Comparison of deuterium incorporation and mass isotopomer distribution analysis for measurement of human cholesterol biosynthesis

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Abstract To compare endogenous cholesterol biosynthesis measured by deuterium incorporation (DI) and mass isotopomer distribution analysis (MIDA), cholesterol fractional and absolute synthetic rates were measured simultaneously by both techniques under identical physiological conditions. Twelve subjects (22 to 39 years of age) underwent a dual stable isotope protocol, involving oral deuterium oxide administration and measurement of incorporation of deuterium into cholesterol coincident with constant infusion of sodium [1-^{13}C]acetate and measurement of the mass isotopomer distribution pattern of newly synthesized cholesterol. Synthesis was determined over 24 h with a 7-h feeding period. Both methods yielded similar measurements of fractional cholesterol synthesis (7.8 ± 2.5% day^{-1} for DI vs. 6.9 ± 2.2% day^{-1} for MIDA). Correlation of fractional synthesis across techniques was strong ($r = 0.84$, $P = 0.0007$). Absolute synthesis rates were also not different at 24 h (13.4 ± 4.3 mg kg^{-1} day^{-1} for DI vs. 11.9 ± 3.6 mg kg^{-1} day^{-1} for MIDA, $r = 0.79$, $P < 0.002$). We conclude that despite different assumptions and analytical requirements, deuterium incorporation and MIDA yield similar rates of cholesterogenesis in humans when measurements are made over 24 h. The decision as to which method to adopt depends on available clinical and analytical facilities.—Di Buono, M., P. J. H. Jones, L. Beaumier, and L. J. Wykes. **Comparison of deuterium incorporation and mass isotopomer distribution analysis for measurement of human cholesterol biosynthesis. *J. Lipid Res.* 2000.** 41: **1516–1523.**

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Evidence is increasing that suppression of endogenous cholesterol synthesis independent of circulating cholesterol concentrations is beneficial in the reduction of cardiovascular disease risk (1–3). Prior to the use of stable isotopes, endogenous cholesterol synthesis could be measured by sterol balance, 3-hydroxy-3-methylglutaryl-CoA reductase activity, cholesterol turnover with [^{14}C]cholesterol, and quantitation of plasma cholesterol precursor levels (4, 5). These methods each possess various limitations, including the requirement for extended measurement periods and/or indirect or overly invasive approaches (4, 5). In contrast, stable isotope methods including deuterium incorporation (DI) and mass isotopomer distribution analysis (MIDA) meet the demand for immediate, direct, and not overly invasive measurement of cholesterol biosynthesis. The fundamental outcome of both methods is the fractional synthesis rate (FSR), which is defined as the fraction of the rapidly turning over cholesterol pool that is newly synthesized from a precursor over a 24-h period. However, each technique relies on different assumptions and thus has its own procedural and theoretical advantages and constraints, as outlined in **Table 1** and detailed in reviews by Jones et al. (4, 5) and Hellerstein and Neese (6, 7).

The fundamental difference in the two techniques lies in their approach to determining the isotopic enrichment of the precursor pool from which cholesterol is synthesized. DI methodology is based on the rate of incorporation of deuterium-labeled water tracer into de novo synthesized cholesterol. The deuterated water tracer equilibrates in total body water and NADPH, that is, a precursor pool from which 22 of the 46 hydrogens in cholesterol derive. Deuterium enrichment of this precursor pool, accessible as plasma water, is analyzed by isotope ratio mass spectrometry (IRMS) (4, 5). Deuterium enrichment of cholesterol is also analyzed by IRMS after isolation and combustion of cholesterol, and reduction to hydrogen gas. In contrast, the MIDA technique is based on incorporation of repeating subunits of acetyl-CoA into the newly synthesized cholesterol poly-

Abbreviations: ASR, absolute synthesis rate; DI, deuterium incorporation; FSR, fractional synthesis rate; GC-MS, gas chromatography-mass spectrometry; HDL, high density lipoprotein; IRMS, isotope ratio mass spectrometry; LDL, low density lipoprotein; MIDA, mass isotopomer distribution analysis; PFB, pentofluorobenzoyl; TLC, thin-layer chromatography.

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TABLE 1. Comparison of two stable isotopic methods of determining human cholesterol synthesis

	Deuterium Incorporation	Mass Isotopomer Distribution Analysis
Tracer	D_2O, inexpensive	[^{13}C]acetate, expensive
Tracer dose	Low	High
Study conditions	Free-living subjects	In-patient
Infusion protocol	Oral bolus with supplemental drinking water over 24 h	24-h intravenous infusion
Sampling protocol	Two blood samples	Serial blood samples requiring indwelling catheter
Sample preparation	Complex multi-step extraction, separation, combustion, and reduction	Routine cholesterol derivatization (pentofluorobenzoyl chloride)
Mass spectrometry	IRMS, high sensitivity, low enrichments	GCMS, low sensitivity, requires high enrichments
Data analysis	Simple mathematical relations	Complex mathematical modeling
Precursor pool	NADPH, total body water	Acetyl-CoA
Determination of precursor enrichment	Plasma water	MID of product
Theoretical issues	Establishing D_{max} (constant labeling of NADPH in different states)	Constant precursor pool labeling and FSR

mer during a constant infusion of ^{13}C-labeled acetate. Fractional cholesterol synthesis is determined from the pattern of excess enrichment among mass isotopomers of de novo synthesized cholesterol. Thus, application of MIDA eliminates the need to measure precursor pool acetyl-CoA enrichment and allows determination of cholesterol synthesis through gas chromatography mass spectrometry (GC-MS) analysis of cholesterol alone and the subsequent application of mathematical modeling (6, 7).

Direct comparisons reveal that DI is in good agreement with the established sterol balance technique in measuring cholesterol synthesis over the long term (8), and with plasma cholesterol precursor concentrations (9, 10) as indices of short-term cholesterogenesis. In general, although DI and MIDA provide similar values for cholesterogenesis in adults (11, 12), the two approaches have never been compared systematically. Because cholesterol synthesis is highly dependent on diet and physiological state (11, 13–16) and each approach relies on different assumptions and constraints, direct intervalidation of the two techniques is warranted. Our objective was thus to compare fractional and absolute endogenous cholesterol biosynthesis using DI and MIDA methodologies simultaneously in human subjects under identical physiological conditions.

MATERIALS AND METHODS

Study design

Twelve subjects underwent a stable isotope infusion protocol designed to measure cholesterol biosynthesis using DI and MIDA simultaneously. Cholesterol synthesis was determined over a 24-h interval with a 7-h feeding period for both methodologies. The study received approval from the Ethics Review Board of the Faculty of Agricultural and Environmental Sciences at McGill University (Montreal, Canada). The aims and procedures of the study as well as potential risks were explained to each subject before obtaining written consent.

Subjects and diets

Subjects (four male, eight female) were judged to be healthy by medical history and physical examination, as well as by a laboratory profile that included fasting circulating lipid concentrations (**Table 2**). Subjects consumed their regular meal at 5:00 PM on day 1 of the study, prior to arriving at the Mary Emily Clinical Nutrition Research Unit on the Macdonald Campus of McGill University. Subjects consumed liquid mixed meals (Ensure™; Ross Laboratories, Montreal, Canada) based on 130% of their resting energy expenditure as determined by the Mifflin et al. predictive equation (17) in equal hourly doses (4.3 ± 0.4 kcal kg^{-1} h^{-1}) between 6:00 AM and 1:00 PM on day 2. The liquid meal contained 30% calories as fat, 45% as glucose, 8% as fructose, and 17% as protein.

Infusions

At 11:00 PM on day 1 (t = 0), a blood sample was drawn to determine baseline water and cholesterol enrichment. Subjects then received an oral bolus of 0.7 g kg^{-1} body water D_2O (Cambridge Isotope Laboratories, Cambridge, MA). Sodium [1-^{13}C]-acetate (99% enriched; Cambridge Isotope Laboratories) was infused at 90 μmol kg^{-1} h^{-1}, using a volumetric infusion pump (IMED, San Diego, CA). Top-up doses of D_2O were given regularly throughout the 24-h protocol to ensure constant body water deuterium enrichment throughout the study (4, 5). At 11:00 PM on day 2, infusion of the labeled acetate was stopped and subjects remained in the Mary Emily Clinical Nutrition Research Unit until 7:00 AM the following morning.

Sampling

The blood-sampling catheter was inserted into a superficial vein on the dorsal surface of the nondominant hand. Blood samples were drawn into EDTA-containing tubes and immediately centrifuged at 1,500 *g* for 10 min. Further blood samples were taken at 15 and 24 h for determination of the cholesterol FSR by DI, and every 2 h for determination of the cholesterol FSR by MIDA. Additional samples were taken after cessation of label administration at 1, 3, 8, 33, and 57 h postinfusion in order to measure the turnover rate of circulating cholesterol. Red blood cells were separated from plasma, and then both were stored at −80°C until processing.

Plasma lipid analysis

Serum cholesterol and lipid concentrations were performed by enzymatic techniques validated by the National Institutes of

TABLE 2. Subject characteristics and plasma lipid concentrations

Subject	Gender	Age	Weight	BMI	Total Cholesterol	HDL-Cholesterol	LDL-Cholesterol	Triglycerides
		yr	*kg*	*kg/m²*		*mmol·L⁻¹*		
1	M	24	95.3	31.2	4.4	1.3	2.9	0.5
2	F	27	72.6	22.9	4.8	2.0	2.6	0.7
3	M	39	73.1	23.1	4.1	1.1	2.3	1.5
4	M	30	84.9	24.1	5.8	1.1	3.7	2.1
5	F	30	50.4	20.5	4.2	1.4	2.3	1.1
6	F	28	49.9	20.8	4.4	0.9	2.7	1.8
7	F	30	49.0	19.9	3.8	1.4	2.2	0.6
8	M	34	93.5	27.9	4.2	1.1	2.2	2.0
9	F	22	53.1	18.8	3.8	1.3	2.2	0.8
10	F	25	51.8	21.6	3.3	1.5	1.5	0.7
11	F	22	49.8	20.8	5.1	0.8	3.4	1.9
12	F	27	57.2	23.3	4.5	1.5	2.3	1.5
Mean ± SD	4 M, 8 F	28 ± 5	65.1 ± 17.2	22.9 ± 3.4	4.4 ± 0.7	1.3 ± 0.3	2.5 ± 0.6	1.3 ± 0.6

Health Lipid Research Clinics. High density lipoprotein (HDL)-cholesterol concentrations were determined after treatment of plasma with dextran sulfate and Mg^{2+} by enzymatic assay (18). Serum low density lipoprotein (LDL)-cholesterol concentrations were calculated from serum total and HDL-cholesterol concentrations, using the equation of Friedewald, Levy, and Frederickson (19).

Cholesterol biosynthesis measurement by deuterium incorporation

The cholesterol FSR by DI was determined as the rate of incorporation of deuterium into erythrocyte membrane free cholesterol over 24 h (5). We have shown previously that the FSR obtained from erythrocyte membrane cholesterol corresponds to the FSR obtained from plasma free cholesterol by the DI method (5, 20). Lipids were extracted by the modified extraction procedure of Folch, Lees, and Sloane Stanley (21) and dried under nitrogen. Free cholesterol was isolated from the extracted lipids, including cholesteryl ester, by thin-layer chromatography (TLC) on silica gel against a free cholesterol standard. Free cholesterol bands were scraped from the TLC plates and the cholesterol was eluted from the silica with hexane–chloroform–diethyl ether 5:2:1 (v/v/v). Free cholesterol was transferred to Pyrex® combustion tubes containing CuO and silver wire. Tubes were subsequently flame sealed under vacuum and cholesterol was combusted to CO_2 and H_2O at 520°C for 4 h. Water resulting from the combustion was cryogenically separated from CO_2 by distillation into Pyrex® tubes containing 50 mg of zinc under vacuum. Tubes were flame sealed under vacuum and the water was reduced at 520°C for 30 min in order to obtain H_2 or HD gas. Deuterium enrichment of the gas was measured on a dual inlet isotope ratio mass spectrometer (IRMS) (VG IsoMass 903D, Cheshire, England). All samples were analyzed in duplicate. Analyses of free cholesterol verified that the enrichment due to deuterium was undetectable by GC-MS, and thus did not confound the MIDA analyses.

Deuterium enrichment in plasma water was measured after dilution of plasma samples with water of known isotopic enrichment to bring the enrichment into the working range of the International Atomic Energy Agency mass spectrometer calibration standards.

The FSR, which represents the fraction of the rapidly turning over free cholesterol pool that is synthesized per day, was calculated as (4, 5)

$$\text{FSR}(\%\ \text{day}^{-1}) = (\delta_{\text{cholesterol}} / \delta_{\text{plasma}}) \times 0.478 \times 24\ \text{h} \times 100\% / t \quad \textit{Eq. 1)}$$

where δ refers to deuterium enrichment of free cholesterol or water above baseline at 24 h, and t refers to the actual measurement period, here 24 h. The factor 0.478 represents the fraction of hydrogen atoms per cholesterol molecule that may become enriched by deuterium, that is, the 22 of 46 hydrogens that derive from H_2O or NADPH (4).

The absolute synthesis rate (ASR) of rapidly exchanging free cholesterol was calculated as its pool size times its fractional synthesis rate (4, 5):

$$\text{ASR}(\text{mg kg}^{-1}\ \text{day}^{-1}) = \text{FSR}(\%\ \text{day}^{-1}) \times M_1/2\ (\text{mg})/\text{body weight (kg)}/100\% \quad \textit{Eq. 2)}$$

$M_1/2$ represents the size of the rapidly exchanging free cholesterol pool, based on the kinetic data of Goodman et al. (22), where the total cholesterol pool $M_1 = 0.287 \times$ body weight (kg) + $0.0358 \times$ total cholesterol concentration (mg dl^{-1}) − $2.40 \times$ TGGP. TGGP is a variable equal to 1, 2, or 3 depending on serum triglyceride concentration (<200, 200–300, or >300 mg dl^{-1}, respectively).

Cholesterol biosynthesis measurement by mass isotopomer distribution analysis

The FSR by MIDA was determined by incorporation of [^{13}C]acetate into de novo synthesized cholesterol over 24 h. Plasma free cholesterol was extracted with a 95% ethanol–acetone 1:1 (v/v) solution, dried under nitrogen, and derivatized to its pentofluorobenzoyl (PFB) derivative by incubating with pentofluorobenzoyl chloride (C_7ClF_5O) and pyridine according to Ostlund et al. (23). The resulting PFB-cholesterol derivative was extracted with petroleum ether, dried under nitrogen, and resuspended in toluene. Triplicate split injections were made on a GC (model 5890; Hewlett Packard, Palo Alto, CA) with a DB-5 capillary column of 0.25-mm i.d., 0.33-μm film thickness, and 10 m in length (J&W Scientific, Folsom, CA). Column temperature was maintained at 250°C for 1 min, then increased at 10°C min^{-1} to 280°C, and maintained at 280°C. Column effluent was admitted to a Hewlett Packard 5988A mass spectrometer operating in negative chemical ion mode with methane reagent gas (1 torr). Ions from m/z 580 corresponding to PFB-cholesterol $[M + 0]^-$ to m/z 584 corresponding to PFB-cholesterol $[M + 4]^-$ were monitored. The mole fractions (MF) 581/(580 + 581 + 582 + 583 + 584), 582/(580 + 581 + 582 + 583 + 584), 583/

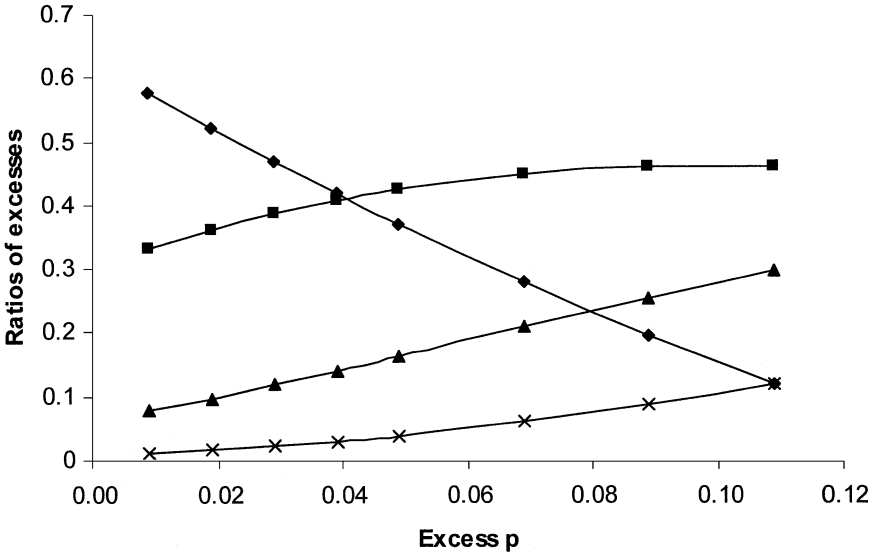

Fig. 1. Ratios among excesses (R_x) versus excess isotopic enrichment of biosynthetic precursor subunit (p) calculated based on probability analysis of theoretical data (see text). $EM_1/\Sigma EM_{1-4}$ (◆) ($R = -4.570p + 0.605$) and $EM_3/\Sigma EM_{1-4}$ (▲) ($R = 2.22p + 0.055$) were used to calculate the actual enrichment of the precursor subunits specific to the pentofluorobenzoyl (PFB) derivative of cholesterol. Also represented are $EM_2/\Sigma EM_{1-4}$ (■) and $EM_4/\Sigma EM_{1-4}$ (×).

(580 + 581 + 582 + 583 + 584), and 584/(580 + 581 + 582 + 583 + 584) were corrected by subtracting the same ratios at baseline in order to obtain molar excesses (EM_{581} to EM_{584}) (12).

The MIDA FSR was determined according to the model of Hellerstein and Neese (12). Briefly, baseline isotopomer frequencies of PFB-cholesterol $[M + x]^-$ were calculated using natural abundances of constituent elements and binomial or polynomial expansion. Using the tracer [1-^{13}C]acetate, C_{max}, the maximum number of ^{13}C atoms that could be incorporated into cholesterol, is 12. The effect of varying excess enrichment of this precursor pool (p) was also calculated with ISOCOM (a generous gift from D. Hachey, Vanderbilt University). As demonstrated previously (12), using ratios among molar excesses in this model eliminates the need to correct for natural abundance. Ratios among molar excesses (R) are determined solely by enrichment of the precursor pool (p) from which they are synthesized. This eliminates the need to isolate or measure the actual precursor molecules. The R of each mass isotopomer of theoretically generated PFB-cholesterol was plotted as a function of p (**Fig. 1**). R_1 ($EM_{581}/\Sigma EM_{581-584}$) and R_3 ($EM_{583}/\Sigma EM_{581-584}$) were chosen to generate equations for p because both functions were linear over the expected range and had a high slope (12). This relation between p and R minimizes the impact of analytical error and of multiple precursor pools if these are present (12). Using these equations, experimentally determined R values were used to determine the enrichment of the precursor pool (p). Estimates of p obtained with R_1 and R_3 were highly correlated ($r = 0.70$, $P = 0.0001$), and therefore an average of R_1 and R_3 was used.

Because of the contribution of dietary cholesterol to the body pool, fractional synthesis can never reach 100%. The asymptote (A*), defined as the theoretical maximum isotopic enrichment of newly synthesized cholesterol expressed as a molar excess, is based on the frequency of each isotopomer of newly synthesized cholesterol for a given p. A* was calculated from molar excesses of theoretically generated PFB-cholesterol (**Fig. 2**). Experimentally, the m + 1 isotopomer was used to calculate A* from the value of p that was determined by equations in Fig. 1. This function (E_1A^*) is relatively flat over a wide range of p values, and therefore if there is concern that precursor enrichment is not constant, then selecting such a relationship between A* and p will minimize the effect of varying p on A* (12). The fraction of cholesterol molecules that were newly synthesized over the infusion was calculated as the ratio of molar excess of m+1 cholesterol (EM_1) to E_1A^* (12):

$$FSR(\%\ day^{-1}) = EM_1 / E_1A^* \times 24\ h / t \qquad Eq.\ 3)$$

where $t = 24$ h.

The ASR was determined as for DI by equation 2.

Statistical analyses

Data were tested for normality before using paired *t*-tests to compare FSR and ASR values obtained by DI with those obtained

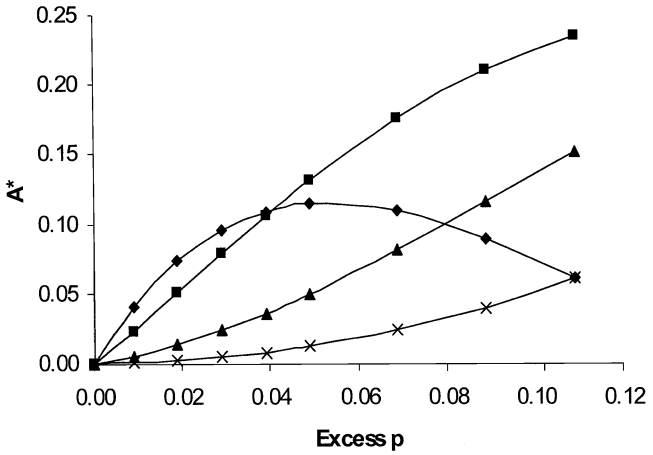

Fig. 2. Asymptote (A*) versus excess p. The asymptote represents the theoretical maximum isotopic enrichment of new molecules expressed as molar excesses ($MF_X/\Sigma MF_{1-4}$). Calculation of the expected frequency of newly synthesized molecules of the PFB derivative of cholesterol was based on the excess mole fraction of m + 1 cholesterol species ($MF_1/\Sigma MF_{1-4}$) (◆) ($A^* = -29.27p^2 + 3.597p + 0.009$). Also represented are $MF_2/\Sigma MF_{1-4}$ (■), $MF_3/\Sigma MF_{1-4}$ (▲), and $MF_4/\Sigma MF_{1-4}$ (×).

TABLE 3. Individual and mean fractional and absolute synthesis rates obtained by deuterium incorporation and mass isotopomer distribution analysis

Subject	FSR		ASR	
	DI	MIDA	DI	MIDA
	% day^{-1}		*mg kg^{-1} day^{-1}*	
1	5.3	4.6	8.7	7.6
2	5.5	5.0	9.4	8.5
3	5,7	4.4	9.4	7.3
4	6.5	6.7	11.5	11.9
5	11.2	6.9	19.8	12.2
6	5.5	6.0	9.8	10.7
7	9.5	10.0	16.4	17.3
8	13.0	11.5	21.1	18.7
9	6.8	5.7	11.5	9.6
10	7.5	8.3	12.4	13.7
11	9.4	7.7	17.8	14.6
12	7.1	5.9	12.5	10.4
Mean ± SD	7.8 ± 2.5	6.9 ± 2.2	13.4 ± 4.3	11.9 ± 3.6

No significant differences were detected between methods for either FSR or ASR ($P > 0.05$).

by MIDA. Correlations between DI- and MIDA-derived indices of synthesis were performed by linear regression. The graphical approach of Bland and Altman (24) was used to plot the difference in FSR as a function of the mean of the two measurements for each subject in order to examine systematic bias. Data were analyzed by SAS (version 6.12; SAS Institute, Cary, NC) and reported as means ± SD. $P < 0.05$ was required for significance.

RESULTS

Subjects tolerated the protocol without reported adverse gastrointestinal or other complaints. Anthropometric and plasma lipid data are provided in Table 2. None of the subjects was hyperlipidemic. Deuterium enrichment of body water remained constant between 15 h (3,958 ± 301‰) and 24 h (3,942 ± 488‰). The MIDA curves used to calculate the isotope content of biosynthetic precursor subunits (p), and the asymptote (A*) representing the theoretical maximum isotopic content of new cholesterol molecules, are depicted in Figs. 1 and 2. These standard curves are consistent with those presented by Hellerstein and Neese for the trimethylsilyl (TMS) derivative of cholesterol (12); however, the PFB derivative has the advantage that the ion analyzed derives only from cholesterol and not the derivatizing agent.

The FSR measured by DI at 24 h was not different from that measured by MIDA (7.8 ± 2.5% day^{-1} vs. 6.9 ± 2.2% day^{-1}) (**Table 3**); however, the correlation was strong ($r = 0.84$, $P < 0.001$) (**Fig. 3A**). The Bland-Altman graph (Fig. 3B) shows an absence of systematic bias. Similarly, the ASR by DI was not different from that by MIDA (13.4 ± 4.3 mg kg^{-1} day^{-1} vs. 11.9 ± 3.6 mg kg^{-1} day^{-1}), and a similar strong correlation was obtained ($r = 0.83$, $P < 0.001$).

DISCUSSION

Despite the emergence of stable isotope techniques for the measurement of in vivo cholesterol biosynthesis in humans (4, 5–7, 12, 25), no systematic cross-comparison has been conducted. Presently, cholesterol synthesis rates by DI and MIDA were simultaneously compared under identical conditions. Over 24 h, DI and MIDA methods yield values for the measurement of human endogenous cholesterol biosynthesis that do not differ and that correlate strongly in normolipidemic subjects. Absolute synthesis indices from each method also corresponded strongly, with estimates of absolute synthesis obtained by DI and MIDA consistent with results previously presented for both methods (typically 11 ± 2 mg kg^{-1} day^{-1} by DI vs. 9 ± 2 mg kg^{-1} day^{-1} by MIDA) (13–16). Closer examination of the FSR comparison shows one subject for whom the difference in FSR between measurements was greater than the 95% confidence limit. When this outlier [indicated by an asterisk (*) in Fig. 3A and B] was removed, then correlation between measurements increased to $r = 0.92$, $P = 0.00005$. Given the narrow physiologic range of cholesterogenesis exhibited in this cohort of normocholesterolemic subjects, this correlation takes on added significance. It is likely that the correlation between methods over a more broad physiologic range would be even stron-

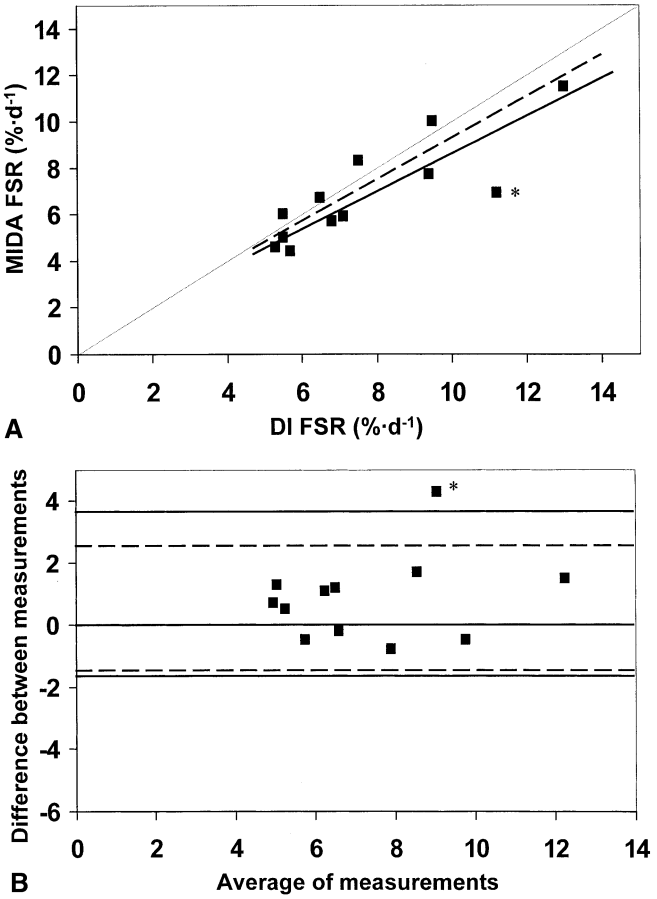

Fig. 3. Fractional synthesis of cholesterol measured at 24 h by deuterium incorporation and MIDA. Correspondence of the two methods expressed as (A) linear regression (—) of FSR, and (B) a Bland-Altman plot, where the difference is plotted as a function of the mean of the two measurements for each subject. When the outlier (*) is removed, linear regression (----) improves to $r = 0.92$, $P < 0.00005$.

ger than that demonstrated here, thus strengthening the agreement between DI and MIDA.

Our primary purpose was to assess cholesterol synthesis over 24 h because of the well-established diurnal rhythmicity (26) and effect of meal timing (27) on cholesterogenesis. However, because the MIDA technique is typically applied over 15 h, and DI studies are typically conducted over 24 h, we took additional blood samples to determine the effect of duration of study. When the two methods in their originally conceived forms are compared, the FSR by DI was greater than the FSR by MIDA (7.8 ± 2.5 vs. 6.3 ± 1.8% day^{-1}, $P < 0.01$) and the two outcomes were correlated ($r = 0.77$, $P < 0.005$). This correlation disappears when the measurement period for both methods is restricted to 15 h ($r = 0.22$, $P = 0.49$), underscoring the importance of 24-h studies to account for circadian effect on cholesterol synthesis.

Analytical constraints of each technique dictated different approaches to measuring isotopic enrichment of free cholesterol in the rapidly exchanging pool. Plasma free cholesterol was derivatized for GC-MS analysis. Because the reaction requires a free hydroxyl group, this analysis is not vulnerable to contamination by cholesteryl ester. In contrast, because IRMS analysis is performed on hydrogen gas derived from combustion, it is important that the free cholesterol isolated by thin-layer chromatography be pure. Cholesterol in erythrocyte membranes is much less vulnerable to contamination by cholesteryl ester than is plasma cholesterol. Previous analysis has shown that plasma and erythrocyte membrane free cholesterol are in isotopic equilibrium (5).

In general, two related difficulties are encountered in the application of the precursor-product relationship: definition of the precise precursor pool from which the product is synthesized and accessibility to measure its enrichment. The complexity of cholesterol synthesis makes definition, isolation, and analysis of the precursor pool particularly problematic. Here the DI and MIDA techniques differ fundamentally in their approach. With each technique, a labeled substrate is introduced into a specific precursor pool, and its incorporation into de novo synthesized cholesterol is then quantified. In the DI technique, deuterated tracer water equilibrates among the intracellular precursors NADPH, and water. Plasma water is a homogeneous representative pool that is accessible for sampling. In contrast, MIDA determines the isotopic content of both product and precursor based on the MID of the product, thus eliminating the need for sampling the acetyl-CoA precursor pool.

Theoretically, the major concern with DI lies in establishing D_{max}, which represents the maximum number of deuterium atoms that can be incorporated into newly synthesized cholesterol. Deuterium can originate from only three sources: 7 directly from water, 15 from NADPH, and 24 from cytosolic acetyl-CoA. Over periods as long as 48 h, protons that derive from water and NADPH are in equilibration with total body water, whereas those originating from acetyl-CoA are unlabeled, thereby limiting D_{max} to 22 of the total 46 hydrogens in cholesterol (4, 5). Beyond 48 h, the acetyl-CoA pool may become labeled due to label recycling and thus contribute to the enrichment of cholesterol (4, 5). D_{max} has been calculated to be as high as 27 (28) or 30 (29) by different methods in rats exposed to deuterium in their drinking water over 1 week. Over the long term, such factors as dietary fat type (30) and ethanol ingestion (31) may also influence D_{max} through changes in NADPH equilibrium (4, 5). D_{max} cannot be determined directly in humans because of the slow turnover of the cholesterol pool, adverse effects of high doses of deuterated water, and the relative insensitivity of GC-MS. Therefore, although the effects of changing metabolic conditions remain to be defined fully, D_{max} is considered to be 22 for studies of cholesterol synthesis over 24 h.

One principal advantage of MIDA is that the enrichment of the precursor pool at the precise site of synthesis is determined uniquely or "fingerprinted" by the pattern of excess enrichments among mass isotopomers of the product, thus eliminating the need to sample the precursor. Theoretically if precursor enrichment is not constant over time or varies at different sites of synthesis, the labeling pattern of the product will be affected, as will estimates of p and FSR. Reviews have assessed the impact of precursor pool nonhomogeneity in the broader context of MID analysis of intermediary metabolism (7, 32). Puchowicz et al. (33) showed in dogs infused with [1,2-^{13}C]-acetate via transhepatic catheters that the enrichment of acetate decreases markedly across the liver due to hepatic production of unlabeled acetate from acetyl-CoA. This may support the existence of multiple acetyl-CoA pools; however, precisely which of these pools would serve as the precursor of cholesterol is unknown. Higher mass isotopomers can become more highly enriched in cultured cells than in vivo, thereby allowing more rigorous analysis of the MID pattern of cholesterol. Kelleher et al. (34) measured cholesterol synthesis by isotopomer spectral analysis, a method based on principles similar to those of MIDA, using high concentrations of [1-^{13}C]acetate in cell culture. The Kelleher et al. analysis showed the MID pattern to be inconsistent with an acetyl-CoA pool of constant enrichment or a constant FSR. Nonetheless, fractional synthesis in the cultured cells did approach 100%, as would be expected. The potential impact of these uncertainties about precursor pools can be minimized by several modeling approaches as discussed by Hellerstein and Neese (7, 12) and as was applied in the present study. Selecting an as near to linear relation between p and R as possible when calculating p from R can minimize the impact of multiple precursor pools. Furthermore, to address the concern that precursor pool enrichment is not constant, selecting a relation between A* and p such that A* is relatively flat over a wide range of p values minimizes the effect of changing precursor pool enrichment. The high correlation of estimates of p obtained from high and low mass ratios (R_3 and R_1) in this study minimizes the practical impact of potential precursor pool nonhomogeneity on calculated cholesterol synthesis.

The fundamental measurement of the FSR can be ex-

TABLE 4. Applications of deuterium incorporation and mass isotopomer distribution analysis in the study of cholesterol biosynthesis in vivo

	Subjects	Summary
Deuterium incorporation		
Jones et al. 1988 (13)	Normal males	Synthesis is sensitive to short term food restriction
Jones et al. 1993 (14)	Normal males	Synthesis is sensitive to changes in meal frequency
Jones et al. 1993 (39)	Normolipidemic adults	ApoE genotype does not influence cholesterogenesis
Cuchel et al. 1997 (35)	Hyperlipidemic males	Declines in plasma lipid levels after Lovastatin treatment are attributable to reduction in synthesis
Mazier and Jones 1997 (30)	Normal males	Synthesis is greater in fed than in fasted state; reduced synthesis is not responsible for effect of different fats on plasma cholesterol concentrations
Jones et al. 1998 (8–10)	Normal adults	Synthesis rates measured by deuterium uptake correspond with those measured by sterol balance and with plasma precursors
Mass isotopomer distribution analysis		
Faix et al. 1993 (15)	Men, menstruating women	Synthesis is 1.5- to 3-fold higher at night and does not vary over the menstrual cycle
Neese et al. 1993 (16)	Rats and humans	Absolute synthesis in women is 568 ± 55 mg/day; women exhibit a diurnal variation
Empen et al. 1997 (36)	Normal adults	Contribution of de novo cholesterol synthesis to bile exceeds that to plasma but is minor in humans
Bjorkhem et al. 1997 (37)	Rats	Hydroxylation facilitates cholesterol transfer across the blood-brain barrier and may be critical for cholesterol homeostasis in the brain
Badsma et al. 1998 (38)	Rats	Contribution of newly synthesized cholesterol is higher to biliary cholesterol than to plasma cholesterol

tended to an absolute synthesis rate by a variety of approaches. We calculated the ASR for both techniques on the basis of the appearance of label in the rapidly exchanging cholesterol pool multiplied by the size of the pool. This approach, also commonly used to calculate plasma protein kinetics, is restricted to the initial monoexponential phase of label incorporation (a condition satisfied in this study). Alternatively, the rate constant of decay of higher mass isotopomers after termination of acetate infusion has been described as another approach to ASR calculation (12). In this study, the decay approach gave ASR values only 10% different from the label incorporation approach (10.7 ± 3.5 mg kg^{-1} day^{-1} for decay vs. 11.9 ± 3.6 mg kg^{-1} day^{-1} for label incorporation). Calculation of the ASR is also dependent on how cholesterol pool size is estimated. The rapidly exchanging cholesterol pool is defined as half the M1 pool, as calculated from the equation of Goodman et al. (22) using serum lipid levels. In this study of normocholesterolemic subjects, cholesterol pool size ranged from 162 to 190 mg/kg, averaging 172 ± 8 mg/kg. Although the existence of an intermediate turning over pool, and a slow turning over pool has been confirmed (22), they do not contribute to the cholesterol FSR over 24 h (4, 5). Both plasma and erythrocyte free cholesterol originate from the rapidly turning over pool described by Goodman et al. (22). Other factors have been used to estimate pool size (12); however, regardless of the approach to calculate the ASR, the fundamental outcome remains the fractional rate of synthesis.

Both deuterium incorporation (5, 10, 13, 14, 30, 35) and MIDA (7, 12, 15, 16, 36–38) have been applied to study cholesterol synthesis under various metabolic conditions (**Table 4**). DI has been evaluated against the cholesterol balance technique, which is considered the standard of choice for measurement of synthesis over the long term (8), showing a strong correlation ($r = 0.745$, $P < 0.0001$) between synthesis measured by sterol balance (17.7 ± 1.7 mg kg^{-1} day^{-1}) and DI (15.9 ± 1.2 mg kg^{-1} day^{-1}). DI is also highly correlated with plasma cholesterol precursors (9, 10) as indices of short-term synthesis. This study is the first direct comparison of DI and MIDA. Technically, DI requires a labor-intensive, lengthy, multistaged off-line preparation of cholesterol before analysis by IRMS. The technique has been refined to minimize invasiveness by administering tracer as an oral bolus and incorporating meal feeding, thereby allowing application in free-living subjects. Procedurally, the MIDA technique as applied here requires an indwelling catheter for tracer infusion. We have eliminated the need for a sampling catheter by streamlining the sampling protocol down to a baseline and a 24-h sample. Although cholesterol derivatization is routine and there is no need to access the precursor pool, GC-MS analysis of the distribution of mass isotopomers is exacting, and elaborate mathematical modeling is necessary. Despite the different assumptions, advantages, and caveats of each isotopic technique, they provided similar and highly correlated estimates of fractional and absolute cholesterol synthesis in humans. Measurements of de novo cholesterogenesis are best made over a 24-h period to account for the inherent diurnal rhythmicity in cholesterol synthesis. The choice between DI and MIDA should be based on the specific study and on available clinical and analytical facilities.

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