of cholesterol that lost the terminal 3 carbons of the side chain afforded values of 273 and 356 mg per day. In addition, it can be seen from the table that, if similar calculations are made from measurements taken during successive 12-hr intervals up to 96 hr, comparable figures are obtained. These data are well within the range reported by a number of investigators using mainly GLC analyses of fecal bile acids (30–33).

Two sources of error in the calculations can be considered: *1*) Recycling of tritium-labeled water and propionate into endogenous cholesterol biosynthesis. This source of radioactivity can be neglected, since less than 5% of the circulating cholesterol pool is synthesized in 24 hr and the radioactivity in body water is such that the amount incorporated into sterols is below the limits of detection (34). In studies utilizing the breakdown of [26-^{14}C]cholesterol for measuring bile acid synthesis, it was found that more than 80% of the propionic acid formed during side chain cleavage was converted to $^{14}CO_2$ (and water) in 5 hr (4); it is likely that a larger conversion of the 3-carbon fragment would have been observed had the period of the experiment been longer. *2*) The second source of error is the turnover of body water. Body water declines with a t½ of 10 days in man (35), a turnover time of 14.4 days; therefore, in 24 hr, the decrease in radioactivity in body water would amount to 7% of the tritium were there no further input of tritiated water from the conversion of cholesterol to bile acids. We have neglected this correction during the 36–48 hr period selected for measurement when the percentage of the radioactivity in body water is increasing rapidly; however, it cannot be neglected if bile acid synthesis from circulating cholesterol were calculated during a later time period where the cholesterol specific activity is low and the input of ^3H_2O from side chain oxidation is correspondingly less.

Estimation of bile acid synthesis by measuring the specific activity of circulating cholesterol and the tritium content of body water has the advantages of convenience and simplicity, obviating the multistep procedures involved in estimating bile acids in feces, the problems of sampling duodenal bile, and the cumbersome technique of collecting expired CO_2 over an extended period of time. It should be pointed out that the technique might possibly be adapted to the use of stable isotopes where deuterium content of cholesterol and body water could be measured by mass spectrometry. We intend to employ the body water method in studies relating to bile acid formation in various hyperlipoproteinemic states and in colon cancer, particularly with regard to various treatment modalities.

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[1] The amount of cholesterol in the circulation that is converted to the sex and adrenocortical hormones, less than 1%, can be neglected for the purposes of this calculation.

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