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


School of Dietetics and Human Nutrition, Faculty of Agricultural and Environmental Sciences, McGill University, Macdonald Campus, Ste-Anne-de-Bellechue, Quebec, H9X 3V9, Canada; Lipid Metabolism Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; School of Nutrition Science and Policy, Tufts University, Medford, MA; and Regis College, Weston, MA

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$_2$O 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 cholesterologenesis 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
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-blindened 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 (D₂O) per kg body water, estimated as 60% of body weight. Blood samples were collected just prior to, and 24 h after, D₂O 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 x 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, then placed at 520°C 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.

Cholesterol synthesis determination using balance

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-blindened 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 (D₂O) per kg body water, estimated as 60% of body weight. Blood samples were collected just prior to, and 24 h after, D₂O 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 x 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, then placed at 520°C 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.

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-stilbesterol, 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 N₂. Samples were saponified by adding 50% KOH and 95% ethanol and incubating for 1 h at 80°C. After cooling in ice, H₂O was added to each sample tube which was then extracted with hexane. The pooled hexane was washed with H₂O and the hexane layer was evaporated under N₂ 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 5x-cholesterol 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 (RTX–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 246°C (0.7°C/min). The injector and detector were set at 250°C and 246°C, respectively. The carrier gas (helium) flow was 1.0 ml/min, the oven temperature was 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, ursodeoxycholic, and cholic acids. Recovery of BA was 71%, determined on the basis of lithocholic acid recovery from fecal samples.

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, ursodeoxycholic, 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, campesteryl, 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>
<thead>
<tr>
<th>Variable</th>
<th>Corn Oil</th>
<th>Beef Tallow</th>
<th>Margarine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%) calories</td>
<td>17.4 ± 0.8</td>
<td>16.1 ± 0.2</td>
<td>16.4 ± 1.6</td>
</tr>
<tr>
<td>Carbohydrate (%) calories</td>
<td>53.0 ± 1.9</td>
<td>51.2 ± 1.6</td>
<td>53.8 ± 2.5</td>
</tr>
<tr>
<td>Fat (%) calories</td>
<td>29.6 ± 1.1</td>
<td>32.7 ± 1.1</td>
<td>29.7 ± 1.13</td>
</tr>
<tr>
<td>SFAs (%) calories</td>
<td>6.41 ± 1.45</td>
<td>13.69 ± 0.58</td>
<td>7.73 ± 0.13</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.09 ± 0.01</td>
<td>0.06 ± 0.05</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.39 ± 0.06</td>
<td>0.87 ± 0.02</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>C16:0</td>
<td>3.86 ± 1.11</td>
<td>8.39 ± 0.32</td>
<td>4.18 ± 0.09</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.57 ± 0.25</td>
<td>3.75 ± 0.27</td>
<td>2.00 ± 0.02</td>
</tr>
<tr>
<td>MUFA (%) calories</td>
<td>10.71 ± 2.36</td>
<td>12.07 ± 0.39</td>
<td>12.16 ± 0.25</td>
</tr>
<tr>
<td>C16:1n-7</td>
<td>0.30 ± 0.07</td>
<td>0.85 ± 0.07</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>10.06 ± 2.33</td>
<td>10.61 ± 0.36</td>
<td>11.65 ± 0.24</td>
</tr>
<tr>
<td>PUFAs (%) calories</td>
<td>9.45 ± 2.55</td>
<td>2.62 ± 0.35</td>
<td>8.30 ± 0.16</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>8.48 ± 3.28</td>
<td>2.44 ± 0.28</td>
<td>7.85 ± 0.18</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.83 ± 0.74</td>
<td>0.23 ± 0.04</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Cholesterol mg/1000 kcal</td>
<td>83 ± 7 (3.11 ± 0.37)</td>
<td>109 ± 12 (3.99 ± 0.47)</td>
<td>77 ± 9 (2.82 ± 0.33)</td>
</tr>
<tr>
<td>β-sitosterol mg/1000 kcal</td>
<td>179 (6.70 ± 0.77)</td>
<td>70 (2.55 ± 0.30)</td>
<td>142 (5.21 ± 0.61)</td>
</tr>
<tr>
<td>Campesterol mg/1000 kcal</td>
<td>56 (2.10 ± 0.25)</td>
<td>28 (1.04 ± 0.12)</td>
<td>53 (1.93 ± 0.23)</td>
</tr>
<tr>
<td>Stigmasterol mg/1000 kcal</td>
<td>21 (0.80 ± 0.09)</td>
<td>12 (0.43 ± 0.05)</td>
<td>22 (0.71 ± 0.09)</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

SFAs, saturated fatty acids; PUFAs, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids.

* Duplicate determinations for one 3-day menu cycle for each diet.
levels after the corn oil diet period were significantly lower ($P < 0.05$) than after the beef tallow and corn oil marginine 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 ± 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 ± 269 mg/day) did not vary significantly ($P = 0.118$) from that obtained using D incorporation (1,362 ± 201 mg/day). Individual data are presented in Table 2.

Table 2. Cholesterol biosynthesis data with different methods and diets

<table>
<thead>
<tr>
<th>Subject</th>
<th>Corn Oil</th>
<th>Beef Tallow</th>
<th>Margarine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D$_2$</td>
<td>D$_2$</td>
<td>D$_2$</td>
</tr>
<tr>
<td></td>
<td>mg/day</td>
<td>mg/day</td>
<td>mg/day</td>
</tr>
<tr>
<td>1</td>
<td>532</td>
<td>428</td>
<td>1181</td>
</tr>
<tr>
<td>2</td>
<td>2316</td>
<td>2002</td>
<td>1799</td>
</tr>
<tr>
<td>3</td>
<td>386</td>
<td>272</td>
<td>910</td>
</tr>
<tr>
<td>4</td>
<td>2425</td>
<td>1982</td>
<td>1614</td>
</tr>
<tr>
<td>5</td>
<td>711</td>
<td>697</td>
<td>745</td>
</tr>
<tr>
<td>6</td>
<td>858</td>
<td>887</td>
<td>1069</td>
</tr>
<tr>
<td>7</td>
<td>2690</td>
<td>1761</td>
<td>978</td>
</tr>
<tr>
<td>8</td>
<td>1494</td>
<td>641</td>
<td>409</td>
</tr>
<tr>
<td>9</td>
<td>2122</td>
<td>2149</td>
<td>1828</td>
</tr>
<tr>
<td>10</td>
<td>2692</td>
<td>1520</td>
<td>1457</td>
</tr>
<tr>
<td>11</td>
<td>1017</td>
<td>703</td>
<td>519</td>
</tr>
<tr>
<td>12</td>
<td>644</td>
<td>449</td>
<td>770</td>
</tr>
<tr>
<td>13</td>
<td>1889</td>
<td>1625</td>
<td>513</td>
</tr>
<tr>
<td>Mean</td>
<td>1506</td>
<td>1163</td>
<td>1059</td>
</tr>
<tr>
<td>±SEM</td>
<td>248</td>
<td>191</td>
<td>135</td>
</tr>
</tbody>
</table>

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

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

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

For the beef tallow diet, total fecal sterol content was 1,456 ± 194 mg/day, of which 293 ± 17 mg/day originated from diet. Mean synthesis rate assessed by sterol balance (1,163 ± 191 mg/day) did not differ significantly ($P = 0.481$) from that assessed using D incorporation (1,059 ± 135 mg/day). Individual data are presented in Table 2. When the mean values of synthesis rates 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 ± 194 mg/day, of which 293 ± 17 mg/day originated from diet. Mean synthesis rate assessed by sterol balance (1,163 ± 191 mg/day) did not differ significantly ($P = 0.481$) from that assessed using D incorporation (1,059 ± 135 mg/day). Individual data are presented in Table 2. When the mean values of synthesis rates 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 margarine diet, total fecal sterol content for 13 subjects was 1,488 ± 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 ± 222 mg/day versus 1,154 ± 148 mg/day ($P = 0.258$) determined 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 ± 125 mg/day) and that of D incorporation (1,183 ± 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 ± 2 × 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-
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).

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).

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 an 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-
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.

The authors thank the staff of the Metabolic Research Unit for the expert care provided to the study subjects. We also gratefully acknowledge the cooperation of the study subjects without whom this investigation would not have been possible. The authors gratefully acknowledge the directed technical assistance of Diane MacDougall, Gayle Namchuk, Maria Wood, Dr. Padmanabhan Nair, and Dr. Gerard Dallal. This project has been funded, at least in part, with Federal funds from the U.S. Department of Agriculture, Agricultural Research Service under contract number 53-3K06-01 and by the Heart and Stroke Foundation of Canada. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement of the U.S. Government.

Manuscript received 24 September 1997 and in revised form 5 January 1998.

REFERENCES


