# [24,25-$^3$H]Cholesterol: presence of tritium at additional sites in the side chain

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**Abstract** In order to measure the distribution of radioactivity present in the side chain of [24,25-$^3$H]cholesterol prepared by a sequence involving catalytic tritiation of 3$\alpha$,5$\alpha$-cyclocholest-24-en-6$\beta$-ol 6-methyl ether, the cholesterol was oxidized to 4-cholesten-3-one, which was then cleaved between C-24 and C-25 to afford the $C_{24}$ alcohol. Oxidation to the corresponding cholenoic acid, followed by alkali equilibration and esterification completed the sequence. It was found that about 20% of the tritium in the labeled cholesterol is not lost when this tracer is physiologically converted to bile acids. Consequently, measurements of bile acid formation using this tracer must be corrected upward by this amount.—**Rosenfeld, R. S., I. Paul, and B. Zumoff.** [24,25-$^3$H]Cholesterol: presence of tritium at additional sites in the side chain. *J. Lipid Res.* 1983. **24:** 781–783.

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Several years ago we prepared [24,25-$^3$H]cholesterol from desmosterol (5,24-cholestadien-3$\beta$-ol) by a method in which the double bond at $C_5$–$C_6$ was protected from hydrogenation (tritiation) by forming the 3$\alpha$,5$\alpha$-cyclocholest-24-en-6-ol 6-methyl ether prior to catalytic reduction (1). We have used this tracer to determine the rate of cholesterol oxidation as a measure of bile acid formation in human subjects (1) and to compare the oxidative metabolism of lipoprotein-bound [24,25-$^3$H]cholesterol and cholesteryl esters in the rat (2).

Recently, a paper appeared that rendered suspect certain preparations of [1,2-$^3$H]cholesterol and [24,25-$^3$H]cholesterol used in in vivo studies (3). While the tracers behaved as radiochemically homogeneous substances in several generally accepted procedures for purification, administration to human subjects was associated with an inexplicable drop in radioactivity not encountered in similarly purified and simultaneously administered [4-$^{14}$C]cholesterol. Such behavior of radiochemically pure [1,2-$^3$H]cholesterol is not consistent since other investigators at about the same time and using material obtained from the same supplier, did not observe this phenomenon (4). Still, the caveat and recommendations of the Rockefeller University group (3), appear to be worthwhile suggestions. The [24,25-$^3$H]cholesterol prepared through the 3,5-cyclosterol derivative showed a radiochemical reliability of greater than 90% (3) and was satisfactory as a cholesterol tracer for our applications (1, 2). However, we were informed that our [24,25-$^3$H]cholesterol might have radioactivity at positions other than at the terminal 3 carbons of the cholesterol side chain,[2] which, if true, would introduce an error in the calculation for the rate of bile acid formation, affording a value lower by the same percentage as that of tritium in positions proximal to C-24. This communication deals with the distribution of radioactivity in the side chain of [24,25-$^3$H]cholesterol prepared by catalytic tritiation of 3$\alpha$,5$\alpha$-cyclocholest-24-en-6-ol.

## EXPERIMENTAL

### Oxidation of [24,25-$^3$H]cholesterol to [$^3$H]cholest-4-en-3-one

Cholesterol, 1.66 g, was treated with dry aluminum *tert*-butoxide in benzene–acetone according to the Oppenauer procedure (5). The product, 1.64 g, was chromatographed on alumina, to yield 0.390 g of 4-cholesten-3-one, m p 86–87°C, after crystalization from acetone–methanol, and 0.96 g of starting material. A second Oppenauer oxidation, recycling the recovered cholesterol, afforded 0.229 g of 4-cholesten-3-one.

### Conversion of [$^3$H]cholest-4-en-3-one to 24-acetoxy-4-cholen-3-one

Oxidative degradation of [$^3$H]cholest-4-en-3-one was performed according to the procedure described by Manley et al. (6). A round-bottom flask containing 619 mg of the labeled unsaturated ketone was cooled to 0°C

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and 40 ml of a freshly made ice-cold solution of 63% trifluorocetic acid (Fisher), 34% of 96% $H_2SO_4$, and 3% of 50% hydrogen peroxide (Fisher) was introduced. The mixture, which turned dark rapidly, was stirred for 4.5 hr at ice-bath temperature. The cold, dark brown mixture was poured on to ice and the ice-water suspension was extracted with benzene. The organic layer was washed with dilute alkali and water until neutral; most of the dark decomposition products were removed in the alkali washes. After drying the benzene solution over $Na_2SO_4$ and evaporating the solvent, 327 mg of oil was recovered. The oily residue was acetylated by refluxing under nitrogen for 19 hr in 50 ml of acetic acid, 2 ml of water, and 1.0 g of sodium acetate. After cooling and diluting with water, a washed hexane extract yielded 250 mg of product. This was chromatographed on alumina to give 74 mg of 4-cholesten-3-one, eluted with benzene–isooctane 3:7, and 154 mg of crystalline material recovered from the benzene eluates. This substance, crystallized from acetone, m p 129–130°C, melted somewhat higher than Manley's product, 123–125°C (6). The molecular peak in the mass spectra, m/e 400.2, was in accord with 24-acetoxy-4-cholen-3-one.

### 24-Hydroxy-4-cholen-3-one

Eighty-five mg of 24-acetoxy-4-cholen-3-one was hydrolyzed overnight at room temperature in dilute ethanolic alkali to afford 72 mg of oily crystals. The substance was crystallized from acetone, m p 133°C; the molecular peak of the 24-trimethylsilyl ether derivative in the mass spectra, m/e 430.4, was identical to the calculated molecular weight.

### Oxidation of 24-hydroxy-4-cholen-3-one to 3-keto-4-cholen-24-oic acid

Sixty-nine mg of 24-hydroxy-4-cholen-3-one was oxidized according to the procedure described by Heusler et al. (7). The usual work-up afforded 70 mg of crystalline material, a portion of which was recrystallized from methanol, m p 180–181°C.

### Equilibration of 3-keto-4-cholen-24-oic acid with alkali

Sixty-two mg of 3-keto-4-cholen-24-oic acid was refluxed in 5% aqueous-ethanolic NaOH for 1 hr, and the solution was extracted with ether before acidification and re-isolation of the acid fraction, 58.9 mg of the starting material. A sample was recrystallized from methanol, m p 181°C.

### Methyl 3-keto-4-cholen-24-oate

Esterification of the $C_{24}$ acid with ethereal diazomethane gave 57.6 mg of oil that crystallized after chromatography on alumina; m p 125°C after crystallization from methanol. In the mass spectrum, the molecular peak, m/e 386.3, corresponded to the molecular weight of methyl 3-keto-4-cholen-24-oate and the spectrum was in accord with that of authentic material.

All crystalline samples were assayed for $^3H$ by liquid scintillation spectrometry.

## RESULTS AND DISCUSSION

The specific activities of the compounds prepared during chemical conversion of [24,25-$^3$H(N)]cholesterol to methyl 3-keto-4-cholen-24-oate are listed in **Fig. 1.** The specific activity of the starting material, side chain-labeled cholesterol, (409 cpm/$\mu$mol) is set at 1.00 and the specific activities of the transformation products are compared with it. Since it is known that the chemical conversion of this labeled cholesterol to 3-hydroxy-5-androsten-17-one results in virtually complete loss of radioactivity (1), all of the $^3H$ is located in the side chain. Oxidative cleavage between C-24 and C-25 (6) to afford the $C_{24}$ alcohol results in the loss of 68% of the radioactivity in the side chain, and oxidation of the primary alcohol to the carboxylic acid is accompanied by an additional loss of 10% of the radioactivity, suggesting that this represents $^3H$ at C-24. When the 3-keto-4-cholen-24-oate was refluxed in alkali and the $C_{24}$ acid was re-isolated, an additional 7% of the radioactivity was lost; this indicates tritium at C-23, $\alpha$ to the carboxyl group.

A

| COMPOUND | RELATIVE SA |
|---|---|
| Cholesterol | 1.00 |
| 4-cholesten-3-one | 0.92 |
| 4-cholesten-3-one; recryst. m.p. 86-87° | 1.02 |
| 24-acetoxy-4-cholen-3-one; recryst. m.p. 129-130° | 0.32 |
| 24-hydroxy-4-cholen-3-one; recryst. m.p. 133° | 0.32 |
| 3-keto-4-cholen-24-oic acid | 0.22 |
| 3-keto-4-cholen-24-oic acid; recryst. m.p. 180-181° | 0.21 |
| 3-keto-4-cholen-24-oic acid; alkali equilibrated; recryst. m.p. 180° | 0.15 |
| methyl-3-keto-4-cholen-24-oate; recryst. m.p. 125° | 0.15 |

B

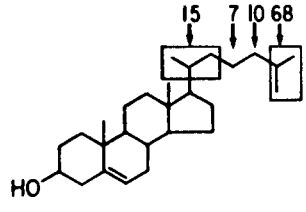


**Fig. 1.** A, Relative specific activities (SA) of chemical transformation products of [$^3$H]cholesterol. B, Location of tritium in the side chain of [$^3$H]cholesterol (in percent of total).

Therefore the residual $^3$H, 15% of the radioactivity in the starting cholesterol, is located around C-20, C-21, C-22. These results are summarized in **Fig. 1.**

That tritium is present at positions other than at the reduced double bond is not surprising since it has long been known that, during catalytic reduction of cholesterol with isotopic hydrogen, hydrogens allylic to the double bond are labilized and can be replaced by isotopic hydrogen (8). In the substance that was tritiated, 3$\alpha$, 5$\alpha$-cyclocholest-24-en-6$\beta$-ol 6-methyl ether, there are eight hydrogen atoms in the allylic positions (C-23, C-26, and C-27), six of which are distal to C-24. Further, it would be expected that introduction of $^3$H at C-24 and C-25 would not be symmetrical since each of the carbons exists in a different steric environment and forms a secondary and tertiary carbon, respectively, on reduction. Lastly, bond migration involving a catalyst-steroid complex at C-5 and C-6 takes place during reduction of cholesterol (8) and it is likely that an analogous situation might occur during introduction of $^3$H into the side chain under the conditions of the catalytic tritiation. This would account for the presence of $^3$H in the C-20, C-21, C-22 portion of the side chain; these positions are chemically and biochemically stable. Although the $^3$H at C-23 is chemically labile during vigorous saponification of the C-24 carboxylic acid, it is stable biologically (9, 10). Therefore, $^3$H at C-20 through C-23 present no problem when the [24,25 $^3$H (N)]cholesterol is used in tracer studies if a correction is made for its presence. While it is possible that $^3$H might redistribute itself between C-24 and C-25 during the trifluoroperacetic acid cleavage of the C-24-C-25 bond, this is operationally irrelevant since during the in vivo conversion of cholesterol to bile acids, the hydrogens distal to and including C-24 (i.e., about 80% of $^3$H) are lost. We have been informed that [24,25-$^3$H(N)]cholesterol prepared in another laboratory by the method described by us (1) possessed essentially the same isotope distribution where the location of the $^3$H was established by a different set of procedures.[2] Therefore, measurements of bile acid formation by the technique reported earlier (1) must be corrected by the amount of residual radioactivity remaining in the shortened side chain, about 20%. While it is probable that all side chain-labeled cholesterol prepared from desmosterol by catalytic tritiation as described (1) will have essentially the same isotope distribution, it is recommended that a sample of each batch so prepared be checked for $^3$H remaining in the side chain after oxidative cleavage of the terminal isopropyl group. Considering that large amounts of labeled cholesterol can be produced and can provide tracer for many studies, this is not an onerous task.

As long as the amount of stably bound $^3$H in the side chain proximal to C-24 is known, [24,25$^3$H(N)]-cholesterol may be safely used in studies relating to oxidative metabolism of the sterol. Such work has been reported in vivo in man (1) and rats (2, 11) and in vitro in rat liver preparations (11), and investigations are currently underway in our laboratories involving lipoprotein metabolism in primates.

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## REFERENCES

1. Rosenfeld, R. S., H. L. Bradlow, J. Levin, and B. Zumoff. 1978. Preparation of [24,25-$^3$H]cholesterol. Oxidation in man as a measure of bile acid formation. *J. Lipid Res.* **19:** 850–855.
2. Miller, L. K., M. L. Tiell, I. Paul, T. H. Spaet, and R. S. Rosenfeld. 1982. Side-chain oxidation of lipoprotein-bound [24,25-$^3$H]cholesterol in the rat: comparison of HDL and LDL and implications for bile acid synthesis. *J Lipid Res.* **23:** 335–344.
3. Davidson, N. O., E. H. Ahrens, Jr., H. L. Bradlow, D. J. McNamara, T. S. Parker, and P. Samuel. 1980. Unreliability of tritiated cholesterol: studies with [1,2-$^3$H]cholesterol and [24,25-$^3$H]cholesterol in humans. *Proc. Natl. Acad. Sci. USA.* **77:** 2255–2259.
4. Mott, G. E., and E. M. Jackson. 1980. Loss of tritium from coprostanone derived from [1,2(n)-$^3$H]cholesterol or [7(n)-$^3$H]cholesterol. *J. Lipid Res.* **21:** 480–484.
5. Oppenauer, R. V. 1955. Organic Syntheses, Collective Vol. 3. John E. C. Horning, editor. John Wiley & Sons, New York. 207–209.
6. Manley, R. P., K. W. Curry, N. C. Deno, and M. D. Meyer. 1980. A one-step conversion of cholest-4-en-3-one to 24-hydroxychol-4-en-3-one. *J. Org. Chem.* **45:** 4385–4387.
7. Heusler, K., J. Kalvoda, Ch. Meystre, P. Wieland, G. Anner, A. Wettstein, G. Cainelli, D. Arigoni, and O. Jeger. 1961. Partialsynthese von Aldosterone. *Helv. Chim. Acta.* **44:** 502–517.
8. Fukushima, D. K., and T. F. Gallagher. 1955. Reduction of unsaturated steroids with deuterium: distribution of isotope and mechanism. *J. Am. Chem. Soc.* **77:** 139–142.
9. Fukushima, D. K., and T. F. Gallagher. 1952. Isotopic distribution in cholesterol after platinum-catalzyed hydrogen–deuterium exchange. *J. Biol. Chem.* **198:** 861–869.
10. Fukushima, D. K., and T. F. Gallagher. 1952. Studies on the platinum-catalyzed exchange of hydrogen isotopes with steroids. *J. Biol. Chem.* **198:** 871–884.
11. Bentzen, C. L., and K. Brendel. 1979. Synthesis of [24,25-$^3$H]cholesterol: a new substrate for determining the rate of cholesterol side chain oxidation. *J. Lipid Res.* **20:** 134–139.