papers and notes on methodology

Measurement of in vivo cholesterol synthesis from ^2H_2O: a rapid procedure for the isolation, combustion, and isotopic assay of erythrocyte cholesterol

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Abstract A rapid preparative scale purification of erythrocyte free cholesterol has been developed for measurements of in vivo cholesterol synthesis from ^2H_2O. The quantity and purity of cholesterol obtained is suitable for combustion, zinc reduction of the water formed, and determination of deuterium isotopic content by gas isotope ratio mass spectrometry. The ability to detect and to quantitate a range of cholesterol synthesis rates is illustrated by measurements on young pigs receiving diets without and with added dietary cholesterol. —**Wong, W. W., D. L. Hachey, A. Feste, J. Leggitt, L. L. Clarke, W. G. Pond, and P. D. Klein.** Measurement of in vivo cholesterol synthesis from ^2H_2O: a rapid procedure for the isolation, combustion, and isotopic assay of erythrocyte cholesterol. *J. Lipid Res.* 1991. **32:** 1049–1056.

Supplementary key words HPLC • cholesterol synthesis • deuterium oxide • human • pig • erythrocytes • mass spectrometry

Nearly 60 years ago, Rittenberg and Schoenheimer (1) showed that cholesterol, during its synthesis, derives 22 hydrogen atoms from body water and NADPH of a total of 46 hydrogen atoms (2). Since the studies of Rittenberg and Schoenheimer, various authors have shown that erythrocyte or plasma free cholesterol is in rapid equilibrium with newly synthesized liver free cholesterol (3–5), and that, in the presence of deuterium oxide, the rate of cholesterol synthesis can be estimated from the increase in deuterium enrichment of plasma or erythrocyte free cholesterol. The deuterium oxide method used to estimate cholesterol synthesis is both rapid and sensitive (6–9).

Nevertheless, this measure of cholesterol metabolism and the effects of drug or diet on whole body cholesterol synthesis rates in humans has had limited use. The isolation of cholesterol from plasma or erythrocytes has required digitonide precipitation (10), thin-layer chromatography (6), or liquid chromatography (8). These procedures are either time-consuming or produce a sample in which the cholesterol is contaminated with other sterols or lipids. In this report, we describe a technique for the purification of milligram quantities of cholesterol from human or porcine erythrocytes by preparative HPLC and the subsequent processing through sample combustion, reduction of the water to hydrogen gas, and determination of isotopic enrichment. We have illustrated the application of the preparative technique to analyses of cholesterol synthesis using deuterium oxide in two groups of piglets fed either a cholesterol-free diet or a diet containing 0.5% cholesterol.

METHODS

Chemicals

Acetic acid, sodium phosphate, cupric oxide wire, and HPLC grade hexane and isopropanol were obtained from Fisher Scientific (Pittsburgh, PA). Methyl tertiary butylether (MTBE) was purchased from Aldrich Chemical (Milwaukee, WI). Cholesterol, triolein, 1,2-dioleylglycerol, 1,3-dioleylglycerol, and Sigmacote were obtained from Sigma Chemical (St. Louis, MO). Silver foil was obtained from Alfa Chemicals and Materials (Danvers, MA), zinc shot from Gallard-Schlesinger Chemical Manufacturing Co. (Carle Place, NY), and deuterium oxide (99.8 atom % ^2H) from Merck Sharp and Dohme (St. Louis, MO).

Abbreviations: HPLC, high performance liquid chromatography; MTBE, methyl tertiary butylether.

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Extraction of lipids from erythrocytes

Red blood cells, obtained from 10 ml of whole blood by centrifugation at 3000 rpm for 10 min using an IEC Centra-8R refrigerated centrifuge (Model 2478, International Equipment Co., Needham, MA), were collected in a 15-ml Vacutainer® coated with freeze-dried sodium heparin (Becton-Dickinson, Rutherford, NJ). After removal of the plasma, the packed cells were transferred to a 50-ml screw-capped centrifuge bottle (Kimble, Vineland, NJ) with 2.5 ml of 0.9% saline solution buffered at pH 7.4 with 10 mM sodium phosphate. Before use, the interior of the 50-ml bottle was coated with Sigmacote to facilitate separation of the organic and aqueous phases in the subsequent steps. To coat the interior, 20 ml of Sigmacote was added to the bottle, which was vortexed, drained, and allowed to air dry. Five ml of isopropanol was added to the cells and the cells were disrupted by sonication for 10 min. Ten ml hexane–isopropanol 4:1 (v/v) was added to the solubilized membranes and the mixture was sonicated for 10 min. An additional 10 ml of the buffered saline was added and the sample was vortexed and then sonicated for another 10 min. The clear hexane layer containing the lipids was transferred to a drying tube. The aqueous layer was extracted again with 10 ml of hexane. The organic layers were combined and dried under a stream of N_2 at 40°C.

Neutral lipids were isolated from the crude lipid extract using a modification of the solid phase extraction procedure described by Hamilton and Comai (11). The lipids were dissolved in approximately 8 ml of hexane–MTBE 200:3 (v/v) and loaded onto a 0.5-g silica solid phase extraction column (Alltech Associates Inc., Deerfield, IL). Triglycerides and free fatty acids were eluted from the column with 10 ml of hexane–MTBE–acetic acid 100:2:0.2 (v/v/v) using a 12-sample vacuum manifold (Alltech Associates Inc., Deerfield, IL). The neutral lipids containing the cholesterol were eluted from the silica column with 10 ml of MTBE–acetic acid 100:0.2 (v/v) and dried under nitrogen at 40°C. Phospholipids were retained on the cartridge, which was discarded after a single use.

Purification of cholesterol by preparative HPLC

The normal phase analytical HPLC method described by Hamilton and Comai (12) was modified for the preparative separation of cholesterol from other neutral lipids. A Waters Delta-prep 3000 preparative HPLC system (Waters Associates, Milford, MA) with a Waters Model 820 Maxima workstation, a Waters Model 490 programmable diode array detector, and a FOXY peak separator/fraction collector (Isco Inc., Lincoln, NE) was used. After dissolving the neutral lipids in 0.5 ml of the mobile phase, hexane–isopropanol–acetic acid 100:2:0.02 (v/v/v), the lipids were loaded onto a Waters μPorasil PrepPak radial compression cartridge column (#38504, 25 mm × 100 mm) contained in a Waters PrepPak cartridge holder (#15814) at 1000 psi. Monoglycerides, diglycerides, and cholesterol were separated using a mobile phase of hexane–isopropanol–acetic acid 100:2:0.02 (v/v/v) at a flow rate of 20 ml/min. Absorbance at 204 nm was monitored. The mobile phase was degassed and purged with helium at 30 ml/min during the analysis, and the μPorasil column was maintained at room temperature. Each chromatogram was allowed to develop for 10 min.

Purity of cholesterol by HPLC

To examine the purity of the chromatographically isolated fractions in the erythrocyte cholesterol fractions from five humans and one pig, the fractions were applied to a Spherisorb silica analytical column (5 μm, 4.6 mm × 250 mm, Custom LC Inc., Houston, TX). Cholesterol was eluted from the analytical column at 3 ml/min with hexane–isopropanol–acetic acid 100:2:0.02 (v/v/v). To optimize separations, the procedure was repeated with the mobile phase containing 1 or 3 vol% of isopropanol. Absorbance was monitored at 204 nm.

Conversion of cholesterol hydrogen to H_2 gas

Approximately 3–5 mg of a cholesterol standard was transferred in a small volume of hexane to a quartz tube (Quartz Scientific, Freeport Harbor, OH). Approximately 3–5 mg of a cholesterol sample collected from the silica solid phase extraction column and the Waters μPorasil PrepPak column was dried under N_2 and also transferred in a small volume of hexane to a quartz tube. The quartz tube (9 mm × 300 mm) was sealed at one end and contained approximately 1 g of cupric oxide wire and 9 mm^2 of silver foil (13). Hexane was removed at 40°C under a stream of nitrogen. The tube containing the cholesterol was attached to a vacuum manifold with a 3/8-in Ultra-Torr union (SS-6-UT-6, Cajon Co., Macedonia, OH), evacuated to $<10^{-2}$ mbar to remove the last traces of the solvent, and sealed with a torch. The sealed tube was heated at 850°C for 1 h and allowed to cool slowly to room temperature. The cooled tube was notched approximately 2 cm from the top with a glass cutter and the notch was aligned with the tapered edge of the lower stainless steel cone of a tube cracker (14). The tube cracker assembly was attached to a transfer system (**Fig. 1**) with a Swagelok 3/8-in port connector (SS-601-PC, Crawford Fitting Co., Solon, OH) and the transfer system was evacuated to $<10^{-2}$ mbar. After the CO_2 and H_2O in the sealed tube were frozen with liquid nitrogen, the tube was opened by flexing the lower end of the flexible tubing of the tube cracker. The CO_2 was released by replacing the liquid nitrogen with a slurry of isopropanol/dry ice at approximately −70°C. The system was again evacuated to $<10^{-2}$ mbar. The transfer system was isolated from the vacuum manifold, the isopropanol/dry ice slurry was re-

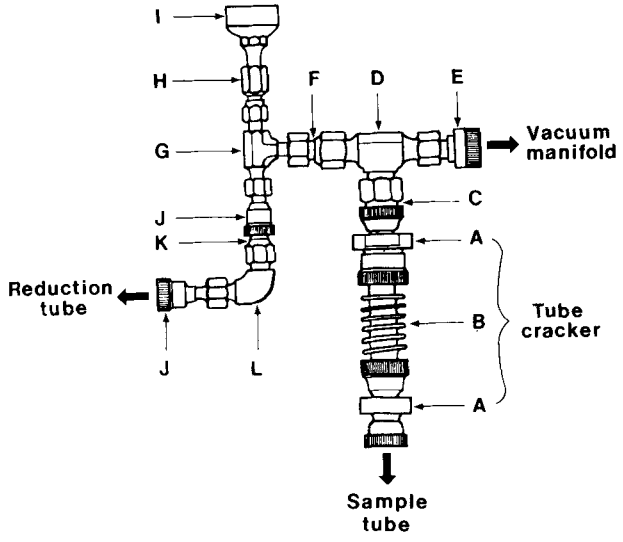

Fig. 1. Tube cracker and transfer assembly: A, 1/2-in to 3/8-in Cajor Ultra-Torr reducing union (SS-8-UT-6-6); B, 1/2-in Cajon flexible tubing (321-8-X-1); C, Swagelok 3/8-in port connector (SS-601-PC); D, Swagelok 3/8-in union tee (SS-600-3); E, 3/8-in Cajon adapter (SS-6-UT-A-6); F, Swagelok 3/8-in to 1/4-in reducing port connector (SS-601-PC-4); G, Swagelok 1/4-in union tee (SS-400-3); H, Cajon 1/4-in fractional tube adapter to 1/8-in female pipe (SS-4-TA-7-2); I, vacuum gauge with 1/8-in male pipe fitting; J, 1/4-in Cajon adapter (SS-4-UT-A-4); K, Swagelok 1/4-in port connector (SS-401-PC); and L, Swagelok 1/4-in union elbow (SS-400-9).

moved, and the H_2O was transferred with liquid nitrogen to a quartz reduction tube containing approximately 250 mg of ≤1 mm zinc shot. The tube cracker and the transfer system was maintained at a temperature of 80°C with a heating tape and a temperature controller (D921K35, Omega Engineering, Inc., Stamford, CT). The H_2O in the reduction tube was reduced to H_2 gas for isotope ratio measurement as described by Wong, Lee, and Klein (15).

Gas-isotope-ratio mass spectrometry

The ^2H:^1H ratio of the hydrogen gas was measured with a Finnigan Delta-E gas-isotope-ratio mass spectrometer (Finnigan MAT, San Jose, CA). The results are expressed in δ^2H units which are defined as follows:

$$\delta^2H, \text{ o/oo} = (R_s/R_{ws} - 1) \times 10^3 \qquad Eq.\ 1)$$

in which R_s and R_{ws} are the ^2H:^1H ratios of the sample and the laboratory working standard, respectively. The measured δ^2H values were normalized against two international water standards, Vienna-Standard Mean Ocean Water (V-SMOW) and Standard Light Antarctic Precipitation (SLAP) according to the procedure of Gonfiantini (16).

Precision of isotope ratio measurements

The deuterium content of a chromatographic grade cholesterol standard was measured after combustion and reduction by gas-isotope-ratio mass spectrometry, before purification, after elution through the silica solid phase extraction column, and after HPLC purification as described above.

Cholesterol synthesis in pigs

Eight 29-day-old genetically obese piglets (17) were studied. The National Research Council's guide for the care and use of laboratory animals was followed. Four of the piglets were fed a diet that contained no cholesterol from the first day after birth. The other four animals were fed a diet that contained 0.5% cholesterol. Baseline blood samples (10 ml) were collected via the anterior vena cava, after which each piglet received a bolus dose of 500 mg ^2H_2O/kg body wt intramuscularly. A bolus dose was used so that the cholesterol synthesized within the first 24 h after the dose would be enriched with ^2H. The deuterium enrichment in the body water of the piglets was maintained at a constant level by intramuscular administration of 70 mg ^2H_2O/kg body wt twice daily. The amount of the daily maintenance doses was based on a fractional turnover rate of body water estimated to be 0.28 d^{-1} for young piglets. One daily post-dose blood sample (10 ml) was collected from each animal for the next 4 days. Free cholesterol was isolated from the erythrocytes, purified, and combusted to CO_2 and H_2O and the H_2O was reduced to H_2 for isotope ratio measurement by the procedures described above.

Cholesterol synthesis computations

A single-pool model was used to compute the fractional synthesis rate of cholesterol from the mean ^2H enrichment in body water ($^2H_{water}$) and the rise in ^2H enrichment of erythrocyte cholesterol ($^2H_{chol}$). The experimental data were fitted to a single exponential equation:

$$^2H_{chol} = 0.4783 \times {}^2H_{water} \times (1 - e^{-kt}). \qquad Eq.\ 2)$$

The value of k (d^{-1}) is the rate constant for the incorporation of ^2H into erythrocyte cholesterol. The constant 0.4783 is the fractional number of hydrogens (22 atoms) in cholesterol (46 total) that are derived from body water during biosynthesis (2). The k values are converted to fractional synthesis rate (FSR) in units of "percent per day" as follows:

$$FSR\ (\%/d) = k \times 24 \times 100. \qquad Eq.\ 3)$$

RESULTS

HPLC

Chromatograms illustrating the separation of cholesterol, triolein, 1,2-dioleylglycerol, and 1,3-dioleylglycerol standards using the Water μPorasil PrepPak cartridge

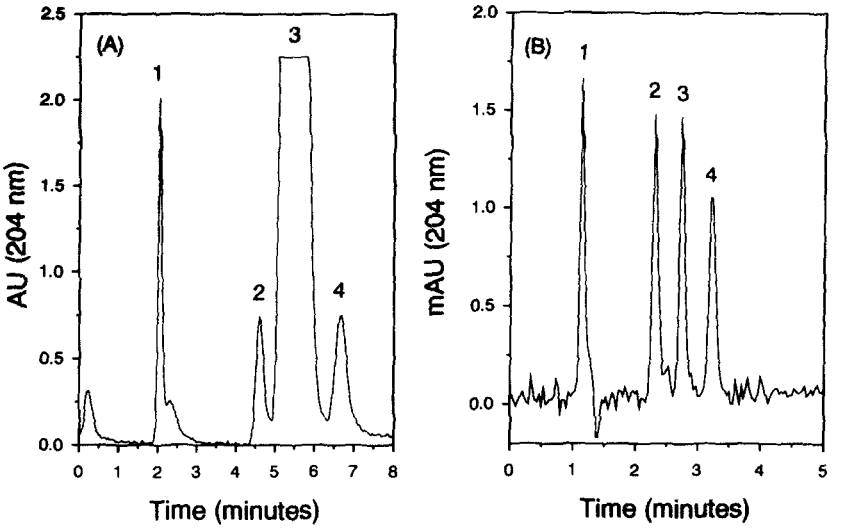

Fig. 2. Chromatograms of known standards: (A) triolein (1, 0.2 mg), 1,3-dioleylglycerol (2, 0.2 mg), cholesterol (3, 6 mg), and 1,2-dioleylglycerol (4, 0.2 mg) through the Waters μPorasil PrepPak cartridge column (25 mm × 100 mm) at 20 ml/min; (B) triolein (1, 4 μg), 1,3-dioleylglycerol (2, 4 μg), cholesterol (3, 2.5 μg), and 1,2-dioleylglycerol (4, 2 μg) through a Spherisorb silica analytical column (5 μm, 4.6 mm × 250 mm) at 3 ml/min. The mobile phase was hexane-isopropanol-acetic acid 100:2:0.02 (v/v/v). Absorbance at 204 nm was monitored.

column and the Spherisorb silica analytical column are shown in **Fig. 2A and 2B,** respectively. At a flow rate of 20 ml/min using the preparative column, triolein, 1,2- and 1,3-dioleylglycerol and cholesterol were eluted within 8 min. As shown in **Table 1,** the alpha factors for the separation of the 1,3-dioleylglycerol-cholesterol pair on the analytical column with mobile phase (hexane-isopropanol-acetic acid) containing 1, 2, and 3 vol% of isopropanol were 1.08, 1.38, and 1.52, respectively. Similar alpha factors were obtained for the 1,3-dioleylglycerol-cholesterol pair using the preparative column (1.19, 1.30, and 1.56). When the cholesterol-1,2-dioleylglycerol pair was separated on the analytical column, the alpha factors obtained for mobile phases containing 1, 2, and 3 vol% of isopropanol were 1.67, 1.30, and 1.17, respectively. Similar alpha factors (1.52, 1.33, and 1.11) were obtained for the separation of the cholesterol-1,2-dioleylglycerol pair on the preparative column. The number of theoretical plates for the separation of cholesterol on the analytical column for mobile phases containing 1, 2, and 3 vol% of isopropanol were 27457, 19984, and 17789, respectively. Plate counts of 14887, 12240, and 10152 were obtained for the preparative separation using mobile phases containing 1, 2, and 3 vol% of isopropanol, respectively.

As shown in **Table 2,** the within- and between-run retention times for the preparative separation of cholesterol using a mobile phase of hexane-isopropanol-acetic acid 100:2:0.02 (v/v/v) were 5.51 ± 0.09 min (CV% = 1.64%), and 5.45 ± 0.09 min (CV% = 1.69%), respectively.

Purity and recovery of cholesterol

The preparative chromatograms of a human and a pig cholesterol extract are shown in **Figs. 3A and 3B,** respectively. Diglycerides were absent from these cholesterol extracts. Similar chromatograms were obtained with the other four human erythrocyte cholesterol extracts. Purity of the cholesterol peaks collected from the Waters μPorasil preparative column was evaluated by eluting aliquots of these cholesterol samples through a Spherisorb silica analytical column using a mobile phase of hexane-iso-

TABLE 1. Optimization of chromatographic separation of known standards

	Analytical Column			Prep Column		
Solvent[a]	A	B	C	A	B	C
Separation factor[b]						
1,3-DG-Chol[c]	1.08	1.38	1.52	1.19	1.30	1.56
Chol-1,2-DG	1.67	1.30	1.17	1.52	1.33	1.11
Theoretical plates/meter[d]	27,457	19,984	17,789	14,887	12,240	10,152

[a]Solvent composition: (hexane-isopropanol-acetic acid (v/v/v)): A, 100:1:0.02; B, 100:2:0.02; C, 100:3:0.02.
[b]Separation factor, $a = k_1/k_2$ where k_1 and k_2 are the capacity factors as described by $k_1 = (V_1 - V_0)/V_0$ and $k_2 = (V_2 - V_0)/V_0$. V_0, V_1, and V_2 are the void volume of the column, the volume of peak 1, and the volume of peak 2, respectively.
[c]DG, dioleylglycerol; Chol, cholesterol.
[d]Theoretical plates per meter = $[5.54(V_e/W_{1/2\ max})^2]$/column length, where V_e is the volume of solvent needed to elute cholesterol and $W_{1/2\ max}$ is the width of the peak at half maximal peak height.

TABLE 2. Reproducibility of retention time of cholesterol for preparative separation

	Retention Time (min)			
	N	Mean	SD	CV%[a]
Within run	33	5.51	0.09	1.64
Between run[b]	122	5.45	0.09	1.69

[a]Coefficient of variation.
[b]Samples separated on 8 different days.

propanol-acetic acid 100:1:0.02 (v/v/v) at 3 ml/min. The procedure was repeated with the mobile phase containing 2 or 3 vol% of isopropanol. Only a single peak with the retention time of cholesterol was obtained in all the chromatograms. Analysis of the HPLC cholesterol fractions by capillary gas chromatography showed only a single sterol peak. **Table 3** summarizes the recovery of cholesterol from five human erythrocyte samples. The percent recovery is based on normal erythrocyte cholesterol concentration of 1.37 mg/g of red blood cells (18). Using our extraction procedures, recovery of erythrocyte free cholesterol was determined to be between 89.1 and 101.5% with a mean value of 94.2 ± 4.5%.

Precision of isotope ratio measurements

The hydrogen isotopic abundance of a chromatographic grade cholesterol before and after purification by a silica preparation column and by preparative HPLC is summarized in **Table 4.** No significant difference was observed between the δ^2H values of the three groups by one-way analysis of variance. The results indicated that hydrogen isotopic abundance of cholesterol can be measured with a precision of approximately 4 ‰ and that no hydrogen isotope fractionation occurred during the extraction and purification procedures.

TABLE 3. Extraction and recovery of erythrocyte cholesterol (Chol) from human blood

Sample	Weight Cells	Chol	Erythrocyte Chol	Recovery[a]
	g	*mg*	*mg/g*	%
A	5.20	6.72	1.29	94.2
B	4.90	6.29	1.28	93.4
C	3.30	4.60	1.39	101.5
D	2.59	3.30	1.27	92.7
E	2.12	2.60	1.22	89.1
Mean	3.62	4.78	1.29	94.2
SD	1.37	1.80	0.06	4.5

[a]Based on the average erythrocyte cholesterol concentration of 1.37 mg/g cell (18).

Cholesterol synthesis in pigs

Incorporation of ^2H_2O into erythrocyte cholesterol of genetically obese pigs on a cholesterol-free diet (n = 4) and on a diet containing 0.5% cholesterol (n = 4) is shown in **Fig. 4.** Cholesterol synthesis of the obese pigs on the cholesterol-free diet, as reflected by the deuterium enrichment in the erythrocyte cholesterol, was significantly higher than that of piglets on the high cholesterol diet. The calculated fractional synthesis rate of cholesterol for the two groups of pigs is summarized in **Table 5.** Piglets on the cholesterol-free diet had a maximal fractional synthesis rate (FSR) of cholesterol (18.85 ± 2.15%/d) while the synthesis of cholesterol by piglets on the diet containing 0.5% cholesterol was almost completely abolished (1.12 ± 0.51%/d).

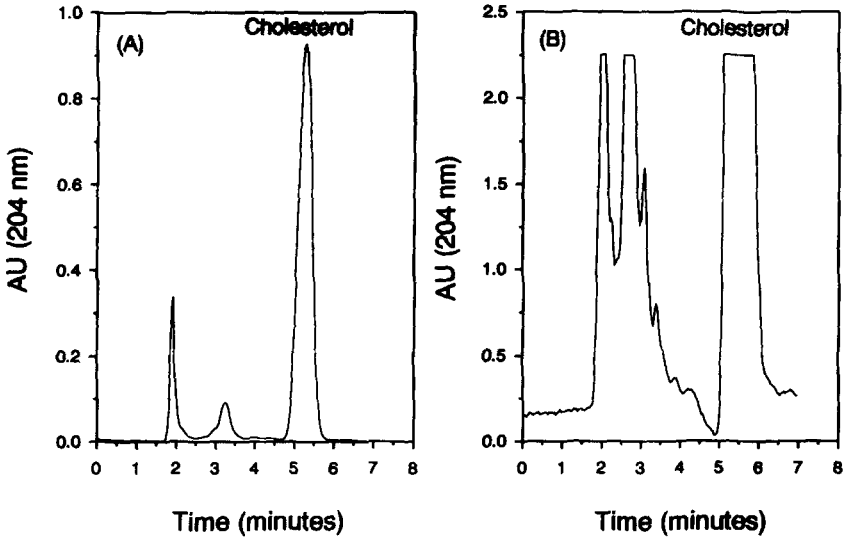

Fig. 3. Chromatograms of (A) a human and (B) a pig erythrocyte cholesterol extracts developed through the Waters μPorasil PrepPak cartridge column with hexane-isopropanol-acetic acid 100:2:0.02 at 20 ml/min. Absorbance at 204 nm was monitored.

TABLE 4. Abundances of deuterium in cholesterol standards before and after purification by solid phase extraction and HPLC

		δ^2H vs V-SMOW/SLAP (‰)	
		Mean	SD
Standard	12	−216.5	7.2
Solid phase extraction	5	−217.3	3.9
Preparative HPLC	6	−216.2	4.0

DISCUSSION

The ability to measure in vivo cholesterol synthesis by incorporation of ^2H_2O has been available for nearly 60 years (1) and has been shown to be both accurate and sensitive (6–9). However, the technique has not gained wide popularity because of the difficulty in sample preparation and of the limited access of instrumentation for accurate and precise hydrogen isotope ratio measurements. With the preparative HPLC technique, after initial lipid extraction, 5 mg of pure cholesterol can be obtained in a single run within 10 min. After combustion, the water is subsequently converted to hydrogen gas for isotope ratio measurement by the zinc reduction procedure described, which has been shown to be rapid and reliable (15). Because water samples are reduced to hydrogen gas in individual reduction vessels using fresh aliquots of zinc shot, memory effects, often present in reduction procedures based on uranium, are eliminated. The reduction vessel also serves as the sample introduction container, thus eliminating the extra step required to transfer the gas before isotope ratio analysis. With 3–5 mg of cholesterol, sufficient hydrogen gas is produced so that an automated multiple sample inlet system (20) can be used. The multiple sample inlet system enables unattended sequential isotope ratio measurement of up to 40 samples and each analysis takes approximately 15 min. The procedures described in the present report enable the processing and analysis of approximately 50 samples per week.

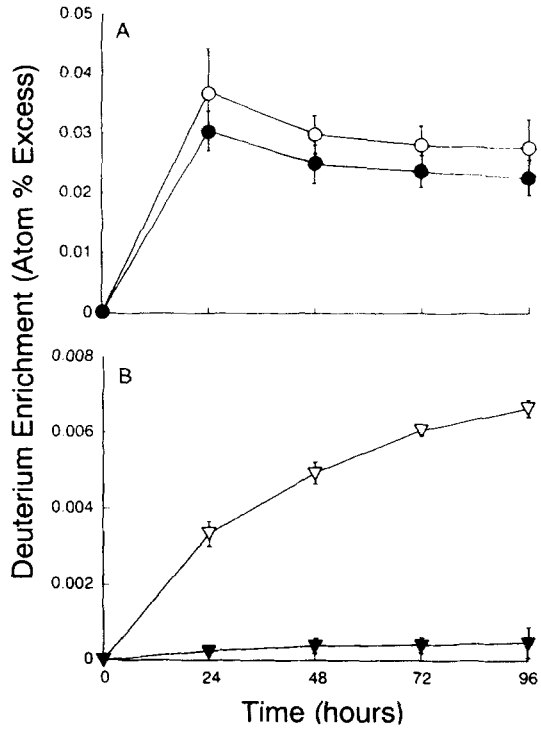

Fig. 4. Deuterium enrichment of plasma water (A) and erythrocyte cholesterol (B) in genetically obese pigs on a cholesterol-free diet (n = 4, open circles and triangles) and on a diet containing 0.5% cholesterol (n = 4, closed circles and triangles). The error bars represent the standard deviations of the average deuterium enrichments at each time point. Omission of the error bars indicates that the standard deviations of the means are within the dimensions of the symbols.

TABLE 5. Fractional synthesis rate (FSR) of genetically obese pigs fed a cholesterol-free diet and a diet containing 0.5% cholesterol

Animal	FSR ± SD	CV[a]
	%/d	%
Cholesterol-free diet		
101	20.23 ± 2.33	11.5
104	20.89 ± 2.09	10.0
105	16.14 ± 1.14	7.1
107	18.15 ± 1.48	8.2
Mean	18.85 ± 2.15	11.4
Diet with 0.5% cholesterol		
402	1.03 ± 0.09	8.8
403	1.47 ± 0.29	19.4
405	1.55 ± 0.27	17.2
406	0.43 ± 0.11	26.0
Mean	1.12 ± 0.51	46.0

[a]Coefficient of variation.

To obtain sufficient cholesterol for isotope ratio measurements, we have routinely collected four samples (40 ml total) from each participant per study: one baseline sample (10 ml) before the ingestion of the ^2H_2O and three post-dose samples (10 ml each) 24-h apart for the next 3 days. Although blood volumes as small as 2.5–3.0 ml can be collected per time point, smaller samples produce smaller quantities of hydrogen gas. Small-volume gas samples must be introduced manually. As a result, there is a concomitant sacrifice in both time and accuracy and precision of isotope ratio measurements. The pediatric hematologist of the Institutional Review Board for Human Investigation at the Texas Children's Hospital has approved this blood sampling schedule for infants weighing 4 kg or more. However, infants should not be restudied more often than every 4–6 weeks.

The normal phase analytical HPLC procedure for the separation of neutral lipids described by Hamilton and Comai (12) was adapted for the purification of milligram quantities of cholesterol isolated from human and pig erythrocytes. Because erythrocytes contain large amounts

of phospholipids whose presence will shorten the lifetime of the preparative column, they were removed using the disposable silica column before preparative HPLC separation. Determination of the alpha factors for the analytical/preparative separation of the 1,3-dioleylglycerol–cholesterol and cholesterol–1,2-dioleylglycerol pairs (Table 1) demonstrated that a mobile phase composition of hexane–isopropanol–acetic acid 100:3:0.02 produced the best separation for the 1,3-dioleylglycerol–cholesterol pair, whereas a mobile phase composition of hexane–isopropanol–acetic acid 100:1:0.02 produced the best separation for the cholesterol–1,2-dioleylglycerol pair. Only the mobile phase of hexane–isopropanol–acetic acid 100:2:0.02 resulted in similar alpha factors for both pairs (1.30 to 1.38). Consequently, the mobile phase composition was selected as the optimum for the separation of the cholesterol from the preceding and subsequent components. This solvent system also permitted rapid separation of cholesterol from the other lipids. We were able to achieve a cycle time of 10 min between samples. In addition, the alpha factors determined for each pair using different mobile phase compositions were simliar between the analytical column and the preparative cartridge indicating that the separation capabilities of both columns were similar. Another important feature of the preparative separation was the reproducibility of the retention time of cholesterol. At a flow rate of 20 ml/min, the within- and between-run precisions (Table 2) of the retention time of cholesterol were 1.64% and 1.69%, respectively. The mean retention times correlated with retention volumes of 110 ± 1.8 ml and 109 ± 1.8 ml for the within- and between-run studies, respectively. These values demonstrate the high precision of this preparative separation.

On the preparative scale, contamination of cholesterol by 1,2- and 1,3-dioleylglycerol is possible as shown in Fig. 1A. However, chromatograms of five human (Fig. 2A) and one pig (Fig. 2B) erythrocyte extracts indicated very low levels of diglycerides and cholesteryl esters in these samples. Analytical HPLC analyses of the cholesterol peaks collected from the preparative column using three different mobile phases all yielded a single peak for cholesterol. A single sterol peak was also obtained when the preparative cholesterol fractions were analyzed by capillary gas chromatography. High purity cholesterol is required for the estimation of in vivo cholesterol synthesis, because contamination by other organic compounds would yield erroneous hydrogen isotope ratios when the samples were combusted.

No hydrogen isotope fractionation was detected during the extraction and purification procedures (Table 4). Although chromatographic fractionation of ^{2}H and ^{3}H is known to occur, the problem is avoided by collecting the entire chromatographic peak for analysis (19).

The two groups of genetically obese pigs fed either cholesterol-free diets or diets containing 0.5% cholesterol illustrate two extremes of cholesterol fractional synthesis rates: from 18% per day to 1% per day. The values in Table 5 also indicate the coefficient of variation associated with these rates based on the protocol sampling schedule (one baseline and three post-dose samples collected at 24-h intervals). At the upper rate, the group variance was 11% and increased to 46% as dietary cholesterol almost completely inhibited new cholesterol synthesis. The large coefficient of variation at a fractional synthesis rate of 1% per day was a consequence of reduced precision in the hydrogen isotope ratio measurement because of minimal incorporation of ^{2}H into cholesterol from body water in the piglets. Assuming similar precision (SD = 2.15% per day), however, in the determination of fractional synthesis rate and an N of 4, the above protocol can detect a statistically significant difference ($P < 0.05$) in fractional synthesis rates between 18% per day and 15% per day. The ability to distinguish such small differences in FSR of cholesterol, coupled with the minimally invasive procedures and absence of radioactivity, should prove useful in future nutritional and pharmacological studies of in vivo cholesterol synthesis rates in all categories of subjects.

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REFERENCES

1. Rittenberg, D., and R. Schoenheimer. 1937. Deuterium as an indicator in the study of intermediary metabolism. XI. Further studies on the biological uptake of deuterium into organic substances, with special reference to fat and cholesterol formation. *J. Biol. Chem.* **121:** 235–253.
2. Dietschy, J. M., and D. K. Spady. 1984. Measurement of rates of cholesterol synthesis using tritiated water. *J. Lipid Res.* **25:** 1469–1476.
3. Hagerman, J. S., and R. G. Gould. 1951. The in vitro interchange of cholesterol between plasma and red cells. *Proc. Soc. Exp. Biol. Med.* **78:** 329–332.
4. Hellman, L., R. Rosenfeld, D. Fukushima, M. Eidinoff, A. Brunschwig, and T. Gallagher. 1953. The major pathways of steroid metabolism in man. *J. Clin. Invest.* **32:** 573–574.
5. London, I. M., and H. Schwarz. 1953. Erythrocyte metabolism. The metabolic behavior of the cholesterol of human erythrocytes. *J. Clin. Invest.* **32:** 1248–1252.
6. Jones, P. J. H., A. M. Scanu, and D. A. Schoeller. 1988. Plasma cholesterol synthesis using deuterated water in humans: effects of short term food restriction. *J. Lab. Clin. Med.* **111:** 627–633.

7. Jones, P. J. H. 1990. Use of deuterated water for measurement of short-term cholesterol synthesis in humans. *Can. J. Physiol. Pharmacol.* **68:** 955–959.
8. Insull, W., D. L. Hachey, W. W. Wong, J. Jiang, B. W. Patterson, L. Clarke, and P. D. Klein. 1989. Acute effects of lovastatin on whole body cholesterol synthesis (WBCS) in man measured by deuterium incorporation. *Arteriosclerosis.* **9:** 741a.
9. Klein, P. D., J. Jiang, W. W. Wong, D. L. Hachey, and W. Insull, Jr. 1990. In vivo demonstration of cholesterol synthesis inhibition by cholesterol feeding in man. *Am. J. Clin. Nutr.* **51:** 516 (abstr).
10. Taylor, C. B., B. Mikkelson, J. A. Anderson, and D. T. Forman. 1966. Human serum cholesterol synthesis measured with the deuterium label. *Arch. Pathol.* **81:** 213–231.
11. Hamilton, J. G., and K. Comai. 1988. Rapid separation of neutral lipids, free fatty acids and polar lipids using prepacked silica Sep-Pak columns. *Lipids.* **23:** 1146–1149.
12. Hamilton, J. G., and K. Comai. 1988. Separation of neutral lipid, free fatty acid and phospholipid classes by normal phase HPLC. *Lipids.* **23:** 1150–1153.
13. Wong, W. W., and P. D. Klein. 1986. A review of techniques for the preparation of biological samples for mass-spectrometric measurements of hydrogen-2/hydrogen-1 and oxygen-18/oxygen-16 isotope ratios. *Mass Spectrom. Rev.* **5:** 313–342.
14. Wong, W. W., L. S. Lee, and P. D. Klein. 1987. Oxygen isotope ratio measurements on carbon dioxide generated by reaction of microliter quantities of biological fluids with guanidine hydrochloride. *Anal. Chem.* **59:** 690–693.
15. Wong, W. W., L. S. Lee, and P. D. Klein. 1987. Deuterium and oxygen-18 measurements on microliter samples of urine, plasma, saliva, and human milk. *Am. J. Clin. Nutr.* **45:** 905–913.
16. Gonfiantini, R. 1984. Report on advisory group meeting on stable isotope reference samples for geochemical and hydrological investigations. Vienna, Austria: International Atomic Energy Agency.
17. Hetzer, H. O., and W. R. Harvey. 1967. Selection for high and low fatness in swine. *J. Anim. Sci.* **26:** 1244–1251.
18. Rose, H. G., and M. Oklander. 1965. Improved procedure for the extraction of lipids from human erythrocytes. *J. Lipid Res.* **6:** 428–431.
19. Klein, P. D. 1966. The occurrence and significance of isotope fractionation during analytical separations of large molecules. *Adv. Chromatogr.* **3:** 3–65.
20. Hachey, D. L., W. W. Wong, T. W. Boutton, and P. D. Klein. 1987. Isotope ratio measurements in nutrition and biomedical research. *Mass Spectrom. Rev.* **6:** 289–328.