# Loss of tritium from coprostanone derived from [1,2(n)-$^3$H]cholesterol or [7(n)-$^3$H]cholesterol

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**Summary** After oral administration of a mixture of [1,2(n)-$^3$H]cholesterol and [4-$^{14}$C]cholesterol to a baboon, fecal coprostanone had a 46% lower $^3$H/$^{14}$C ratio than the dose administered. Loss of $^3$H by enolization of the 3-ketone could account for the decrease in $^3$H/$^{14}$C. If [7(n)-$^3$H]cholesterol was administered instead of [1,2(n)-$^3$H]cholesterol a 23% loss of $^3$H from coprostanone was found. Procedures requiring measurement of $^3$H-coprostanone derived from [1,2(n)-$^3$H]- or [7(n)-$^3$H]cholesterol could be seriously in error unless an appropriate correction for loss of $^3$H is made.—**Mott, G. E., and E. M. Jackson.** Loss of tritium from coprostanone derived from [1,2(n)-$^3$H]cholesterol or [7(n)-$^3$H]cholesterol. *J. Lipid Res.* 1980. **21:** 480–484.

**Supplementary key words** fecal neutral steroids · cholesterol absorption

Radioactive cholesterol is commonly used to measure cholesterol absorption (1, 2) and cholesterol balance in animals (3). We assessed the potential for loss of tritium during these procedures by mixing [1,2(n)-$^3$H]cholesterol or [7(n)-$^3$H]cholesterol with [4-$^{14}$C]cholesterol and orally administering the labeled sterols to two baboons. We then compared the $^3$H/$^{14}$C of the fecal neutral steroids with the $^3$H/$^{14}$C of the cholesterol administered. We also determined the radioactivity in the steroid products of $^3$H-labeled and $^{14}$C-labeled cholesterol and β-sitosterol metabolism by a microorganism in pure culture. These experiments indicate that significant losses of $^3$H occur from [1,2(n)-$^3$H]- and [7(n)-$^3$H]coprostanone which can cause large errors in measurements of cholesterol absorption or isotopic balance.

## MATERIALS AND METHODS

### Radiolabeled sterols

[1α,2α(n)-$^3$H]cholesterol (Batch 21 and 23), [4-$^{14}$C]cholesterol (Batch 100), and [4-$^{14}$C]β-sitosterol (Batch 10) were obtained from Amersham-Searle Corp. (Arlington Heights, IL). [7(n)-$^3$H]Cholesterol (Lot #998-224) and [22,23-$^3$H]β-sitosterol (Lot #951-029) were obtained from New England Nuclear Corp. (Boston, MA). Each sterol was purified with 0.5 mg unlabeled cholesterol or β-sitosterol by TLC on silica gel G with diethyl-ether–hexane 70:30 (v/v). Steroid standards and supplies for GLC were purchased from Applied Science (State College, PA).

### Biological test for purity of labeled cholesterol

We also determined the purity of the [1,2-$^3$H]- and [4-$^{14}$C]cholesterol in a biological experiment. After TLC purification of the [$^3$H]- and [$^{14}$C]cholesterol as described above $23.92 \times 10^6$ dpm of [1,2-$^3$H]cholesterol and $4.26 \times 10^6$ dpm of [4-$^{14}$C]cholesterol ($^3$H/$^{14}$C

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography. The following are systematic names of steroid trivial names used in the text: cholesterol, cholest-5-en-3β-ol; coprostanol, 5β-cholestan-3β-ol; coprostanone, 5β-cholestan-3-one; β-sitosterol, 24β-ethyl-cholest-5-en-3β-ol; β-sitostanol, 24β-ethyl-5β-cholestan-3β-ol; β-sitostanone, 24β-ethyl-5β-cholestan-3-one.

= 5.61) were injected intravenously into a 30 kg baboon. The injection medium was 5 ml of serum obtained from the baboon prior to injection and filtered through a 0.22 micron Millipore (Millipore Corp., Bedford, MA) filter. The radioactive sterols were dissolved in 100 $\mu$l of acetone and added rapidly to the serum with mixing which was continued overnight. The serum $^{3}H/^{14}C$ ratios 1, 2, 3, and 11 days following injection were 5.66, 5.69, 5.58, and 5.54, respectively. Bile aspirated from the duodenum through a fiber optic scope on day 11 had a $^{3}H/^{14}C$ of 5.59. We concluded that the similar metabolism and TLC purity of the [$^{3}$H]- and [$^{14}$C]cholesterol indicated these were of suitable purity for further metabolic experiments.

### Administration of radioactive cholesterol to baboons

A dose of [1,2-$^{3}$H]cholesterol and [4-$^{14}$C]cholesterol was repurified by TLC immediately before use as described above. An aliquot was counted for radioactivity to determine the $^{3}H/^{14}C$ of the dose administered and the remainder ($85.56 \times 10^{6}$ dpm $^{3}$H and $14.28 \times 10^{6}$ dpm $^{14}$C, $^{3}H/^{14}C = 5.99$) was added in 100 $\mu$l of chloroform–acetone 1:1 (v/v) to a ball of feed and fed to an adult baboon. A dose of [7-$^{3}$H]cholesterol and [4-$^{14}$C]cholesterol was prepared in the same manner and given to a second animal. The dose was $88.20 \times 10^{6}$ dpm $^{3}$H and $14.12 \times 10^{6}$ dpm of $^{14}$C, $^{3}H/^{14}C = 6.25$. The adult males (~25 Kg each) used for these experiments were maintained on a diet containing 1.7 mg cholesterol/Kcal with 40% of the calories as fat (31% of calories from lard). At the time that the isotope dose was administered, a second feed-ball containing 100 mg Carmine (alum lake) was given as a fecal flow marker. The feces were collected when the red color of the flow marker was observed and twice during the following day. Each of the three fecal collections was homogenized separately in a blender with an equal volume of water. Approximately 2 g of each homogenate was weighed and extracted.

### Analysis of fecal radioactivity

Lipids were extracted from the homogenates with chloroform–methanol 2:1 (v/v) according to the procedure of Folch, Lees, and Sloane Stanley (4). The lower phases were evaporated to dryness on a rotary evaporator and applied to silica gel G thin-layer plates (0.25 mm thickness) which were developed in a system of diethyl ether–hexane 70:30 (v/v). The TLC plates were sprayed lightly with 0.02% dichlorofluoroscein and the bands corresponding to coprostanone (Band 1), coprostanol (Band 2), and cholesterol (Band 3) standards were visualized under UV light. The bands were scraped and eluted with chloroform–methanol 2:1. GLC confirmed that there was no significant overlap among these three fractions. Half of the eluate was taken for radioactivity counting and half for saponification. The amount of radioactivity was determined in Scintisol (Isolabs Inc., Akron, OH) with a Searle Isocap 300 Counter (Searle Analytic Inc., Des Plaines, IL). Counting efficiencies of the organic extracts were calculated from [$^{3}$H]- and [$^{14}$C]toluene standards quenched with fecal extracts using an external standard channels ratio. Counting efficiencies of aqueous samples were calculated using the internal standards, [$^{3}$H]- and [$^{14}$C]toluene. Aliquots of TLC fractions were evaporated to dryness in 16 × 150 mm screw-capped culture tubes, dissolved in 3.2 ml redistilled ethanol, and saponified with 0.35 ml 10 N NaOH at 80°C for 1 hr. After cooling, 1.0 ml distilled water was added to each tube and the non-saponifiable fraction was extracted into 3 × 5 ml portions of redistilled petroleum ether (bp 36–44°C). The radioactivity was determined in aliquots of the petroleum ether extracts of the saponified samples and in portions of the aqueous phase from each extract after neutralization with HCl.

### Culture experiment

We also estimated the loss of radioactivity by bacterial metabolism of labeled cholesterol and $\beta$-sitosterol. An organism (*Eubacterium* 403) which we isolated from baboon feces was grown in a lecithin medium (5) containing 1 mg/ml of the radioactive sterols. A mixture of [1,2-$^{3}$H]cholesterol, ($1.79 \times 10^{6}$ dpm), and [4-$^{14}$C]cholesterol, ($2.59 \times 10^{5}$ dpm), dissolved in chloroform–methanol 2:1 (v/v) was added to 2 ml of culture medium. The medium was lyophilized to remove the solvent, resuspended with water, and autoclaved. The culture medium was placed in an anaerobic chamber, inoculated with the organism and incubated at 35°C for 7 days. The samples were saponified and extracted as described by Miettinen, Ahrens, and Grundy (6). The organism was also incubated in a culture containing $3.6 \times 10^{6}$ dpm of [22, 23-$^{3}$H]$\beta$-sitosterol and $5.5 \times 10^{5}$ dpm of [4-$^{14}$C]$\beta$-sitosterol and treated in the same manner as the culture containing labeled cholesterol. Control cultures were prepared identically, but were not inoculated with the organism.

## RESULTS

### Animal experiment with [1,2-$^{3}$H]cholesterol + [4-$^{14}$C]cholesterol

Of the total radioactivity extracted from the fecal samples, an average of 0.46% of the $^{3}$H and 0.03% of

TABLE 1. Ratio of $^3H$ to $^{14}C$ of fecal neutral steroids derived from an oral dose of [1,2-$^3H$]cholesterol and [4-$^{14}C$]cholesterol[a]

| Steroid | Lipid Extract | Saponified Lipid Extract |
|---|---|---|
| | $^3H/^{14}C$[b] | |
| Cholesterol | 6.32 ± 0.33 | 6.28 ± 0.34 |
| Coprostanol | 5.72 ± 0.13 | 5.63 ± 0.06 |
| Coprostanone | 4.49 ± 0.38 | 3.24 ± 0.04 |

[a] $^3H/^{14}C$ of dose = 5.99.
[b] Mean ± S.D. of three fecal collections.

the $^{14}C$ were found in the upper phase (4). After saponification of each steroid band from TLC and petroleum ether extraction, 19% of the $^3H$ and 2% of the $^{14}C$ from Band 1 were found in the aqueous layer. Less than 1% of the total $^3H$ or $^{14}C$ from Bands 2 and 3 were found in the aqueous phase after saponification. A mean of 24% of the total $^{14}C$ radioactivity recovered after saponification was coprostanone, 56% coprostanol, and 20% cholesterol. Before saponification, about 10% of the radioactivity was in other bands which were predominantly sterol esters. Recovery of $^{14}C$ radioactivity from the TLC plates averaged 85%.

The $^3H/^{14}C$ of the three neutral steroid fractions before and after saponification are shown in **Table 1.** The $^3H/^{14}C$ of coprostanone from the unsaponified material in the lipid extract was approximately 25% lower than the $^3H/^{14}C$ of the dose administered (4.49 versus 5.99). After saponification, the ratio of $^3H$ to $^{14}C$ in coprostanone was 45.9% lower than the starting dose (3.24 versus 5.99). This suggests that substantial loss of $^3H$ from [1,2-$^3H$]coprostanone by exchange with water occurs in the bowel. These losses are enhanced by saponification.

Some consistent yet unexplained differences also were observed in the $^3H/^{14}C$ of cholesterol and coprostanol recovered from the feces compared to the ratio of $^3H$ to $^{14}C$ in the dose administered (Table 1). These differences were minor, relative to the change in the coprostanone $^3H/^{14}C$, and were not affected by saponification.

TABLE 2. Ratio of $^3H$ to $^{14}C$ of fecal neutral steroids derived from an oral dose of [7-$^3H$]cholesterol and [4-$^{14}C$]cholesterol[a]

| Steroid | Lipid Extract | Saponified Lipid Extract |
|---|---|---|
| | $^3H/^{14}C$[b] | |
| Cholesterol | 6.86 ± 0.30 | 6.71 ± 0.27 |
| Coprostanol | 6.03 ± 0.04 | 6.00 ± 0.09 |
| Coprostanone | 5.51 ± 0.10 | 4.87 ± 0.09 |

[a] $^3H/^{14}C$ of dose = 6.25.
[b] Mean ± S.D. of three fecal collections.

### Animal experiment with [7-$^3H$]cholesterol + [4-$^{14}C$]cholesterol

In the upper (aqueous) phase (4), a mean of 0.44% of the total $^3H$ and 0.24% of $^{14}C$ was recovered. After saponification and petroleum ether extraction of TLC Band 1, a mean of 8.7% of the $^3H$ counts and 3.1% of the $^{14}C$ counts were found in the aqueous phase. Less than 2.5% of the $^3H$ or $^{14}C$ counts from TLC Bands 2 and 3 were found in the aqueous layer after saponification. Approximately 21% of the total $^{14}C$ radioactivity was coprostanone, 65% coprostanol, and 12% cholesterol. Prior to saponification 15–25% of the radioactivity was found predominantly in the sterol ester band.

The ratio of $^3H$ to $^{14}C$ of the neutral steroids isolated after administration of [7-$^3H$]cholesterol and [4-$^{14}C$]-cholesterol are shown in **Table 2.** Coprostanone isolated from the lipid extract had a 12% lower $^3H/^{14}C$ than the starting dose (5.51 versus 6.25). Since negligible amounts of $^3H$ were found in the upper phase (4), the loss of $^3H$ from coprostanone occurred prior to lipid extraction. After saponification the ratio of $^3H/^{14}C$ was 22% lower than the starting dose (4.87 versus 6.25). A higher $^3H/^{14}C$ of cholesterol and lower ratio in coprostanol than that of the dose administered was also observed with this combination of labeled sterols.

### Experiment with *Eubacterium* 403

The radioactivity in the steroids isolated from the cholesterol-containing culture had the following distribution: 58% coprostanol, 0.45% coprostanone, and the remainder cholesterol. Coprostanone isolated from this culture had a low $^3H/^{14}C$ (4.67) compared to the $^3H/^{14}C$ of cholesterol (6.69) in the control medium (**Table 3**). In the β-sitosterol-containing culture, about 41% of the β-sitosterol was converted to β-sitostanol and 0.34% to β-sitostanone. No important

TABLE 3. Metabolism of radiolabeled cholesterol and β-sitosterol by *Eubacterium* 403

| | Substrates | | | |
|---|---|---|---|---|
| | Cholesterol[b] | | β-Sitosterol[c] | |
| TLC Bands[a] | Control[d] | Culture | Control[d] | Culture |
| | $^3H/^{14}C$ | | $^3H/^{14}C$ | |
| Band 3 | 6.69 | 6.83 | 6.78 | 6.99 |
| Band 2 | | 6.54 | | 6.47 |
| Band 1 | | 4.67 | | 6.74 |

[a] Band 3—cholesterol or β-sitosterol; Band 2—coprostanol or β-sitostanol; Band 1—coprostanone or β-sitostanone.
[b] [1,2-$^3H$]cholesterol + [4-$^{14}C$]cholesterol.
[c] [22,23-$^3H$]β-sitosterol + [4-$^{14}C$]β-sitosterol.
[d] Uninoculated control.

differences were observed in the $^{3}H/^{14}C$ of β-sitostanone isolated from the culture with [$^{3}H$]- and [$^{14}C$]β-sitosterol as substrates. This suggests that no significant loss of $^{3}H$ from [$^{3}H$]β-sitostanone occurs when the precursor is [22,23-$^{3}H$]β-sitosterol. The ratio of $^{3}H$ to $^{14}C$ of cholesterol was slightly higher and the ratio in coprostanol lower in the inoculated culture compared to the $^{3}H/^{14}C$ of the control culture. Similar differences were observed between the $^{3}H/^{14}C$ of β-sitosterol and β-sitostanol. These differences correspond to those observed for cholesterol and coprostanol in vivo (See Tables 1 and 2).

## DISCUSSION

The loss of $^{3}H$ from coprostanone derived from either [1,2-$^{3}H$]cholesterol or [7-$^{3}H$]cholesterol is primarily a result of enolization of $^{3}H$ from either the 2 or 4 position. The tritium distribution in [1,2(n)-$^{3}H$]cholesterol used in these experiments was reported by Amersham Corp. to be 42.3% in the 1α position, 45.1% in the 2α position, and the remaining 12.6% in other unspecified positions (personal communication). Therefore, if complete exchange of the 2α-$^{3}H$ occurred by enolization during isolation of coprostanone in our baboon experiment with [1,2-$^{3}H$]-cholesterol and [4-$^{14}C$]cholesterol, the $^{3}H/^{14}C$ of coprostanone should be 45.1% lower than the starting material. This value is very close to the 45.9% lower ratio of $^{3}H$ to $^{14}C$ we actually observed in coprostanone after saponification (Table 1).

In another study with 20 baboons we used [1,2-$^{3}H$]-cholesterol to measure cholesterol absorption and also determined the mass of fecal neutral steroids by GLC. The $^{3}H$ specific radioactivity of coprostanone was only 57% of the overall $^{3}H$ specific radioactivity, i.e., total neutral steroids counts ÷ total mass of cholesterol + bacterial metabolites.[1] This result could be explained by a loss from coprostanone of approximately 43% of the tritium which is comparable to the 45.9% loss we observed in the present experiment with [1,2-$^{3}H$]cholesterol.

Smaller but significant losses of isotope also occurred from nominally labeled [7(n)-$^{3}H$]cholesterol. In this material approximately 85% of the $^{3}H$ is in the 7 position with the remaining 15% largely in the 4 position.[2] Tritium in the 4 position would be readily enolized from coprostanone in the same manner as the 2 position. The 22% lower ratio of $^{3}H$ to $^{14}C$ in coprostanone (saponified) in the experiment with [7-$^{3}H$]-cholesterol + [4-$^{14}C$]cholesterol is 7% lower than one would expect by enolization of tritium from the 4 position alone. Since both the 4 and 7 hydrogens are allylic to the double bond of cholesterol they would be susceptible to loss from oxidation or alkali treatment. However, the loss of $^{3}H$ from [7(n)-$^{3}H$]cholesterol appears to be small since less than 2.4% of the radioactivity from Band 3 is found in the aqueous phase after saponification which lowers the $^{3}H/^{14}C$ from 6.86 to 6.71.

The enol of coprostanone is much less stable than the ketone and therefore is not usually seen in GLC analysis of coprostanone. However, if the enol is silylated one can trap coprostanone as the TMS enol ether. Both the $\Delta^{2}$ and $\Delta^{3}$ enols can be formed and have been separated by GLC and identified by mass spectrometry (7). We formed these TMS enol ethers by heating coprostanone overnight at 70°C with TRI-SIL-TBT (Pierce Chem. Corp., Rockford, IL). The identities of the TMS ethers were confirmed by separation of the isomers by GLC and mass spectral analysis.[3] The slightly alkaline TRI-SIL-TBT was more effective in producing the TMS enol ethers than TRI-SIL-BSA (Pierce Chem. Corp., Rockford, IL). It is apparent that the alkaline conditions of saponification in the present experiments enhanced enolization and loss of $^{3}H$ from either the 2 or 4 positions of coprostanone.

The higher $^{3}H/^{14}C$ of cholesterol and lower $^{3}H/^{14}C$ of coprostanol in these in vivo experiments with [1,2-$^{3}H$]- or [7-$^{3}H$]cholesterol and [4-$^{14}C$]cholesterol could result from a preferential metabolism of [4-$^{14}C$]cholesterol to coprostanol by the intestinal bacteria. This explanation is supported by the results of the culture experiment. Both cholesterol and β-sitosterol had slightly higher $^{3}H/^{14}C$ and the 5β-reduction products lower $^{3}H/^{14}C$ than the cholesterol or β-sitosterol of the control cultures. One cannot completely rule out the possibility of a minor $^{3}H$ impurity which migrates with cholesterol on TLC or a $^{14}C$ impurity which is metabolized to a product migrating with coprostanol on TLC. However, the in vivo test of purity experiment discussed under Materials and Methods does not suggest significant isotopic impurities in the radiolabeled cholesterol.

Using [7-$^{3}H$]- or [1,2-$^{3}H$]cholesterol the measurement of percent cholesterol absorption would be in error by the fractional loss of tritium from coprostanone multiplied by the percentage of coprostanone mass in the total fecal neutral steroids (cholesterol + coprostanol + coprostanone). However, if the specific radioactivities of the three steroid fractions

[1] Mott, G. E., and E. M. Jackson. Unpublished observations.
[2] Personal communication. New England Nuclear Corp.
[3] Weintraub, S. T., and G. E. Mott. Unpublished observations.

are dissimilar before the enolization process occurs, a correction based on the mass of coprostanone would be in error. A more sensitive correction for loss of $^3H$ from coprostanone could be achieved by dividing the dpm recovered in coprostanone by (1 − the fractional loss of $^3H$). Adding that result to the dpm in Bands 2 and 3 (coprostanol and cholesterol) would give an accurate estimate of the total $^3H$ in the extract. If the percent coprostanone were 30% of the fecal neutral steroids and the loss of $^3H$ from [1,2-$^3H$]coprostanone were 50%, the estimation of percent cholesterol absorption would be 15% too high. The errors in baboons are generally much smaller since we have found a mean of 4.5% coprostanone (by GLC) in the total neutral steroids (cholesterol + bacterial cholesterol products) of 50 juvenile animals fed a low cholesterol diet and 10.4% coprostanone in 47 young baboons fed a diet containing 1 mg cholesterol/Kcal. However, the percentage coprostanone was higher (~23%) in another experiment with several adult baboons ingesting a very high cholesterol diet.[1]

We recommend a correction for any data which depend on recovery of radioactivity from [1,2(n)-$^3H$]- or [7(n)-$^3H$]coprostanone. This correction may be accomplished as described above but may vary with the source and batch number of labeled sterols. We believe that [4-$^{14}C$]cholesterol would be preferable to either of the [$^3H$]cholesterol substrates available if only a single labeled sterol is required. However, some cholesterol absorption methods require both cholesterol and β-sitosterol labeled with different isotopes (1). Borgstrom's recommendation (1) to use [4-$^{14}C$]-cholesterol and [22,23-$^3H$]β-sitosterol is probably the best combination of sterols for these studies. Cholesterol absorption methods not requiring measurement of fecal coprostanone such as the serum isotope ratio method (2) would not be affected by loss of $^3H$ from coprostanone.

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