[^{13}C]cholesterol as a tracer for studies of cholesterol metabolism in humans

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Abstract The investigation of cholesterol metabolism in humans would be facilitated by the availability of a tracer that is not radioactive. However, to be useful such a tracer must be detectable in the large pool of body cholesterol over an extended time. To meet these requirements we used synthetic [23,24,25,26,27-$^{13}C_5$]cholesterol with detection by isotope ratio mass spectrometry (IRMS), a technique in which the ratio of $^{13}CO_2/^{12}CO_2$ can be determined with high precision in the combustion products of a biological sample. [^{13}C]cholesterol (65 mg) and [^{14}C]cholesterol (15 μCi) were solubilized in Intralipid and injected simultaneously (IV) into three normal subjects, and plasma samples were obtained over the ensuing 10 weeks. Cholesterol was isolated from plasma and either counted for radioactivity or combusted to CO_2 and analyzed by IRMS. [^{14}C]cholesterol and [^{13}C]cholesterol tracer concentrations in plasma were very similar. [^{13}C]cholesterol kinetic parameters calculated using a standard two-compartment cholesterol turnover model and a new three-compartment minimal model were 103 ± 10.5 SD % of those computed from corresponding [^{14}C]cholesterol data. These results show that [$^{13}C_5$]cholesterol can be used as a practical tracer for cholesterol metabolic studies lasting many weeks.—**Ostlund, R. E., Jr., and D. E. Matthews.** [^{13}C]cholesterol as a tracer for studies of cholesterol metabolism in humans. *J. Lipid Res.* 1993. **34:** 1825–1831.

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A principal focus of clinical cardiovascular disease research over the last two decades has been the relation of circulating levels of cholesterol and lipoproteins to coronary heart disease risk. However, only about 9% of the body cholesterol burden is found in plasma (1), and much less work has been done on whole body cholesterol metabolism. For example, the relation of whole body cholesterol metabolism to coronary heart disease has not yet been studied systematically. Although several methodological difficulties remain to be resolved, an important limiting factor in human investigation is the need to use cholesterol labeled with radioactive isotopes. Because radioactive cholesterol can be detected in the plasma for over a year after it is administered, the study of families, normal younger individuals, and women with childbearing potential is fraught with ethical concern and these groups have been substantially excluded from clinical investigation. Moreover, few such subjects will consent to the use of radioactive materials.

An alternative is the use of cholesterol labeled with stable isotopes. Although theoretically attractive, the detection of stable isotopic enrichment of cholesterol is more difficult than simple radioactive counting and it is not clear whether cholesterol metabolic studies lasting many weeks are even possible. To address this question we have used a synthetic cholesterol labeled with ^{13}C in five positions using detection by IRMS and have compared its turnover to [^{14}C]cholesterol. The results show that the [^{13}C]cholesterol tracer was still readily detectable in plasma 70 days after administration and that parameters of cholesterol turnover could be computed equally well with either the stable or radioactive tracer.

METHODS

Tracers

[23,24,25,26,27-$^{13}C_5$]cholesterol (**Fig. 1**) was synthesized by Dr. Alfred Ajami of Tracer Technology, Inc. The material had isotopic purity of 98.8% at each labeled position. As supplied the material also contained 26% [23,24,25-$^{13}C_3$]-26,27-bis-norcholesterol, a phytosterol lacking two terminal methyl groups. This was removed before analysis by HPLC as described below. [4-^{14}C]cholesterol was obtained from Amersham.

Abbreviations: IRMS, isotope ratio mass spectrometry; HPLC, high performance liquid chromatography; APE, atom % excess; dpm, disintegrations per minute; GC-MS, gas chromatography-mass spectrometry.

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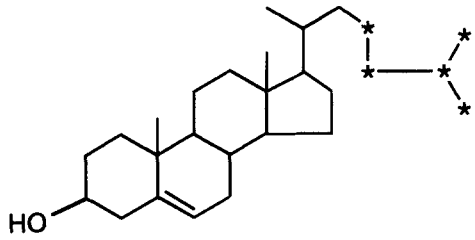

Fig. 1. Structure of [^{13}C]cholesterol.

Patient studies

Labeled [^{13}C]cholesterol was dissolved at 5 mg/ml in USP ethanol in a sterile container; water was added to 10% by volume in order to potentiate the virucidal activity of ethanol, and the solution was allowed to stand at room temperature for at least 6 h. The solvent was then sterilely removed by lyophilization through a 0.22-μm filter and the cholesterol residue was dissolved at 20 mg/ml in ethanol and passed through a 0.22-μm solvent-resistant filter. Aliquots were tested for sterility by culture and dried aliquots for pyrogenicity using the Limulus assay, and the stock was kept at −70°C. In previous reports [^{14}C]cholesterol was prepared for intravenous infusion by dispersing ethanolic stock solutions in saline or serum. These methods are not useful for stable isotope studies because of the larger mass of tracer given and they also have the disadvantage of resulting in substantial losses of insoluble material on syringes and intravenous tubing which must then be washed routinely with solvent to allow calculation of the exact amount of cholesterol injected. In the present work we solubilized the tracer cholesterols in Intralipid. On the day of the experiment an aliquot (approximately 4 ml) of the ethanolic [^{13}C]cholesterol stock was warmed to 37°C and [^{14}C]cholesterol was added to it in a small volume of ethanol. The warm ethanolic solution of cholesterol was added dropwise to 4 volumes of warm 10% Intralipid. After 5 min at 37°C and 15 min at room temperature the mixture was passed through a 0.8-μm particulate filter. The filtration was included only as a precaution to prevent possible injection of particulate cholesterol, but it was not strictly necessary as more than 94% of the labeled cholesterol passed through the filter. An aliquot containing approximately 65 mg [^{13}C]cholesterol and 15 μCi [^{14}C]cholesterol was drawn into a tared syringe and weighed. The syringe contents were injected over 10 min into a running saline infusion and the syringe was then washed several times with saline. With this method cholesterol was quantitatively solubilized and less than 0.4% of the tracer [4-^{14}C]cholesterol was found in the syringe and intravenous lines after washing with ethanol. Blood samples of 5 ml were taken over 10 weeks for a total of 28. The protocol was approved by the Human Studies Committee of Washington University.

Detection of tracer

Weighed aliquots (approximately 20 mg) of the Intralipid–cholesterol mixture that was infused were either counted or added to 2 ml of 10 mg/ml natural cholesterol for determination of the amount of [^{13}C]cholesterol infused by isotope dilution as previously described for sodium [^{13}C]bicarbonate (2). Plasma samples (0.5 ml) were saponified and the nonsaponifiable lipids were quantitatively extracted and counted as previously described (3) and then divided by plasma cholesterol mass (4) to yield specific activity. For determination of [^{13}C]cholesterol enrichment 1.0-ml plasma samples were saponified and aliquots of nonsaponifiable material containing approximately 1 mg of cholesterol were taken up in 17.5 μl tetrahydrofuran and injected into a 4.6 × 250 mm octadecyl silica HPLC column and eluted with methanol. Approximately 0.5 mg of the purified cholesterol fraction was transferred to a 15-cm section of Vycor high temperature glass tubing that previously had been sealed at one end and contained 250 mg of 48–100 mesh CuO. Residual solvent was removed by lyophilization and the tubes were sealed with a flame under a vacuum of 80 mTorr and combusted by heating to 860°C for 4 h. The tubes were then broken under vacuum into the inlet of a V-G SIRA 12 isotope ratio mass spectrometer (IRMS) for determination of the ratio of $^{13}CO_2/^{12}CO_2$. Data were expressed as ^{13}C atoms divided by the sum of ^{12}C + ^{13}C atoms times 100 (atom % ^{13}C). The detection limit of the method was 0.00005 atom % ^{13}C. **Fig. 2** is a standard curve generated by mixing weighed amounts of natural cholesterol containing 1.08413 atom % ^{13}C (determined by IRMS) with tracer cholesterol containing 19.17 atom % ^{13}C. The line generated by plotting measured atom % (vertical axis)

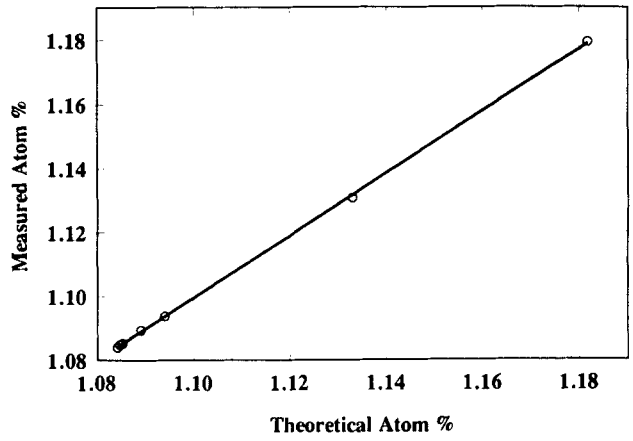

Fig. 2. Standard curve for IRMS detection of cholesterol. Each point is the mean of triplicate determinations.

against the theoretical atom % (horizontal axis) was linear with a correlation coefficient of 0.999 and a slope of 0.976. The precision of measurement was independent of the amount of ^{13}C present over the range of values found in patient material; in seven different standards analyzed in triplicate the average standard deviation of replicate measurements was 0.00023 atom %. Atom % excess ^{14}C (APE) was computed as the atom % ^{13}C in the sample minus atom % ^{13}C in the plasma cholesterol of the subject taken before isotope infusion.

Calculations

Isotope concentration in plasma at various time points was expressed as fraction of injected dose/mg plasma cholesterol. For radioactive experiments this was computed as specific activity (dpm/mg cholesterol) of plasma cholesterol/total dpm infused. For stable isotope experiments it was computed as plasma cholesterol APE divided by the product of APE of the infused sample times the mg of tracer cholesterol given (determined by isotope dilution). Parameters of a two-compartment model and of a new three-compartment minimal model (5) were computed with the Simulation, Analysis, and Modeling (SAAM) computer program distributed by the Resource Facility for Kinetic Analysis at the University of Washington (**Fig. 3**). The minimal model (Fig. 3B) is a three-compartment model in which only four parameters are computed: the mass of compartment 1, the fractional rate of cholesterol excretion L(0,1), and average forward and reverse rate constants for the transfer of cholesterol between the central and peripheral compartments. The minimal model retains the accuracy inherent in a traditional three-compartment model while substantially increasing the precision of parameter estimation. The minimal model is especially useful in the analysis of 10-week cholesterol turnover studies such as those reported here.

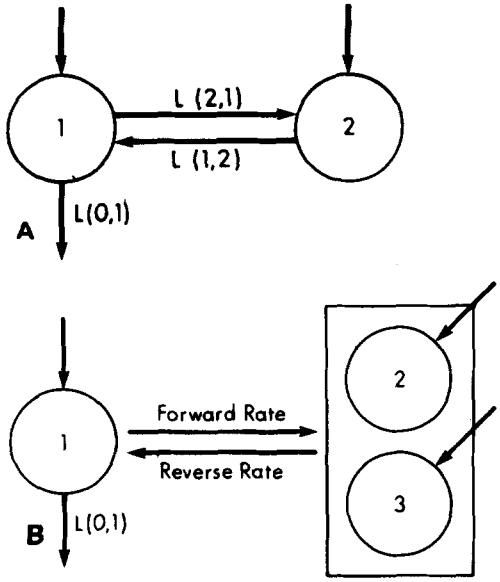

Fig. 3. Models used to calculate cholesterol turnover parameters (5). A: two-compartment model. B: three-compartment minimal model.

TABLE 1. Baseline cholesterol enrichment over time

Subject	Diet	Weeks	Fasting Specimens	Atom % ^{13}C + SD
1	Step 1	17	No	1.09003 ± 0.00028
2	Step 1	12	No	1.09269 ± 0.00007
3	Ad lib	13	Yes	1.08866 ± 0.00018
4	Ad lib	5	Yes	1.09118 ± 0.00029

Plasma was collected every 2–4 weeks for the indicated time period (5–9 samples) and ^{13}C enrichment was determined by IRMS in duplicate.

RESULTS

Approximately 1% of all carbon atoms in the biosphere are ^{13}C. **Table 1** shows that the baseline ^{13}C content of plasma cholesterol was quite constant over time in a given individual, with standard deviations of less than 0.0003 atom % during multiple plasma sampling over a 5–17 week period. Variation in fasting and nonfasting plasma samples was similar. The mean value for ^{13}C content in plasma cholesterol in four individuals was 1.09064 ± 0.00171 atom % with values ranging from 1.08866 to 1.09269 atom %. This suggested that long-term cholesterol turnover studies might be feasible by measuring tracer ^{13}C enrichment over the unique and constant natural ^{13}C abundance in plasma cholesterol of a given individual.

Cholesterol turnover studies were therefore performed in the three subjects listed in **Table 2** with simultaneous intravenous injection of [^{14}C]cholesterol and [^{13}C]cholesterol. **Fig. 4** shows the results for [^{13}C]cholesterol in subject 1 in whom 62.2 mg of that tracer was given. Baseline ^{13}C was 1.09088 atom % and the enrichment above that level is plotted on the ordinate as Atom % Excess ^{13}C

TABLE 2. Characteristics of subjects undergoing double-label cholesterol turnover studies

Subject	Sex	Age	Body Mass Index	Cholesterol			Triglyceride
				Total	LDL	HDL	
			kg/m²		*mg/dl*		*mg/dl*
1	M	61	27.0	182	129	49	66
2	M	65	25.2	195	126	65	47
3	F	65	22.4	198	111	73	91

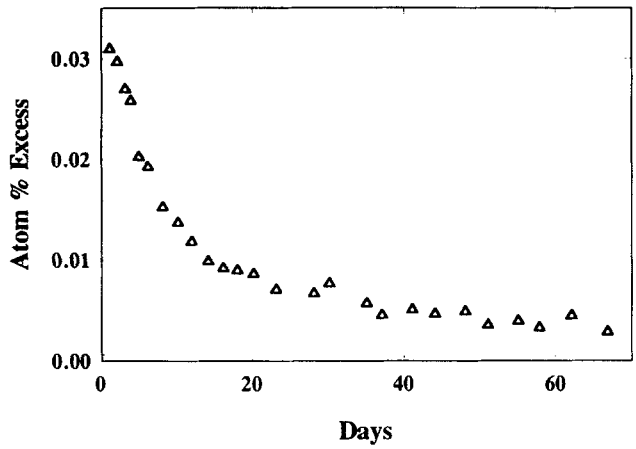

Fig. 4. Metabolism of [^{13}C]cholesterol in subject 1.

(APE). One day after tracer injection the ^{13}C content of plasma cholesterol had risen to 1.12197 atom % or 0.03109 APE. Over the next 10 weeks the level declined to 1.09385 atom %, an enrichment of 0.00297 APE. Although small, this enrichment was 10-fold higher than the largest standard deviation for a single individual found in Table 1 (which includes both analytical and biological variation) and 13-fold higher than the average standard deviation of replicate standards (analytical error). Thus, the enrichments of plasma cholesterol found 10 weeks after infusion appeared to be meaningful.

The simultaneous ^{14}C and ^{13}C studies were compared in **Fig. 5** where the tracer concentration in plasma is plotted as the fraction of the injected dose per mg plasma cholesterol at various times. The data points agree well and many are exactly superimposable. Parameters of whole body cholesterol metabolism were calculated independently from the data of each tracer and are presented in detail in **Table 3.** The top portion of the table gives parameters of a two-compartment cholesterol turnover model (Fig. 3A) and the bottom portion those of a three-compartment minimal model (Fig. 3B). There were marked differences between subjects in some, but not all, parameters. However, within each subject the parameters calculated using the two tracers agreed well. For all subjects and all parameters the [^{13}C]cholesterol parameter values were 103 ± 10.5 SD % of those determined from the [^{14}C]cholesterol data. However, as noted in Table 3, there were small but statistically significant differences between tracers in some parameters. In both models the ^{13}C tracer gave a computed pool 1 mass slightly lower than the ^{14}C tracer (7.6–8.2% difference). Examination of Fig. 5 shows that this might have resulted in part from a 7.0% increase in the relative abundance of the ^{13}C tracer with respect to the ^{14}C tracer during the first 3 days of the experiment (Fig. 5, $P = 0.004$ by analysis of variance.) Small but statistically significant differences between ^{13}C and ^{14}C data were also noted in parameters L(2,1) and L(1,2) of the two-compartment model and in the forward rate of the minimal model (Table 3). The maximum mean difference observed was 13%.

DISCUSSION

Over 50 years ago Rittenberg and Schoenheimer labeled cholesterol and fatty acids in animals with deu-

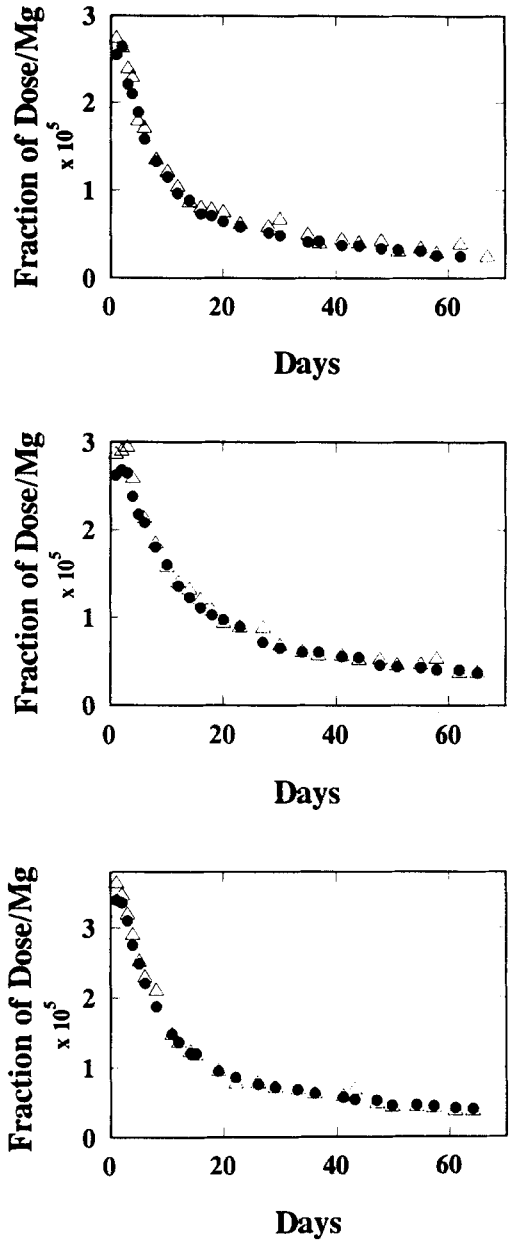

Fig. 5. Metabolism of [^{14}C]cholesterol (closed circles) and [^{13}C]cholesterol (open triangles) given IV simultaneously in three normal subjects. Ordinate is the fraction of the injected dose per mg of total plasma cholesterol.

TABLE 3. Parameters of cholesterol turnover models (Fig. 3) computed using either [^{14}C]cholesterol or [^{13}C]cholesterol data

Parameter	Subject 1	Subject 2	Subject 3	Intra-Individual: ^{13}C–^{14}C Parameter Difference	Intra-Individual: ^{13}C Parameter/ ^{14}C Parameter
				mean ± SD	*mean percent ± SD*
Two-compartment model					
Pool 1 mass (g)				−2.2 ± 0.7[a]	92.4 ± 2.0[a]
^{14}C	31.4	32.3	24.3		
^{13}C	29.7	29.3	22.3		
L(0,1) (pools/day)				0.0012 ± 0.0043	103.6 ± 9.0
^{14}C	0.0547	0.0336	0.0454		
^{13}C	0.0512	0.0357	0.0504		
L(2,1) (pools/day)				0.0063 ± 0.0012[a]	110.8 ± 1.9[a]
^{14}C	0.0718	0.0417	0.0670		
^{13}C	0.0794	0.0470	0.0730		
L(1,2) (pools/day)				0.0058 ± 0.0011[a]	113.1 ± 2.5[a]
^{14}C	0.0566	0.0334	0.0471		
^{13}C	0.0624	0.0382	0.0540		
Three-compartment minimal model					
Pool 1 mass (g)				−2.4 ± 0.7[a]	91.8 ± 1.1[a]
^{14}C	31.7	32.3	24.2		
^{13}C	29.1	29.3	22.5		
L(0,1) (pools/day)				0.0003 ± 0.0033	101.3 ± 7.5
^{14}C	0.0484	0.0331	0.0458		
^{13}C	0.0449	0.0353	0.0479		
Forward rate				0.0075 ± 0.0022[a]	112.4 ± 1.1[a]
^{14}C	0.0753	0.0416	0.0660		
^{13}C	0.0851	0.0470	0.0733		
Reverse rate				−0.0011 ± 0.0079	99.4 ± 18.7
^{14}C	0.0430	.0324	0.0475		
^{13}C	0.0445	.0375	0.0375		

[a] $P < 0.05$ for significance of intra-individual differences between ^{13}C and ^{14}C parameters.

terium, then a newly discovered isotope, and performed the very first cholesterol turnover experiments (6). However, relatively few stable isotope studies of cholesterol metabolism have subsequently been published. Further work has confirmed that feeding deuterated water and measuring deuterium enrichment in plasma cholesterol can yield significant information about the endogenous cholesterol fractional synthetic rate (7–9). However, the expense, inconvenience, and possible side effects of continuously labeling all endogenous molecules makes this approach less desirable than the use of cholesterol specifically labeled in vitro and then administered orally or intravenously. Several isotope derivatives of cholesterol have been prepared. [^{18}O]cholesterol was tested but the label exchanged with unlabeled oxygen during intestinal bacterial metabolism (10). However, cholesterols labeled with ^{13}C or ^{2}H have been useful in human studies.

Work by Hachey and collaborators (11) in 1973 showed that [26,26,26,27,27,27-$^{2}H_6$]cholesterol could be used as an intravenous tracer in dogs. Similarly, in a pioneering series of publications Ferezou, Chevallier, and colleagues (12–16) demonstrated that [2,2,3,4,4,6,7,7-$^{2}H_8$]cholesterol could be used in short-term metabolic studies. The analytical method used was gas chromatography–mass spectrometry (GC–MS), a facile technique but not a sensitive one. The detection limit of GC–MS is a dilution of tracer cholesterol in natural cholesterol of about 1/4000 (13). As a result, cholesterol turnover could be studied for only 1–4 weeks after infusion of 100 mg tracer cholesterol, whereas it is desirable to follow cholesterol turnover for at least 10 weeks. Using GC–MS we were able to measure cholesterol absorption in 3-day human studies with dual cholesterol tracers enriched by 5 and 6 mass units when a dose was used that resulted in a dilution of approximately 1/1600 in body cholesterol (17). The difficulty experienced in using GC–MS to monitor cholesterol enrichment for longer periods is related principally to the extremely large pool of natural cholesterol present. For example, the rapidly exchanging pool of human cholesterol is approximately 24 g and total body cholesterol is estimated at 73 g or more in normal subjects (1).

IRMS is a much more sensitive analytical method than GC–MS. Ferezou, Coste, and Chevallier (14) used IRMS for detection of [3,4-$^{13}C_2$]cholesterol for up to a week after isotope administration. In preliminary experiments we also used [3,4-$^{13}C_2$]cholesterol (18) but found that the doses required were too large to be practical for either long-term cholesterol turnover studies or short-term cho-

lesterol absorption experiments (data not shown). Our solution to this problem was to use tracer cholesterol enriched by 5 mass units (rather than 2). This allowed IRMS detection of tracer cholesterol at a dilution in natural cholesterol of more than 1/60,000. Such a dilution corresponds to an enrichment of 0.0003 atom %, the level of biological + analytical variation shown in Table 1. Using [$^{13}C_5$]cholesterol and IRMS it was possible to follow cholesterol turnover for at least 70 days after an intravenous injection of 65 mg (Figs. 4 and 5).

With [$^{13}C_5$]cholesterol data the compartmental parameters of cholesterol metabolism could be calculated as readily as with data derived from the [^{14}C]cholesterol tracer injected simultaneously (Table 3, Fig. 5). Overall, parameters were very similar using the two tracers (Table 3, right column), and this was true for both two-compartment and three-compartment minimal model estimates. Such heavy labeling of a tracer molecule with ^{13}C has not been used previously for biological studies and the relatively normal metabolism of our tracer cholesterol is consistent with the normal biological activity of octadeuterated cholesterol previously reported (16). Thus, cholesterols enriched from 5–8 mass units appear to be suitable for biological systems and greatly facilitate the analytical methods.

However, two limitations need to be kept in mind when applying stable isotopic methods to cholesterol turnover studies. First, the stability of baseline plasma cholesterol ^{13}C enrichments over time in subjects consuming their usual diets is important to define the conditions needed for these outpatient studies (Table 1). The possibility that variations in diet or manipulation of endogenous cholesterol biosynthesis might affect baseline cholesterol enrichment needs to be considered in future experiments. Second, Table 3 shows that quantitatively small but statistically significant differences were seen between certain parameters computed with radioactive and stable isotopic data. The reason for this is not known, but it could have arisen either because of an isotope effect or through differences in post-study processing of analytical samples. With regard to the latter, the ^{13}C method has the advantage of using as primary data only weights and mass spectrometric carbon isotope ratios (which are independent of quantitative recovery or pipetting), neither of which is prone to laboratory error. In contrast, the radioactive method as performed here depends upon accurate pipetting of plasma, quantitative recovery of radioactive counts from plasma, and determination of cholesterol concentration (which has a ±3% standard deviation). Each method relies on the purity of its labeled cholesterol tracer. However, in the ^{13}C method milligram amounts of tracer are available that can be purified easily and analyzed by conventional physical methods such as GC–MS and nuclear magnetic resonance spectroscopy. In contrast, the radioactive tracer cannot be analyzed routinely by these techniques and assessment of its purity is further hampered by the production over weeks to months of radiation-catalyzed decay products that migrate similarly to authentic cholesterol chromatographically but are not cholesterol (L. H. Krut and R. E. Ostlund, Jr., unpublished observations). [^{13}C]cholesterol is subject only to slow air oxidation and can be repurified by most chromatographic procedures such as HPLC as used in this study.

In metabolic studies using stable isotopes the recycling of isotopic materials into newly synthesized molecules is often appreciable and can limit the method. However, in the case of cholesterol, the potential for recycling appears to be quantitatively quite small as shown by the following calculations. The principal pathway by which the cholesterol label enters the general metabolic carbon pool is during bile acid synthesis when the three terminal carbons of the side chain are released as propionic acid (Fig. 1) of which 80% appears in the breath as CO_2 (19). If 1 mmol of body cholesterol/day is converted to bile acids then $3 \times 0.8 = 2.4$ mmol/day of expired CO_2 are produced from the liberated cholesterol side chain. This constitutes 0.015% of the 16 moles of CO_2 produced daily by general resting metabolism. Assuming that the acetyl-CoA precursor for cholesterol biosynthesis has a similar enrichment, one can calculate that newly synthesized cholesterol on the day after isotope injection could have at most 0.03 atom % excess ^{13}C (Fig. 4) × 27/5 labeled carbon atoms × 0.00015 = 0.000024 atom % excess due to recycling. Because about 5% of pool 1 cholesterol turns over per day (Table 3) the additional enrichment of plasma cholesterol due to recycling would be at most 0.0000012 atom % excess on day 1. This is small compared to a measured enrichment of 0.03 atom % excess on day 1 and 0.003 atom % excess 10 weeks after infusion (Fig. 4).

The current work shows that [^{13}C]cholesterol turnover experiments have the potential to be useful in metabolic and cardiovascular risk factor research. The lack of radiation exposure may allow more healthy subjects to be studied and the exact relation of whole body cholesterol metabolism to cardiovascular disease to be defined more completely.

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