notes on methodology

1,2-^{3}H-Cholesterol as a tracer in studies of human cholesterol metabolism

PETER D. S. WOOD, DAPHNE MYERS, YUEN-LING LEE, RYUZO SHIODA, and LAURANCE W. KINSELL

Institute for Metabolic Research, Highland General Hospital, Oakland, California 94606

SUMMARY 1,2-^{3}H-Cholesterol was fed to a subject together with 4-^{14}C-cholesterol at a known ^{3}H/^{14}C ratio. The ratio was satisfactorily preserved in cholesterol recovered from plasma, red cells, and bile, and in bile acids of bile.

Isotopic fractionation was seen during thin-layer chromatographic isolation of cholesterol. In work with 1,2-^{3}H-cholesterol or its metabolites care should be taken to recover chromatographic bands in their entirety.

KEY WORDS cholesterol · 1,2-^{3}H-cholesterol · 4-^{14}C-cholesterol · thin-layer chromatography · isotope effect · plasma · red blood cells · bile acids · man

IN STUDIES of human cholesterol metabolism it is often very useful to employ tritium-labeled cholesterol in addition to the well established 4-^{14}C-cholesterol. Double-label studies of this kind may be particularly valuable when the fate of ingested cholesterol must be distinguished from that of endogenous cholesterol. A number of investigators have used generally-labeled ^{3}H-cholesterol, which has certain disadvantages, including (*a*) uncertainty regarding the resistance of this material to some degree of tritium loss by exchange, either in vivo or during analytical procedures; and (*b*) the undoubted loss of tritium from the side-chain and nucleus during conversion of cholesterol to bile acids, the magnitude of which cannot be easily calculated since generally-labeled ^{3}H-cholesterol is probably not uniformly labeled.

The availability of cholesterol labeled with tritium on the nucleus at the metabolically inactive 1 and 2 positions prompted a test of this material for suitability as a tracer both in vivo and during laboratory handling.

Preparation of Radioactive Cholesterol for Dietary Incorporation. 30 μc of 4-^{14}C-cholesterol (Volk Radiochemical Co., Burbank, Calif., stated to show "99.5% radiopurity by TLC"; specific activity 63.8 μc/mg) and 120 μc of 1,2-^{3}H-cholesterol (New England Nuclear Corp., Boston, Mass., supplied with a scanned paper chromatogram showing one peak of radioactivity; specific activity 32.8 mc/mg) were added to 10 mg of carrier cholesterol (Eastman Organic Chemicals, Rochester, N.Y.), and the mixture was purified by precipitation and washing of the digitonide. Cholesterol was released from the complex, and purified further by TLC on Silica Gel G with petroleum ether–diethyl ether–acetic acid 60:40:1 as solvent. Cholesterol was recovered by elution of the appropriate band with diethyl ether. A study of this preparation by means of TLC in chloroform on Silica Gel G impregnated with silver nitrate showed that 98% of both ^{14}C and ^{3}H were present in the cholesterol region, while less than 1% of each isotope was present in the cholestanol region.

The purified preparation was counted in a Packard Tri-Carb liquid scintillation spectrometer with settings appropriate for doubly-labeled samples (^{14}C efficiency, 56%; ^{3}H efficiency, 21%).

Administration of Labeled Material. A female subject (who had previously undergone sterilization by tubal ligation) was maintained on a quantitatively constant formula diet in the metabolic ward for 31 days. The formula was administered in six equal daily portions fed at 2 hr intervals during the day. The diet provided each day: protein, 90 g; carbohydrate, 240 g; fat (palmitate–oleate triglyceride with 50% of each fatty acid), 120 g, in which was dissolved 750 mg of pure cholesterol containing about 1.7 μc of 1,2-^{3}H-cholesterol and about 0.5 μc of 4-^{14}C-cholesterol with a ^{3}H/^{14}C ratio of 3.40:1. The ^{3}H and ^{14}C specific activities of cholesterol in the total diet were determined.

Collection of Samples. Blood samples were taken twice a week from a forearm vein. Duodenal bile samples were obtained each week by intubation of the fasting subject followed by intravenous injection of purified cholecystokinin (Cecekin; Vitrum, Stockholm, Sweden) to promote bile flow.

Analysis of Samples. Plasma lipids were extracted and washed by the procedure of Folch, Lees, and Sloane Stanley (1); free cholesterol and cholesteryl esters were recovered separately by TLC. Red blood cells were separated from five blood samples and washed free from plasma; after Folch extraction and washing, the free cholesterol was isolated by TLC. Bile samples were extracted and washed by the Folch procedure. The lower layer from the washing step yielded free cholesterol after TLC, while the upper (aqueous methanol) layer, after saponification under pressure, TLC, and a methylation step, yielded pure samples of cholic, chenodeoxycholic, and deoxycholic methyl esters. At each stage individual steroid bands were removed from thin-layer plates in their entirety; removal of only the central portions of bands was avoided.

Abbreviation: TLC, thin-layer chromatography.

TABLE 1 MEAN VALUES FOR $^{3}H/^{14}C$ RATIO IN STEROIDS RECOVERED FROM A SUBJECT FED 1,2-^{3}H-CHOLESTEROL AND 4-^{14}C-CHOLESTEROL DAILY FOR 31 DAYS

	RBC	Plasma Cholesterol		Bile			
	FC	Free	Esterified	FC	MC	MCDC	MDC
No. of samples	5	9	9	4	4	4	3
Mean ratio $^{3}H/^{14}C$	3.37	3.39	3.39	3.31	3.36	3.35	3.32
Mean % loss of ^{3}H	0.9	0.3	0.3	2.6	1.2	1.5	2.1

The $^{3}H/^{14}C$ ratio in fed cholesterol was 3.40:1.
Abbreviations: RBC, red blood cells; FC, free cholesterol; MC, methyl cholate; MCDC, methyl chenodeoxycholate; MDC, methyl deoxycholate.

The mass of free and esterified cholesterol was determined by weighing (2) and that of bile acid methyl esters by spectrophotometry (3). The ^{3}H and ^{14}C content of samples was determined to a standard deviation of less than 1%. Quenching due to addition of samples to the counting fluid was negligible, as judged by internal standardization; any quenching due to the counting fluid itself was held constant by standardizing the procedure by which the recovered steroid samples—and the labeled cholesterol fed—were dissolved in scintillation fluid and counted.

Results. The ratio of $^{3}H/^{14}C$ in the cholesterol fed, and the mean ratios found in the various steroid fractions recovered from the subject, are shown in Table 1. Fig. 1 shows the result of plotting for each plasma sample the ratio of the specific activity in plasma free cholesterol to the specific activity of the dietary cholesterol, with respect to each of the two isotopes.

Isotope Effect during TLC of Purified, Doubly-Labeled Cholesterol. A mixture was made of carrier cholesterol (Eastman Organic Chemicals), 1,2-^{3}H-cholesterol, and 4-^{14}C-cholesterol. This was purified by the following steps: (*a*) bromination (4) and crystallization of the cholesteryl dibromide, first from diethyl ether and then from methanol; (*b*) debromination of the dibromide

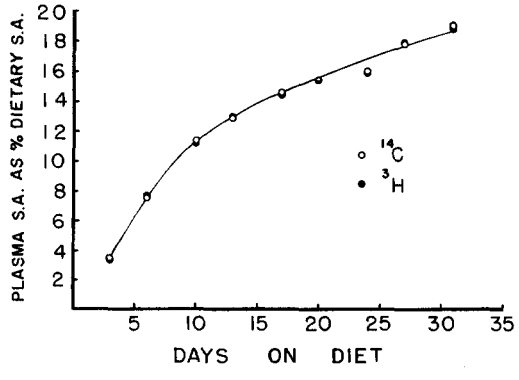

FIG. 1. Specific activity of plasma free cholesterol as a percentage of the specific activity of dietary cholesterol for two isotopes. $^{3}H/^{14}C$ ratio in dietary cholesterol was 3.40:1. The ^{3}H specific activity was 1476 dpm/mg and the ^{14}C specific activity 5017 dpm/mg.

(4) and crystallization of the resulting cholesterol from methanol; (*c*) TLC of the product on Silica Gel G in petroleum ether–diethyl ether–acetic acid 50:50:1, followed by recovery of the material from the cholesterol band; and (*d*) final crystallization of this material from methanol, and drying. The purified cholesterol gave: ^{3}H specific activity, 60,350 dpm/mg; ^{14}C specific activity, 20,000 dpm/mg; ratio $^{3}H/^{14}C$, 3.018:1.

Estimates of polar impurities (TLC on Silica Gel G) and of saturated sterols (TLC on Silica Gel G impregnated with silver nitrate) made on this material gave the following results (percentage of total radioactivity): polar impurities, ^{3}H 0.37, ^{14}C 0.43; saturated sterols, ^{3}H 0.15, ^{14}C 0.19.

A 5 mg sample of this material was applied as a streak to a thin-layer plate coated with Silica Gel G (300 μ in thickness) and developed in petroleum ether–diethyl ether–acetic acid 60:40:1. After being dried briefly the plate was sprayed with Rhodamine 6G and the cholesterol band was located under UV light. Six 3 mm strips of the adsorbent were removed with the aid of a razor blade from the area of the band, starting just ahead of the band and finishing just behind the trailing edge (Fig. 2). The cholesterol was eluted from each portion of adsorbent with diethyl ether and the $^{3}H/^{14}C$ ratio determined.

Discussion. An ideal radioactive tracer molecule should be identical in all its properties with the inactive molecule, except for its radioactivity and molecular weight. 4-^{14}C-Cholesterol has proved to be a very satisfactory tracer for cholesterol. The ^{14}C-atom is firmly attached to the nucleus; there is no evidence of discrimination between the inactive and 4-^{14}C-labeled molecule within the animal body (isotope effect); and in analytical systems significant separation of 4-^{14}C-labeled and unlabeled steroids apparently does not occur (5, 6). A comparison of the behavior of 4-^{14}C-cholesterol and 1,2-^{3}H-cholesterol during in vivo transformations and in subsequent analytical procedures was therefore thought to be of importance before any extensive investigational use is made of the tritium-labeled material. The data presented in Table 1 show only

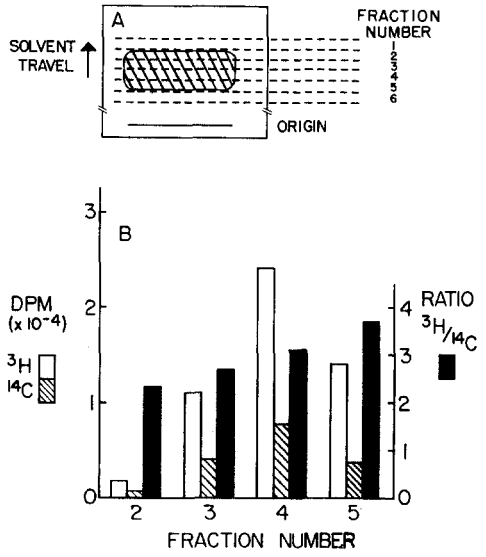

FIG. 2. Isotope effect during TLC of cholesterol labeled with ^{3}H and ^{14}C. *A*. Thin-layer plate showing the division of the band (detected with Rhodamine 6G) and its immediately adjacent areas into six fractions. *B*. ^{3}H and ^{14}C radioactivity present in four of the six fractions and ratio of $^{3}H/^{14}C$ across the band. The $^{3}H/^{14}C$ ratio of cholesterol applied to the plate was 3.018:1. Radioactivity present in fractions 1 and 6 was negligible.

very small losses of tritium relative to ^{14}C for the steroids investigated in blood and bile from a single human subject. There is no reason to think that isotopic material administered by the intravenous route would be any more severely treated within the body. Thus 1,2-^{3}H-cholesterol appears to be satisfactory (after purification) with respect to (*a*) firmness of attachment of tritium atoms at the 1 and 2 nuclear positions; and (*b*) freedom from isotope effects in vivo and during the particular analytical procedures used here. Put in another way, the use of either isotope, administered by the same route, should lead to the same experimental conclusions. This is clearly true for the study shown in Fig. 1, where a curve is obtained from which the contribution of dietary cholesterol to plasma cholesterol (in the steady state) may be obtained by the method of Wilson and Lindsey (7).

However, since it has been reported recently that certain 1,2-^{3}H-labeled steroids are partially separable from the inactive or ^{14}C-labeled steroids during paper chromatography (5) and column chromatography (6), the behavior of 1,2-^{3}H-cholesterol in the thin-layer chromatographic systems used in the present study was investigated. It is clear (Fig. 2) that tritium is not distributed across the cholesterol band in the same way as ^{14}C (and presumably also differs from inactive cholesterol in this respect).

Considerable effort was made to ensure the purity of the doubly-labeled cholesterol employed in this chromatographic study; conventional purification procedures and tests for the more likely contaminants were used. It thus seems most unlikely that tritium-labeled impurities could have been responsible for the observed distribution of radioactivity on the thin-layer chromatogram. Support for this contention is provided by the preservation of the $^{3}H/^{14}C$ ratio in each of three bile acids recovered in the in vivo study, since this would be most improbable if the cholesterol fed had contained significant proportions of any radioactive impurity.

Although the tritiated material is all found within the confines of the band of inactive cholesterol, it tends to be concentrated towards the trailing edge of the band. Thus the removal and recovery of only a part of the total band is likely to yield misleading values for ^{3}H specific activity and for the ratio of $^{3}H/^{14}C$, which may lead to interpretive errors in dual-label studies. Although the behavior of free cholesterol only was studied here, it seems probable that similar chromatographic effects occur with thin-layer separation of 1,2-^{3}H-labeled metabolities of cholesterol.

We therefore concluded that 1,2-^{3}H-cholesterol is a satisfactory tracer for cholesterol in human metabolic studies with respect to the steroids recovered from blood and bile, providing care is exercised to circumvent isotope effects during separatory and analytical procedures.

This work was supported in part by PHS Research Grant HE-09346 from the National Institutes of Health, U.S. Public Health Service, and in part by grants from the Corn Products Company and the Safflower Council of the National Institute of Oil Seed Products. Acknowledgment is also made to the California Heart Association for the award of a Summer Student Research Associateship (to Mrs. Myers); and to the Alameda County Heart Association for a Research Fellowship (to R. Shioda).

Manuscript received 12 December 1966; accepted 15 March 1967.

REFERENCES

1. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
2. Wood, P. D. S., R. Shioda, and L. W. Kinsell. 1966. *Lancet.* **ii:** 604.
3. Sjövall, J. 1959. *Clin. Chim. Acta.* **4:** 652.
4. Fieser, L. F. 1953. *J. Am. Chem. Soc.* **75:** 5421.
5. Gold, N. I., and J. F. Crigler, Jr. 1966. *J. Clin. Endocrinol.* **26:** 133.
6. Cejka, V., and E. M. Venneman. August 1966. *Atomlight.* New England Nuclear Corp. Boston. No. **55:** 1.
7. Wilson, J. D., and C. A. Lindsey, Jr. 1965. *J. Clin. Invest.* **44:** 1805.