Validation of deuterium incorporation against sterol balance for measurement of human cholesterol biosynthesis

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Abstract　**To examine the validity of the deuterium (D) incorporation technique for measurement of human cholesterol synthesis rates, D uptake from D_2O into cholesterol was compared to sterol balance in 13 subjects each under three controlled diet settings. Subjects (age 62 ± 3.6 yr, body weight 74 ± 4.0 kg, BMI 27 ± 1.4) consumed weight maintenance diets enriched in either corn oil, beef tallow, or stick corn oil margarine over a 5-week period. During the final week of the study period, subjects were given 1.2 g/ D_2O per kg body water. D enrichment was measured in plasma water and total cholesterol over 24 h. Also, during the final week, dietary intake and fecal elimination rates of cholesterol were assessed over one 6-day period to calculate sterol balance. There was no significant difference (t = 0.858, P = 0.397) between D incorporation into cholesterol (1,183 ± 92 mg/day) and sterol balance (1,316 ± 125 mg/ day). Among diets, net cholesterol biosynthesis measured by D incorporation agreed (r = 0.745, P = 0.0001) with values derived from sterol balance. The degree of association between methods was not influenced by the wide range of fatty acid composition of the diet fat.▪▪ These data demonstrate the utility of the simple, non-restrictive deuterium incorporation method as a reliable means of determining cholesterol biosynthesis in free-living humans.**—Jones, P. J. H., L. M. Ausman, D. H. Croll, J. Y. Feng, E. A. Schaefer, and A. H. Lichtenstein. **Validation of deuterium incorporation against sterol balance for measurement of human cholesterol biosynthesis.** *J. Lipid Res.* 1998. **39:** 1111–1117.

Supplementary key words cholesterol • biosynthesis • deuterium • sterol balance • diet fat • human • polyunsaturated fat • saturated fat • *trans* fatty acid

Cholesterol biosynthesis contributes substantially to whole body cholesterol pools (1); however, its regulation has been difficult to study in humans, in part due to methodological limitation. Techniques for measurement of cholesterol synthesis in humans include sterol balance, i.e., difference between dietary intake and fecal excretion (2–12), cholesterol precursor level assessment (12–19), as well as in vitro (19, 20) and in vivo (21–40) tracer incorporation approaches. More recent tracer approaches, namely deuterium (D) incorporation (32, 33, 35–40) and mass isotopomer distribution analysis (MIDA) (25–27), have been suggested as possessing advantages of accuracy and ease of use compared with other existing methods (25). Deuterium incorporation is less invasive and disruptive than MIDA for both the study subject and investigator. However, this approach has not been systematically and simultaneously evaluated against the classic sterol balance. It is important to know whether newer tracer techniques for determining cholesterol synthesis rate are accurate from the standpoint of their appropriateness for application to studying factors known to influence human cholesterol metabolism. In particular, whether the techniques agree under differing dietary situations is important information.

The purpose of the present study, therefore, was to compare D incorporation for measuring cholesterol synthesis against the classic cholesterol balance technique in human subjects consuming controlled diets differing in fatty acid composition that result in different blood cholesterol levels. It was hypothesized that there would be no difference between these methods of cholesterogenesis measurement among dietary periods where the relative composition of the fat intake varies.

METHODS

Subjects

Healthy volunteers (7 females, 6 males, mean age 62 yrs, age range 44–74 yrs) with low density lipoprotein (LDL) cholesterol levels higher than 3.37 mmol/L were screened for presence of hepatic, renal, and cardiac dysfunction before admission to the

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study. Subjects were non-smokers and were not taking lipid-lowering drugs, beta blockers, diuretics, or hormones. Females were post-menopausal. The protocol was approved by the Human Investigation Review Committee of New England Medical Center and Tufts University.

Protocol

Each subject underwent three 32-day dietary periods. Subjects were provided with experimental solid food diets containing 15 and 30% of kcal as protein and fat, respectively, and 80 mg cholesterol/1000 kcal. Two-thirds of the dietary fat was derived either from corn oil, beef tallow, or stick corn oil margarine, allocated in semi-randomized order using a double-blinded study design. Fatty acid and non-saponifiable lipid compositions of the diets are shown in Table 1.

In the Metabolic Research Unit of the Jean Mayer USDA Human Nutrition Research Center on Aging, foods and beverages were prepared for consumption on site or for take-out. Subjects reported to the unit on at least three occasions per week to obtain meals. During the final week of each dietary phase, three fasting blood samples were obtained for lipid level determinations. Caloric intakes of subjects were tailored to individual requirements, as verified by ability to maintain body weight. When necessary, adjustments to caloric intakes were made during the initial 10-day period of each dietary phase.

During week 5 of each study phase, D uptake was measured over 24 h after administration of 1.2 g deuterium oxide (D_2O) per kg body water, estimated as 60% of body weight. Blood samples were collected just prior to, and 24 h after, D_2O dosing for plasma total cholesterol and water D enrichment measurement. Dietary cholesterol intake and fecal excretion rates were determined over 6 days during wk 5 of each dietary trial to quantify cholesterol synthesis measurement by sterol balance.

Plasma lipid levels

Fasting blood samples from week 5 were collected in tubes containing EDTA (0.1%). Plasma was separated and assayed for total cholesterol level using enzymatic procedures (41).

Cholesterol synthesis determination using deuterium incorporation

Methods have been reported in detail previously (36, 38–40). Briefly, lipids were extracted from 2–3 ml plasma in duplicate. Extracts were saponified, then dissolved in chloroform and separated on thin-layer silica (TLC) gel plates (20 × 20 cm, 250 mm, Whatman Inc, Clifton, NJ). TLC plates were developed in petroleum ether–diethyl ether–acetic acid 135:15:1.5 (v/v/v) and air dried. Lipid fractions were visualized; free cholesterol was then removed from the plate and eluted from the silica using hexane–chloroform–diethyl ether 5:2:1 (v/v/v). Extracts containing cholesterol were transferred to Pyrex combustion tubes containing cupric oxide and silver wire. Tubes were sealed under vacuum after removal of solvent, then placed at 520°C for combustion of the cholesterol. Combustion product water was transferred by vacuum distillation into a second tube containing zinc reagent.

Plasma water samples were distilled into Pyrex tubes containing zinc and sealed under vacuum. Water derived from both cholesterol and plasma was reduced at 520°C before analysis of product hydrogen gas D enrichment by isotope ratio mass spectrometry (IRMS) (VG Isomass 903D, Cheshire, UK). Mean internal and external precision (SD) levels of the IRMS were 0.18 and 2.0 per mil ($^0/_{00}$), respectively. The sample H_3^+ contribution was corrected for electronically. The instrument was calibrated using water standards of known isotopic composition. Samples for each subject were analyzed concurrently using a single set of standards.

Cholesterol fractional synthesis rates (FSR) were determined as incorporation of precursor D into plasma total cholesterol relative to the maximum theoretical enrichment using the linear regression model. Model considerations have been described previously (40). As the central, rapid turnover pool requires months to attain plateau D enrichment in humans (32), the initial 24 h uptake rate closely represents the initial turnover value. Assuming that the value of 0.81 was the ratio of D atoms per carbon atom, maximum attainable enrichment was calculated as the body water pool enrichment corrected for the fraction of protons in de novo synthesized cholesterol that derive from water, relative to non-water sources (34, 42).

Non-saponifiable lipid contents

Non-saponifiable lipids of the diets were analyzed after lipid extraction, saponification with KOH, and TLC separation. Bands containing the non-saponifiable lipid components were scraped from TLC, eluted, and methylated using trimethylsiloxane reagents (41). Levels were measured using gas–liquid chromatography (Hewlett Packard model 5890, Palo Alto, CA) with a 30 m × 0.25 mm i.d. dimethyl polysiloxane column (Restek Corp., Mississauga, Ont). Sitosterol, campesterol, and other peaks were identified using authentic standards, and quantitated by comparison with alpha-5-cholestane internal standard.

Cholesterol synthesis determination using balance

For each diet, 3-day food aliquots were analyzed for cholesterol content (Hazelton Laboratory, Madison, WI). To obtain total daily cholesterol intake, the daily calorie intake of each person was multiplied by the cholesterol content of the diet per calorie. In addition, single 3-day aliquots for each diet were analyzed for β-sitosterol, campesterol, and stigmasterol. Food samples (100 mg) in duplicate were extracted three times in 10 ml chloroform–methanol 2:1 (v/v), centrifuged (3,000 rpm), and the supernatants were pooled. KCl (7.5 ml) was added to each 30 ml pooled extractant and the mixture was shaken vigorously. The chloroform layer was evaporated under N_2. Samples were saponified by adding 50% KOH and 95% ethanol and incubating for 1 h at 80°C. After cooling in ice, H_2O was added to each sample tube which was then extracted with hexane. The pooled hexane was washed with H_2O and the hexane layer was evaporated under N_2 and stored at −70°C for further analysis. Food plant sterol levels were analyzed as described for fecal sterol analysis using gas chromatography and the resultant values were used for all calculations.

Subjects were instructed to collect complete stool specimens for 3-day periods. Entire specimens were placed in individual plastic bags immediately after defecation, frozen on dry ice, and held at −70°C until analysis. Frozen samples were weighed, lyophilized to dryness, and reweighed. The dry samples were ground to a fine powder, mixed together in a food processor, and duplicate aliquots were taken for analysis of fecal neutral and acidic sterols.

Fecal neutral sterols (NS) and bile acids (BA) were extracted and isolated according to the method of Ausman et al. (43) using duplicate 200 mg fecal sample aliquots and 5α-cholestane as a recovery standard for the neutral sterols. Fecal NS extractants were dissolved in hexane, then separated and quantified by gas–liquid chromatography (Autosystem, Perkin Elmer, Norwalk, CT) using a capillary column (RT$_X$®–225, 15 meter, 0.25 mm ID, 0.25 μm film thickness, Restek Corporation, Bellefonte, PA). Samples were analyzed under the following conditions: 1.0 μl NS sample in hexane was injected into the column at 220°C oven temperature. After remaining at this temperature for 1

min, the oven temperature was increased to 238°C (20°C/min), held for 1.0 min, and further increased to 248°C (0.7°C/min). The injector and detector were set at 250° and 252°C, respectively. The carrier gas (helium) flow was 20 psi with the inlet splitter set at 4:1. Standards used were cholesterol, cholestanol, coprostanol, and coprostanone. Peak identification and purity of peaks were confirmed by GC–MS analysis. The average recovery of the 5α-cholestane internal standards in these experiments was 75.6%.

Fecal BA were quantified as described by Setchell et al. (44) with the following modifications. Dried and methylated BA extracts were silylated with Tri-sil, containing 77% pyridine, 15.4% hexamethyldisilazane, and 7.6% trimethylchlorosilane (Pierce Chemical Company, Rockford, IL). Samples were dried under nitrogen, resuspended in hexane, and centrifuged at 300 rpm for 5 min to settle the precipitates. Fecal BA were separated and quantified by GC using a capillary column ($RT_X^®$–1, 30 meter, 0.25 mm ID, 0.25 μm film thickness, Restek Corporation) under the following conditions: 1.0 μl derivatized BA sample was injected into the column at 200°C oven temperature. After a 0.5 min hold period, the temperature was gradually increased to 236°C (10°C/min), then increased to 246°C (0.4°C/min), and then immediately increased to 256°C (0.2°C/min) and held for 1.0 min. The injector and detector were both set at 280°C. The helium carrier gas flow was 16.4 ml/min with the inlet splitter set at 5:1. Standards included lithocholic, deoxycholic, chenodeoxycholic, ursodexoycholic, and cholic acids. Recovery of BA was 71%, determined on the basis of lithocholic acid recovery from fecal samples.

Statistical analyses

Statistical Analysis System version 6.08 (SAS Institute Inc., Cary, NC) was used for statistical analysis. Effects of method of measurement on synthetic rates were assessed separately in each diet using paired *t*-tests. Two-way ANOVA for repeated measures (Proc GLM), followed by Tukey's *t*-tests, was used to assess the effects of different diets and methods on the resulting cholesterol synthesis values. Correlation statistics were used to measure the relation between D incorporation-derived indices of synthesis and those from sterol balance. In addition, agreement between methods was assessed using a graphical approach developed by Bland and Altman (45). Results are expressed as mean ± SEM.

RESULTS

The fatty acid composition and non-saponifiable lipid contents of the three diets are compared in **Table 1**. The corn oil diet was relatively rich in linoleic acid, while the beef tallow diet contained higher levels of oleic and stearic acids. The corn oil-based margarine diet was intermediate with respect to saturated and polyunsaturated fatty acid content. In addition, the dietary intake of cholesterol and plant sterols is shown in Table 1. Dietary β-sitosterol, campesterol, and stigmasterol were 2- to 2.5-fold higher in the corn oil diet than the beef tallow diet, with intermediate levels in the margarine diet. Similar differences occurred in actual amount of sterol consumed per kilogram body weight.

Subject age averaged 62 ± 3.6 years. Mean body weight, height, and body mass index (BMI) were 74 ± 4.0 kg, 167 ± 2.8 cm, and 27 ± 1.4 kg/m², respectively. After consumption of corn oil, beef tallow, and corn oil margarine enriched diets, plasma total cholesterol levels were 5.01 ± 0.13, 5.64 ± 0.20, and 5.31 ± 0.15 mmol/L, respectively (f = 3.57, $P = 0.04$). Among them, the plasma total cholesterol

TABLE 1. Composition of the diet and sterol intakes

Variable	Corn Oil	Beef Tallow	Margarine
Protein (% calories)	17.4 ± 0.8	16.1 ± 0.2	16.4 ± 1.6
Carbohydrate (% calories)	53.0 ± 1.9	51.2 ± 1.6	53.8 ± 2.5
Fat (% calories)	29.6 ± 1.1	32.7 ± 1.1	29.7 ± 1.13
SFAs (% calories)	6.41 ± 1.45	13.69 ± 0.58	7.73 ± 0.13
C12:0	0.09 ± 0.01	0.06 ± 0.05	0.07 ± 0.01
C14:0	0.39 ± 0.06	0.87 ± 0.02	0.32 ± 0.01
C16:0	3.86 ± 1.11	8.39 ± 0.32	4.18 ± 0.09
C18:0	1.57 ± 0.25	3.75 ± 0.27	2.00 ± 0.02
MUFAs (% calories)	10.71 ± 2.36	12.07 ± 0.39	12.16 ± 0.25
C16:1n–7	0.30 ± 0.07	0.85 ± 0.07	0.23 ± 0.01
C18:1n–9	10.06 ± 2.33	10.61 ± 0.36	11.65 ± 0.24
PUFAs (% calories)	9.45 ± 2.55	2.62 ± 0.35	8.30 ± 0.16
C18:2n–6	8.48 ± 3.28	2.44 ± 0.28	7.85 ± 0.18
C18:3n–3	0.83 ± 0.74	0.23 ± 0.04	0.35 ± 0.01
C20:4n–6	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
Cholesterol[a] mg/1000 kcal	83 ± 7	109 ± 12	77 ± 9
(mg/kg·day)	(3.11 ± 0.37)	(3.99 ± 0.47)	(2.82 ± 0.33)
β-sitosterol[a] mg/1000 kcal	179	70	142
(mg/kg·day)	(6.70 ± 0.79)	(2.55 ± 0.30)	(5.21 ± 0.61)
Campesterol[a] mg/1000 kcal	56	28	53
(mg/kg·day)	(2.10 ± 0.25)	(1.04 ± 0.12)	(1.93 ± 0.23)
Stigmasterol[a] mg/1000 kcal	21	12	22
(mg/kg·day)	(0.80 ± 0.09)	(0.43 ± 0.05)	(0.71 ± 0.09)

Values are mean ± SD.
SFAs, saturated fatty acids; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids.
[a]Duplicate determinations for one 3-day menu cycle for each diet.

TABLE 2. Cholesterol biosynthesis data with different methods and diets

Subject	Corn Oil		Beef Tallow		Margarine	
	STEBAL	D$_2$	STEBAL	D$_2$	STEBAL	D$_2$
	mg/day		*mg/day*		*mg/day*	
1	532	—	428	1181	380	876
2	2316	2032	2001	1789	1629	1598
3	186	561	272	910	357	695
4	2425	2383	1982	1614	2782	1477
5	711	812	697	725	621	—
6	858	—	887	1069	1333	1239
7	2690	1888	1761	978	1703	1311
8	1494	678	641	409	820	523
9	2121	1245	2149	1828	2083	1768
10	2692	2201	1520	1457	2124	2090
11	1017	1499	703	519	552	795
12	644	919	449	770	813	997
13	1889	772	1625	513	1452	479
Mean	1506[a]	1362	1163	1059	1281[b]	1154
±SEM	248	201	191	135	212	148

STEBAL, sterol balance; D$_2$, deuterium incorporation.

[a] Mean ± SEM without subject 1 and 6 for paired comparison is 1653 ± 269; n = 13.

[b] Mean ± SEM without subject 5 for paired comparison is 1336 ± 222; n = 13.

levels after the corn oil diet period were significantly lower ($P < 0.05$) than after the beef tallow and corn oil margarine periods.

Cholesterol biosynthesis data were compared among diets independently and in a pooled fashion. Values were expressed as net production rates per day and production rates were adjusted for subject body weight. During consumption of the corn oil diet, total fecal sterol content for 13 subjects was $1,734 \pm 251$ mg/day, of which 229 ± 13 mg/day originated from diet. Using the paired comparison (n = 11), mean synthesis rate assessed by sterol balance ($1,653 \pm 269$ mg/day) did not vary significantly ($P = 0.118$) from that obtained using D incorporation ($1,362 \pm 201$ mg/day). Individual data are presented in **Table 2**. Similarly, when data from each method were expressed per kg body weight, there was no significant difference ($P = 0.127$) between mean values of synthesis rate determined using sterol balance (22.19 ± 3.29 mg/kg·day) and D incorporation (17.93 ± 1.92 mg/kg·day).

For the beef tallow diet, total fecal sterol content was $1,456 \pm 194$ mg/day, of which 293 ± 17 mg/day originated from diet. Mean synthesis rate assessed by sterol balance ($1,163 \pm 191$ mg/day) did not differ significantly ($P = 0.481$) from that as assessed using D incorporation ($1,059 \pm 135$ mg/day). Individual data are presented in Table 2. When the mean values of synthesis rates expressed per kg body weight were compared, there was no significant difference ($P = 0.504$) between the sterol balance (15.67 ± 2.50 mg/kg·day) and the D incorporation (14.26 ± 1.68 mg/kg·day) methods.

For the margarine diet, total fecal sterol content for 13 subjects was $1,488 \pm 215$ mg/day, of which 207 ± 12 mg/day originated from diet. Using paired comparison, mean synthesis rate assessed by sterol balance was $1,336 \pm 222$ mg/day versus $1,154 \pm 148$ mg/day ($P = 0.258$) deter-

mined using D incorporation. Individual data are shown in Table 2. When biosynthesis rates were expressed per unit body weight, there was no significant difference between mean values ($P = 0.229$) derived from the sterol balance (17.88 ± 2.69 mg/kg·day) and the D incorporation (15.33 ± 1.72 mg/kg·day) methods.

Because there were no significant differences between the two methods of measuring synthesis for any of these diets, data for all subjects for all three diets were pooled in a two-way repeated measures ANOVA. Diet (f = 4.62, $P < 0.0196$) but not method (f = 1.40, $P = 0.259$) was a significant main effect in this analysis. Therefore, the mean value of sterol balance ($1,316 \pm 125$ mg/day) and that of D incorporation ($1,183 \pm 92$ mg/day) were not significantly different. The correlation of values obtained from D incorporation and those from sterol balance across the three diets, expressed as net synthesis, was good ($r = 0.745$, $P = 0.0001$) and is shown in **Fig. 1**. When the data were expressed per kg body weight, there was also no significant difference (f = 1.43, $P = 0.254$) between the mean value of sterol balance (15.74 ± 1.02 mg/kg·day) and that of D incorporation (16.64 ± 1.35 mg/kg·day). The correlation coefficient between methods was 0.651 ($P = 0.0001$).

Figure 2 shows a plot of the differences of the two methods against their mean. The relative bias estimated by the mean difference between sterol balance and D incorporation was -187 mg/day and the 95% limits of agreement, defined as the bias $\pm 2 \times$ SD of the difference, $-1,237$ mg/day and 863 mg/day.

DISCUSSION

Despite the emergence of modern stable isotope methods for measurement of cholesterol biosynthesis in vivo in humans (25, 27, 32, 33, 35–40), their systematic evaluation against the cholesterol balance technique, considered as the standard of choice for measurement of synthesis, has not been previously conducted. We present new evidence indicating that the D incorporation method, although perhaps slightly underestimating synthesis rate across diets, yields values that correspond well to those obtained using sterol balance. These findings indicate that D incorporation may serve as a more easily applicable method for reliable determination of synthesis compared with the greater labor-intensiveness and inconvenience of sterol balance.

One of the chief questions in the present pursuit was whether dietary perturbations might influence the reliability of the D incorporation method. Consumption of polyunsaturated fats is accompanied by a rise in cholesterol synthesis versus certain other dietary fats (37, 38). However, this increase has not always been observed using other methods (9, 10, 46). In particular, Fernandez, Yount, and McNamara (46) showed in guinea pigs that correspondence between cholesterol synthesis measured using sterol balance and tritium incorporation differed between methods in a way that depended on the compo-
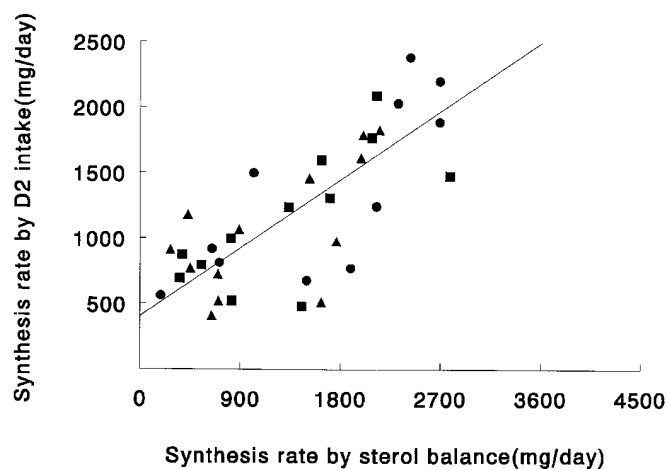
Fig. 1. The correlation of subjects' cholesterol synthesis between sterol balance and deuterium incorporation ($r = 0.745$; $P = 0.0001$; n = 13). The results from each subject are depicted by a different symbol (● corn oil; ▲ beef tallow; ■ corn oil margarine).

sition of dietary fat consumed. Variations in fat oxidation rate were postulated to alter the D/H incorporation ratio and possibly confound the accuracy of the method (42). Fats that are rapidly utilized for energy may thus produce a shift in the D/H ratio relative to a fat oxidized more gradually, which would result in an artifactual shift in synthesis rate. The present results show no systematic shift in the comparison between two methods across three diet fats differing considerably in fatty acid composition. From these data it can be suggested that any influence of fat type on D/H incorporation ratio is small. The present comparison of synthesis across dietary fats showing no significant difference, using either method, further attests to the robustness of the D incorporation technique.

Biosynthesis rates for cholesterol assessed using either method, but particularly sterol balance, were generally
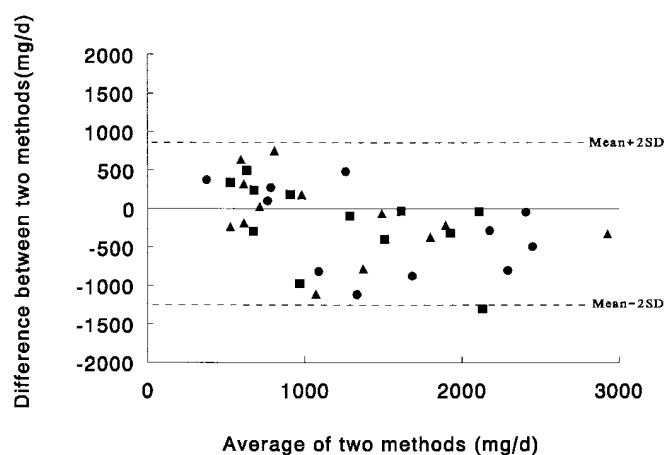
Fig. 2. The differences between sterol balance and deuterium incorporation against mean of the two measurements for each subject's cholesterol synthesis data; (–––), mean difference, upper and lower 95% limits of agreement (2SD) between methods (n = 13). The results from each subject are depicted by a different symbol (● corn oil; ▲ beef tallow; ■ corn oil margarine).

greater than those previously reported using the individual techniques (25–36). The higher values may relate to greater mean age of individuals in this study versus others. The possibility that biosynthesis increases with age is a concept that has not been fully explored in humans. Alternatively, the higher values may be related to the relatively low levels of dietary cholesterol intakes resulting in an increased endogenous synthesis rate.

With each technique, in a few cases we observed cholesterol biosynthesis values below 500 mg/day, which are considered to be unreasonable. However, after omitting values under 500 mg/day for both sterol balance and D incorporation, further correlation analysis between the two techniques still yielded a good agreement ($r = 0.725$, $P = 0.0001$). Bland and Altman (45) suggested that measuring agreement between the methods may be more informative than using associative analysis as potential error can exist even when data produce high correlation. Such an approach is based on examination of the distribution of differences between the results of the two methods which is not clearly apparent using a correlation approach.

Figure 2 shows that D incorporation underestimates cholesterol synthesis, relative to sterol balance, by on average about 200 mg/day. The limits of agreement in Fig. 2 indicate large individual differences between two measurements in some cases. This trend towards underestimation in synthesis measured using D incorporation was fairly consistent across both subjects and diets. This tendency suggests an error in the derivation of fractional or absolute cholesterol biosynthesis rates using D incorporation. This error may result from incorrect assumptions regarding the D/H incorporation ratio, the time required to reach plateau for deuterated water, or the derivation of total cholesterol pool size. Pool size estimation is based on a somewhat subjective principle using anthropometric and circulating lipid level data (23). In addition, it has been postulated that cholesterol biosynthesis in nonhepatic tissues represents a major proportion of total body production. Deuterated cholesterol in deeper pools may not equilibrate instantaneously with the rapid turnover plasma M_1 pool. Thus, the apparent underestimation of the D uptake method could be due to a lag in appearance of label over the 24-h measurement window used in these studies. Perhaps the good agreement between methods observed in the present experiment is because, in the primate, most cholesterol synthesis occurs in those extrahepatic tissues that are rapidly, rather than slowly, exchanging with the circulating free cholesterol pools.

Certain of the error terms observed between the two methods presently compared are also doubtlessly attributable to limitations of cholesterol balance. Although long regarded as the "gold standard" for determining cholesterol synthesis, cholesterol balance possesses potential inherent errors in both intake and excretion components. Endogenous biosynthesis rate is generally expressed as the difference between cholesterol intake and fecal sterol excretion, specifically, cholesterol and bile sterols (2–12). When intake is precisely quantified, as in the present ex-

periment, and fecal excretion is verified, sterol balance will provide an accurate value for biosynthesis once internal sterol pools have equilibrated. In the present study, 5 weeks should be considered as sufficient duration for equilibration of sterol input and output across each diet. However, the reliability of the cholesterol intake component of the balance equation depends on the accuracy of food-recording instruments, which have been shown to underestimate true values (47).

Accuracy of the sterol balance method is also dependent on completeness of stool collections. Gas–liquid chromatography reliability is important to ensure that dietary or fecal plant sterols are not inadvertently identified as cholesterol. In addition, although data from the current study demonstrate reasonable accuracy and precision of D incorporation against cholesterol balance, the two methods measured synthesis over differing time windows. The level of agreement between methods was considered impressive particularly given that D incorporation was carried out during only a short interval immediately after cessation of the sterol balance period.

An alternate method for measuring human cholesterol biosynthesis is MIDA. MIDA enables study of polymer synthesis from repeating monomeric subunits (25–28). Precursor subunits labeled with stable isotope are constantly infused as relative abundances of mass isotopomers of the product are determined using gas chromatography–mass spectrometry. Fractional synthetic rate is assessed from the observed pattern, and concentration of excess isotopomer frequencies in the synthesized polymer in relation to the expected statistical distribution yields the isotopic enrichment of the true precursor pool. Typical net cholesterol synthesis rates of 600 mg/d are obtained using MIDA (25). Although measurement of cholesterol synthesis by MIDA possesses advantages over older techniques, potential drawbacks also exist. Particularly, the large amount of tracer required may perturb the pool size of tracee. Also, a prolonged intravenous infusion protocol is required. Lastly, there is some suggestion that the labeled acetate precursor pool enrichment may vary across subcellular compartments (30, 31).

In contrast, D incorporation does not suffer from these drawbacks. The isotope is given in true tracer quantities orally. Deuterated water is inexpensive, highly available, and safe at the doses provided (48). Theoretical considerations have been extensively reviewed for use of tritium in animals (34, 42), suggesting that the selection of a 24-h measurement interval for cholesterogenesis determination in the free circulatory pool reflects a reasonable representation of the mean of organ synthesis rates. Moreover, preliminary indications suggest successful application of selected ion mass spectrometry for detection of D enrichment in cholesterol (49). Such improvements to the method would reduce the labor-intensiveness and cost associated with the measurement. This improved ease of application, together with the present demonstration of good agreement with a reliable reference technique, should make D uptake a valuable method for investigating effects of physiological and dietary actions on cholesterol biosynthesis.

In summary, present results represent the first report of validation of the deuterium incorporation methodology for measurement of human cholesterogenesis across diets varying chiefly in fatty acid pattern, but not amount. It is concluded that this approach serves as a comparable means of assessing cholesterol synthesis in human studies investigating the influence of dietary factors on cholesterol synthesis.∎

REFERENCES

1. Dietschy, J. M. 1984. Regulation of cholesterol metabolism in man and other species. *Klin. Wochenschr.* **62:** 338–345.
2. Grundy, S. M., and D. W. Bilheimer. 1984. Inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase by mevinolin in familial hypercholesterolemia heterozygotes: effects on cholesterol balance. *Proc. Nat. Acad. Sci. USA.* **81:** 2538–2542.
3. Avigan, J., and D. Steinberg. 1965. Sterol and bile acid excretion in man and the effects of dietary fat. *J. Clin. Invest.* **44:** 1845–1856.
4. Moore, R. B., J. T. Anderson, H. L. Taylor, A. Keys, and I. D. Frantz, Jr. 1968. Effect of dietary fat on the fecal excretion of cholesterol and its degradation products in man. *J. Clin. Invest.* **47:** 1517–1534.
5. Connor, W. E., D. T. Witiak, D. B. Stone, and M. L. Armstrong. 1969. Cholesterol balance and fecal neutral steroid and bile acid excretion in normal men fed dietary fats of different fatty acid composition. *J. Clin. Invest.* **48:** 1363–1375.
6. Grundy, S. M. 1975. Effects of polyunsaturated fats on lipid metabolism in patients with hypertriglyceridemia. *J. Clin. Invest.* **55:** 269–282.
7. Nestel, P. J., N. Havenstein, H. M. Whyte, T. J. Scott, and L. J. Cook. 1973. Lowering of plasma cholesterol and enhanced sterol excretion with the consumption of polyunsaturated ruminants fats. *N. Engl. J. Med.* **288:** 379–382.
8. Nestel, P. J., N. Havenstein, Y. Homma, T. W. Scott, and L. J. Cook. 1975. Increased sterol excretion with polyunsaturated-fat high-cholesterol diets. *Metab. Clin. Exp.* **24:** 189–198.
9. Shepherd, J., C. J. Packard, S. M. Grundy, D. Yeshurun, A. M. Gotto, Jr., and O. D. Taunton. 1980. Effects of saturated and polyunsaturated fat diets on the chemical composition and metabolism of low density lipoproteins in man. *J. Lipid Res.* **21:** 91–99.
10. Grundy, S. M., and E. H. Ahrens, Jr. 1970. The effects of unsaturated dietary fats on absorption, excretion, synthesis, and distribution of cholesterol in man. *J. Clin. Invest.* **49:** 1135–1152.
11. McNamara, D. J., R. Kolb, T. S. Parker, H. Batwin, P. Samuel, C. D. Brown, and E. H. Ahrens, Jr. 1987. Heterogeneity of cholesterol homeostasis in man. Response to changes in dietary fat quality and cholesterol quantity. *J. Clin. Invest.* **79:** 1729–1739.
12. Glatz, J. F., and M. B. Katan. 1993. Dietary saturated fatty acids in-

crease cholesterol synthesis and faecal steroid excretion in healthy men and women. *Eur. J. Clin. Invest.* **23:** 648–655.

13. Pappu, A. S., D. R. Illingworth, and S. Bacon. 1989. Reduction in plasma low-density lipoprotein cholesterol and urinary mevalonic acid by lovastatin in patients with heterozygous familial hypercholesterolemia. *Metab. Clin. Exp.* **38:** 542–549.

14. Pappu, A. S., and D. R. Illingworth. 1989. Contrasting effects of lovastatin and cholestyramine on low-density lipoprotein cholesterol and 24-hour urinary mevalonate excretion in patients with heterozygous familial hypercholesterolemia. *J. Lab. Clin. Med.* **114:** 554–562.

15. Popjak, G., G. Boehm, T. S. Parker, J. Edmond, P. A. Edwards, and A. M. Fogelman. 1979. Determination of mevalonate in blood plasma in man and rat. Mevalonate "tolerance" tests in man. *J. Lipid Res.* **20:** 716–728.

16. Parker, T. S., D. J. McNamara, C. D. Brown, R. Rolb, E. H. Ahrens, Jr., A. W. Alberts, J. Tobert, J. Chen, and P. J. DeSchepper. 1984. Plasma mevalonate as a measure of cholesterol synthesis in man. *J. Clin. Invest.* **74:** 795–804.

17. Parker, T. S., D. J. McNamara, C. Brown, O. Garrigan, R. Kolb, H. Batwin, and E. H. Ahrens, Jr. 1982. Mevalonic acid in human plasma: relationship of concentration and circadian rhythm to cholesterol synthesis rates in man. *Proc. Natl. Acad. Sci. USA.* **79:** 3037–3041.

18. Miettinen, T. A. 1982. Diurnal variation of cholesterol precursors squalene and methyl sterols in human plasma lipoproteins. *J. Lipid Res.* **23:** 466–473.

19. Bjorkhem, I., T. Miettinen, E. Reihner, S. Ewerth, B. Angelin, and K. Einarsson. 1987. Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. *J. Lipid Res.* **28:** 1137–1143.

20. Carulli, N., A. Tripodi, and F. Carubbi. 1989. Assay of HMG-CoA reductase activity in the evaluation of cholesterol synthesis in man. *Clin. Chim. Acta.* **183:** 77–81.

21. Goodman, D. S., and R. P. Noble. 1968. Turnover of plasma cholesterol in man. *J. Clin. Invest.* **47:** 231–241.

22. Ferezou, J., J. Rautureau, T. Coste, E. Gouffier, and F. Chevallier. 1982. Cholesterol turnover in human plasma lipoproteins: studies with stable and radioactive isotopes. *Am. J. Clin. Nutr.* **36:** 235–244.

23. Goodman, D. S., F. R. Smith, A. H. Seplowitz, R. Ramakrishnan, and R. B. Dell. 1980. Prediction of the parameters of whole body cholesterol metabolism in humans. *J. Lipid Res.* **21:** 699–713.

24. Schwartz, C. C., L. A. Zech, J. M. VandenBroek, and P. S. Cooper. 1993. Cholesterol kinetics in subjects with bile fistula. Positive relationship between size of the bile acid precursor pool and bile acid synthetic rate. *J. Clin. Invest.* **91:** 923–938.

25. Hellerstein, M. K. 1995. Methods for measurement of fatty acid and cholesterol metabolism. *Curr. Opin. Lipidol.* **6:** 172–181.

26. Lee, W. N., S. Bassilian, Z. Guo, D. Schoeller, J. Edmond, and E. A. Bergner. 1994. Measurement of fractional lipid synthesis using deuterated water (^2H_2O) and mass isotopomer analysis. *Am. J. Physiol.* **266:** E372–E383.

27. Neese, R. A., D. Faix, C. Kletke, K. Wu, A. C. Wang, C. H. Shackleton, and M. K. Hellerstein. 1993. Measurement of endogenous synthesis of plasma cholesterol in rats and humans using MIDA. *Am. J. Physiol.* **264:** E136–E147.

28. Kelleher, J. K., A. T. Kharroubi, T. A. Aldaghlas, I. B. Shambat, K. A. Kennedy, A. L. Holleran, and T. M. Masterson. 1994. Isotopomer spectral analysis of cholesterol synthesis: applications in human hepatoma cells. *Am. J. Physiol.* **266:** E384–E395.

29. Kelleher, J. K. 1995. Isotopomer spectral analyses: encountering variable pool size enrichment. *Ann. Biomed. Eng.* **23** (Suppl. 1): S72.

30. Landau, B. R., C. A. Fernandez, S. F. Previs, K. Ekberg, V. Chandramouli, J. Wahren, S. C. Kalhan, and H. Brunengraber. 1995. A limitation in the use of mass isotopomer distributions to measure gluconeogenesis in fasting humans. *Am. J. Physiol.* **269:** E18–E26.

31. Previs, S. F., C. A. Fernandez, D. Yang, M. V. Soloviev, F. David, and H. Brunengraber. 1995. Limitations of the mass isotopomer distribution analysis of glucose to study gluconeogenesis. Substrate cycling between glycerol and triose phosphates in liver. *J. Biol. Chem.* **270:** 19806–19815.

32. Schoenheimer, R., and D. Rittenberg. 1935. Deuterium as an indicator in the study of intermediary metabolism. *J. Biol. Chem.* **111:** 163–192.

33. 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.

34. Jeske, D. J., and J. M. Dietschy. 1980. Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using [^3H]water. *J. Lipid Res.* **21:** 364–376.

35. Jones, P. J., A. S. Pappu, D. R. Illingworth, and C. A. Leitch. 1992. Correspondence between plasma mevalonic acid levels and deuterium uptake in measuring human cholesterol synthesis. *Eur. J. Clin. Invest.* **22:** 609–613.

36. Jones, P. J., A. M. Scanu, and D. A. Schoeller. 1988. Plasma cholesterol synthesis using deuterated water in humans: effect of short-term food restriction. *J. Lab. Clin. Med.* **111:** 627–633.

37. Jones, P. J., A. H. Lichtenstein, and E. J. Schaefer. 1994. Interaction of dietary fat saturation and cholesterol level on cholesterol synthesis measured using deuterium incorporation. *J. Lipid Res.* **35:** 1093–1101.

38. Jones, P. J., A. S. Pappu, L. Hatcher, Z. C. Li, D. R. Illingworth, and W. E. Connor. 1996. Dietary cholesterol feeding suppresses human cholesterol synthesis measured by deuterium incorporation and urinary mevalonic acid levels. *Arterioscler. Thromb.* **16:** 1222–1228.

39. Mazier, M. J., and P. J. H. Jones. 1997. Dietary fat saturation and feeding state modulate rates of cholesterol synthesis in normolipidemic men. *J. Nutr.* **127:** 332–340.

40. Jones, P. J., C. A. Leitch, and R. A. Pederson. 1993. Meal-frequency effects on plasma hormone concentrations and cholesterol synthesis in humans. *Am. J. Clin. Nutr.* **57:** 868–874.

41. Jones, P. J., A. H. Lichtenstein, E. J. Scheafer, and G. L. Namchuk. 1994. Effect of dietary fat selection on plasma cholesterol synthesis in older, moderately hypercholesterolemic humans. *Arterioscler. Thromb.* **14:** 542–548.

42. Dietschy, J. M., and D. K. Spady. 1984. Measurement of rates of cholesterol synthesis using tritiated water. *J. Lipid Res.* **25:** 1469–1476.

43. Ausman, L. M., J. A. Johnson, C. Guidry, and P. P. Nair. 1993. Fecal bile acids and neutral sterols in the cotton-top tamarin (*Saguinus oedipus*). *Comp. Biochem. Physiol.* **105B:** 655–663.

44. Setchell, K. D., A. M. Lawson, N. Tanida, and J. Sjovall. 1983. General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. *J. Lipid Res.* **24:** 1085–1100.

45. Bland, A. E., and D. G. Altman. 1986. Statistical methods for assessing agreement between two measures of clinical measurement. *Lancet.* **I:** 307–310.

46. Fernandez, M. L., N. Y. Yount, and D. J. McNamara. 1990. Whole body and hepatic cholesterol synthesis rates in the guinea-pig: effect of dietary fat quality. *Biochim. Biophys. Acta.* **1044:** 340–348.

47. Martin, L. J., W. Su, P. J. Jones, G. A. Lockwood, D. L. Tritchler, and N. F. Boyd. 1996. Comparison of energy intake determined by food records and doubly labelled water in women participating in a dietary-intervention trial. *Am. J. Clin. Nutr.* **63:** 483–490.

48. Jones, P. J., and S. T. Leatherdale. 1991. Stable isotopes in clinical research: safety reaffirmed. *Clin. Sci.* **80:** 277–280.

49. Diraison, F., C. Pachiaudi, and M. Beylot. 1997. Measuring lipogenesis and cholesterol synthesis in humans with deuterated water: use of simple gas chromatographic/mass spectrometric techniques. *J. Mass Spectrom.* **32:** 81–86.