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Measuring Lipogenesis and Cholesterol Synthesis in Humans with Deuterated Water: Use of Simple Gas Chromatographic/Mass Spectrometric Techniques

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Lipogenesis and cholesterol synthesis can be studied by measuring the incorporation into fatty acids and cholesterol of deuterium from deuterated water. This has been previously achieved in human subjects using low levels of deuterium enrichment in plasma water, and thus in fatty acids and cholesterol. For the measurement of enrichment in lipids, this required the use of isotope ratio mass spectrometry, a tedious and time-consuming technique. It is shown that these measurements can be performed using the much simpler gas chromatography/mass spectrometry if higher, but always safe, deuterium enrichments in plasma water are obtained. Normal subjects ingested deuterated water in order to obtain stable enrichment in plasma water of 0.3% during a 60 h period. Enrichment in palmitate of plasma triglycerides (TG) plateaued (0.6–0.76%) whereas plasma cholesterol enrichment increased progressively [0.32 ± 0.08% (12 h) to 0.78 ± 0.18% (60 h)]. Endogenous synthesis was estimated to contribute, in post-absorptive subjects, 8–10% of the plasma TG pool and 3–5% of plasma free cholesterol pool. These data agree with results obtained previously using isotope ratio mass spectrometry. The present method will be useful for studies of normal and abnormal lipid metabolism in humans.

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INTRODUCTION

Measurement of the incorporation of deuterium into lipids[1,2] during administration of deuterated water (^2H_2O) is an attractive method for determining the fractional synthetic rate of fatty acids and cholesterol.[3–8] ^2H_2O is a non-radioactive tracer that can be ingested orally and the enrichment of the precursor, body water, can be easily measured.[6] Lipid synthesis can also be measured using ^{13}C-labelled acetate and the mass isotopomer distribution analysis of the lipids synthesized,[9–11] but the need for intravenous infusion of the tracer makes this method inconvenient. Deuterated water has been used for studies of lipogenesis and cholesterol synthesis *in vitro*[12–14] and *in vivo* in rats[15,16] and also in humans.[3–8] Studies *in vitro* and in rats were mainly aimed at determining the average maximum number of deuterium atoms that can be incorporated in the molecules synthesized, taking in account the different pathways of deuterium incorporation and the possible isotope discrimination against deuterium; high levels (i.e. >2%) of deuterium enrichment in water were attained in these studies and the enrichment by excess deuterium of fatty acids and cholesterol was determined by gas chromatography/mass spectrometry (GC/MS).

During studies in humans,[3–8] deuterium enrichment in plasma triglycerides (TG) and cholesterol was measured by isotope ratio mass spectrometry (IRMS). This required the extensive purification of TG and cholesterol, combustion of the purified compounds and reduction of the water produced before measuring by IRMS the deuterium to hydrogen ratio, delicate, tedious and time-consuming procedure. This was necessary because the very small increase in the deuterium to hydrogen ratio attained in TG and cholesterol (about 0.002%) could not be measured by GC/MS, even if one takes into account the total number of hydrogen atoms present in fatty acids or cholesterol (i.e. the increase in the $m_1:m_0$ ratio would have been <0.1%). Deuterium enrichment in the palmitate of TG was barely detectable by GC/MS in a study by Hackey *et al.*[17] However, in all these experiments,[3–8,14] the deuterium enrichment in plasma water was only 0.04–0.08%. Higher enrichment levels (0.3–0.5%) of water have been used in humans[18,19] during studies aimed at measuring gluconeogenesis, and appeared well tolerated. Theoretical

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calculations show that, under these conditions, deuterium enrichment in plasma cholesterol and in the fatty acid part of plasma TG should be measurable by GC/MS. Since this would greatly simplify the measurement of lipogenesis and cholesterol synthesis in humans, we tested this possibility in this work.

EXPERIMENTAL

Subjects and methods

Pyridine, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and bis(trimethylsilyl)trifluoroacetamide were supplied by Pierce (Rockford, IL, USA). Other solvents and reactifs were obtained from Merck (Darmstadt, Germany) or Prolabo (Paris, France). Deuterated water (^2H_2O, 99.8 atoms%) was purchased from Eurisotop (Gif-sur-Yvette, France).

Nine healthy subjects (five women and four men, aged 22–46 years) with no personal or family history of diabetes or lipid disorders participated in the study. They gave their informed consent after full explanation of the nature, aim and possible risks of the study. None was taking any medication, all had a normal body mass and normal total cholesterol (3.90 ± 0.25 mmol l^{-1}) and TG (0.62 ± 0.15 mmol l^{-1}) levels (measured by enzymatic methods). They consumed their usual isocaloric diet the days before and throughout the study, except when indicated.

Two studies were performed. In the first (study 1) four subjects, after an initial blood sampling, drank in the evening a loading dose of deuterated water (3 g ^2H_2O kg^{-1} body water) in two equal portions (one half at 20:00 h and the other at 22:00 h) followed by water containing 4.5 g ^2H_2O l^{-1} over the following 60 h in order to maintain body water deuterium enrichment at plateau (the subjects consumed between 3 and 4 l of water). Blood samples were collected 12, 20, 36, 44 and 60 h after the loading dose (i.e. at 08:00 h during three days and at 16:00 h during two days). Samples collected at 08:00 h were taken in the post-absorptive state, before breakfast. In the second study (study 2), five subjects drank in the evening the same loading dose of deuterated water followed until the next evening by water enriched with ^2H_2O (4.5 g ^2H_2O l^{-1} drinking water); they fasted until 18:00 h the next day. Blood samples were collected before the loading dose and at 08:00 and 18:00 h the following day (12 and 22 h after the loading dose).

Plasma obtained by centrifugation was split into two parts. One was stored at -20 °C until analysis and the other was used for purification of very low-density lipoproteins (VLDL) by ultracentrifugation. A 4 ml volume of plasma was mixed with 3 ml of a 1.0063 g l^{-1} solution of NaCl in EDTA. For samples collected in the afternoon, chylomicrons were first removed by a 30 min centrifugation at 25 000 rpm at 25 °C (70.1 Ti rotor and L60 Beckman ultracentrifuge). The upper 1 ml fraction containing chylomicrons was removed by aspiration and 1 ml of fresh NaCl solution in EDTA was added to the remaining 6 ml. VLDL were isolated by a 22 h centrifugation at 110 000 *g* at 17 °C. The upper fraction containing VLDL was collected by aspiration and stored at -20 °C until further analysis.

Lipids from plasma (1 ml) or from the VLDL fraction were extracted by the method of Folch *et al.*[20] Extracted lipids were dissolved in ethanol–chloroform (1:2 v/v) and spotted on silica gel G thin-layer plates (Merck). The plates were developed with hexane–diethyl ether–acetic acid (80:20:1, v/v). Free and esterified cholesterol, TG and fatty acids were visualized using fluorescein vapour against a standard and scraped off the silica gel plate. Cholesterol was eluted twice from the silica with diethyl ether and the eluate was washed with water before evaporation to dryness. Esterified cholesterol was hydrolysed by heating at 60 °C for 60 min in 6 M KOH and ethanol (2 ml). Cholesterol was next extracted with chloroform before evaporation to dryness. The trimethylsilyl derivative of cholesterol was prepared with pyridine (50 µl) and BSTFA (50 µl). Fatty acids were methylated and fatty acids of TG transmethylated by the method of Morrison and Smith.[21] Glucose was purified from the neutralized perchloric acid extract of plasma by sequential ion-exchange chromatography before preparation of its bisbutylboronate acetate derivative.[22] The bis(*tert*-butyldimethylsilyl) derivative of plasma lactate was prepared as described previously.[23]

All samples were injected into an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 25 m fused-silica capillary column (OV-1701; Chrompack, Raritan, NJ, USA), interfaced with an HP 5871 A mass spectrometer (Hewlett-Packard) working in the electron impact mode (70 eV). The carrier gas was helium. GC/MS procedures for glucose and lactate have been published previously.[18,19] For cholesterol the operating conditions were injector temperature 290 °C (split mode), oven temperature 270 °C and ions of *m/z* 367, 368 and 369 were selectively monitored (dwell time 100 ms), and for palmitate methyl ester the conditions were temperature injector 250 °C (split mode), oven temperature 210 °C and ions of *m/z* 269, 270 and 271 were monitored (dwell time 100 ms). The mass spectrometer parameters were set manually before each series of samples in order to obtain, for the range of ion masses selectively monitored, the best sensitivity and the best resolution between consecutive masses for palmitate or for cholesterol. Samples of unlabelled molecules were run with the biological samples and the linearity of the response of the mass spectrometer to increasing enrichment was verified by measuring calibration graphs for ^{13}C-enriched palmitate or cholesterol. All samples (standard and biological samples) were injected in triplicate. Special care was taken in order to obtain comparable ions peak areas (i.e. $<20\%$ difference) between the standard and biological samples,[24] diluting the sample or adjusting the volume injected if necessary. The relative standard deviations were 5–8% in the range of enrichments obtained in this study (0.3–1%); they increased to 10–15% when the enrichments were only 0.1–0.2%. The lowest enrichment measurable was 0.1% for both palmitate and cholesterol.

Deuterium enrichment in plasma water was determined by IRMS (Optima; Fisons Instruments, Middlewich, UK).[25] Basal plasma samples were distilled for 4

min and reduced over zinc reagent (Indiana University Foundation, Bloomington IN, USA) at 500 °C for 30 min. Subjects' drinking water and enriched plasma samples were diluted (1:300 and 1:100, respectively) with reference water then reduced over zinc reagent at 500 °C for 30 min. The hydrogen produced was analysed on a triple-inlet IRMS. The ^2H to ^1H ratio for each sample was measured six times against International Standard Mean Ocean Water (SMOW) (International Atomic Energy Agency, Vienna, Austria) reference. The results, expressed as $\delta‰$ ^2H:

$$\delta‰ = 1000\left(\frac{^2H/^1H_{sample} - ^2H/^1H_{reference}}{^2H/^1H_{reference}}\right)$$

were converted into atom per cent (AP) $[^2H/(^2H + ^1H)]$ for each sample, taking the dilution into account when necessary. The enrichment in drinking water and plasma samples was expressed as atom per cent excess (APE):

$$APE = AP_{sample} - AP_{basal}$$

where AP_{basal} is the value for plasma water before tracer administration.

Calculations

Enrichments of palmitate and cholesterol were calculated from the observed spectral intensities of the ions monitored using matrix correction.[13] The correction matrix was obtained by running samples of natural palmitate and cholesterol. This correction matrix gives the abundance of molecules having incorporated one or two excess deuterium atoms expressed relative to the abundance of molecules with no excess deuterium (i.e. results are in molar ratios: m_i/m_0). These values were then converted into molar excess, i.e. the ratio of molecules having incorporated one or two excess deuterium atoms to the total number of molecules $[m_i/(m_0 + m_1 + m_2)]$. From this mass isotopomer distribution, we then calculated the average number of deuterium atoms incorporated per molecule (IE_{obs}): $IE_{obs} = m_1 + 2m_2 + \cdots + nm_n$. The comparison of this observed deuterium enrichment (IE_{obs}) with the enrichment (IE_{exp}) that would have been obtained if all palmitate or cholesterol molecules were from endogenous synthesis gives the contribution (F), as a percentage, of synthesis to the pool of molecules sampled:[13] $F = IE_{obs}/IE_{exp}$. IE_{exp} is given by $IE_{exp} = pN$, where p is the deuterium enrichment in plasma water measured by IRMS and N is the maximum number of deuterium atoms that can be incorporated into the molecules synthesized and is estimated, based on previous studies,[16] to be 22 for plasma palmitate and 27 for plasma cholesterol. When a constant enrichment in the precursor pool (plasma water in this study) is obtained, F is the percentage of the product pool (plasma TG palmitate or plasma cholesterol) produced by synthesis during the time elapsed between the loading dose of the tracer and the collection of sample. F can be constant, if isotopic equilibrium in the product pool has been obtained, or increases with time, if isotopic equilibrium has not yet been achieved. In the latter case, a fractional synthetic rate (FSR = percentage of synthesis/time unit) can be calculated if the increase of F is linear with time: $FSR = F/\text{time}$.

Results are shown as individual values or as averages with standard errors of the mean (SEM). Comparisons were performed by two-ways analysis of variance or Student's t-test for paired or unpaired values.

RESULTS

No subjects experienced any side-effects after ingestion of deuterated water, particularly no vertigo. Figure 1 shows that stable levels of deuterium enrichment in plasma water around 0.30–0.34% were obtained during both studies, despite a trend for a slight decline during study 1. The evolution of deuterium enrichment in plasma lipids is shown in Fig. 2. With the plasma water enrichment obtained in these studies, we observed only singly labelled molecules of palmitate and cholesterol (i.e. only increases in m_1/m_0 were observed; there was no increase in m_2/m_0). During the first study the enrichment in free cholesterol was $0.32 \pm 0.08\%$ 12 h after the loading dose of deuterated water; it increased progressively to reach $0.78 \pm 0.18\%$ ($p < 0.01$ *vs.* 12 h) at 60 h. During the second study, this enrichment in free cholesterol was slightly, but not significantly, higher at 12 h ($0.38 \pm 0.07\%$) and increased to $0.54 \pm 0.06\%$ at 20 h ($p < 0.05$ *vs.* 12 h).

Deuterium enrichment of esterified cholesterol was detectable only 36 h after the loading dose and remained much lower than in free cholesterol (final value at 60 h in study one: $0.15 \pm 0.02\%$). During study 1 we compared the enrichment of palmitate of total TG and of VLDL TG and found no significant differences (Fig. 2) The enrichment was about 0.60% at 12 h; contrary to what was observed for cholesterol, this enrichment plateaued during the following 48 h. During study 2, only total TG palmitate enrichment was measured. This enrichment was $0.54 \pm 0.11\%$ at 12 h and decreased in these fasting subjects to $0.17 \pm 0.04\%$ at 22 h.

Table 1 shows the individual F values for the pools of plasma free cholesterol and TG palmitate sampled at 12

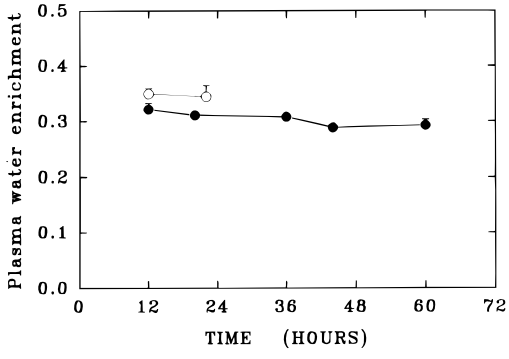

Figure 1. Evolution of deuterium enrichment in plasma water during studies 1 (●) and 2 (○).

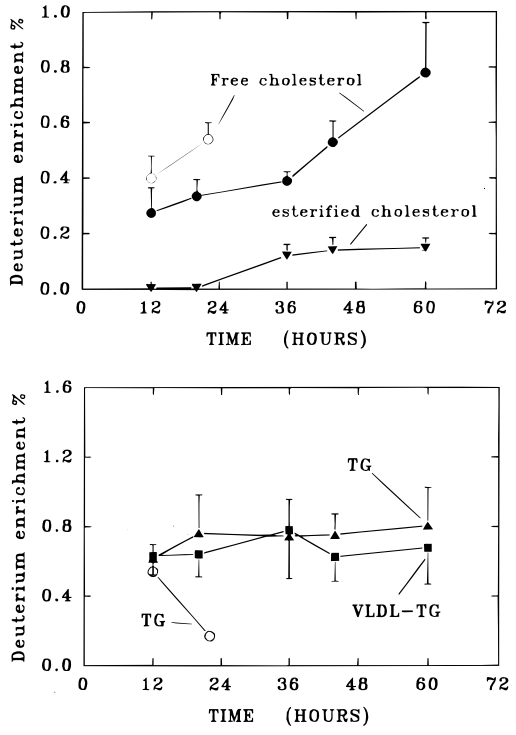

Figure 2. Deuterium enrichment in plasma cholesterol (top) and in the palmitate of total TG or VLDL TG (bottom) during studies 1 (closed symbols) and 2 (open symbols).

h and Table 2 the evolution of F during the two studies. During the first study, F increased progressively throughout the study for free cholesterol whereas it remained between 9 and 13% for TG palmitate. During study 2 (fasting subjects), F also increased slightly for free cholesterol but decreased for TG palmitate.

During study 1 we also measured deuterium enrichment in the palmitate of the plasma FFA pool and in plasma glucose and lactate. No deuterium excess was detectable in the palmitate of FFA at 12 h. However, enrichment appeared at 20 h and then plateaued between 0.45 and 0.55%. As expected, glucose and lactate were also enriched at 12 h and this enrichment subsequently remained stable (Fig. 3).

Table 1. Individual F values (contribution of endogenous synthesis, as a percentage) for plasma free cholesterol and TG palmitate, measured in the post-absorptive state (12 h after the loading dose of deuterated water)

Free cholesterol[a]	5.9, 5.1, 2.3, 2.8, 6.5, 3.3, 2.8, 4.3, 5.5
TG palmitate[a]	10.8, 6.6, 9.7, 7.3, 4.3, 8.4, 11.8, 6.3, 4.4

[a] Mean ± SEM: free cholesterol, 4.3 ± 0.5; TG palmitate, 7.7 ± 0.8.

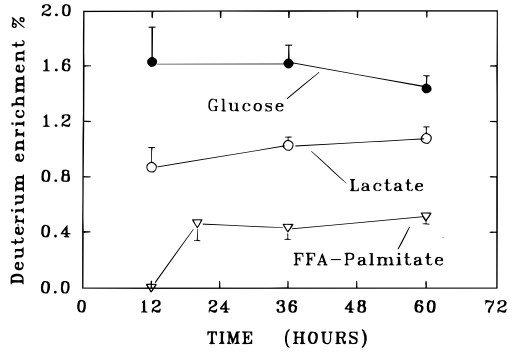

Figure 3. Deuterium enrichment in the palmitate of plasma free fatty acids (FFA palmitate) and in plasma glucose and lactate during study 1.

DISCUSSION

The present results show that deuterium enrichment in plasma cholesterol and in palmitate of TG can be measured by GC/MS with administration of deuterated water. This needed an enrichment in plasma water (0.3%) higher than that in previous studies.[3–8,17] (In an additional study, we tried (data not shown) to lower this enrichment in plasma water to about 0.1%, but under these conditions most of the enrichments in plasma lipids were too low to allow accurate measurements.) The doses of deuterated water we used were 3–6 times higher than those used in previous studies of lipogenesis.[3–8,17] These doses were, as in the reports of Landau *et al.*,[18,19] well tolerated and no side-effects were observed. The enrichment levels attained in plasma water remained two orders of magnitude lower than those (25%) giving toxic effects in mice and one order of magnitude lower than levels achieved during several days or weeks in rats[15,16] without evidence of side-effects. Therefore, we think that the dose of deuterated water that we used is safe for studies in humans, as least in non-pregnant subjects and in adults. The possibility of measuring deuterium appearance in lipids without using the tedious and time-consuming procedure of combustion and reduction of the water produced for IRMS analysis should greatly simplify further studies of lipogenesis and cholesterol synthesis in humans. Moreover, this allows one to measure deuterium enrichment in the fatty acid part of TG and not in the whole TG molecule as with the combustion–IRMS procedure. IRMS was used in this work to measure deuterium enrichment in plasma water. However, a simpler procedure using GC/MS to measure deuterium enrichment in plasma water above 0.1% has been proposed and will also simplify the analytical procedures.[26]

The evolution of the enrichments in palmitate of either total plasma TG or VLDL TG and in plasma cholesterol that we observed during study 1 is essentially comparable to those reported previously,[4,6–8] i.e. near plateau enrichment in TG palmitate 12 h after the loading dose and a progressive rise of enrichment in plasma free cholesterol with a delayed and smaller rise in esterified cholesterol. If we consider the entry of labelled palmitate or cholesterol from endogenous synthesis in the plasma pool as equivalent to a tracer infusion, these evolutions are consistent with previously published kinetic data. In post-absorptive normotriglyceridaemic subjects, the fractional turnover rate and half-life of plasma TG have been reported to be

Table 2. Percentage contribution of endogenous synthesis (*F*) to the plasma pools of TG palmitate and of free cholesterol[a]

	Study 1				
	12 h	20 h	36 h	44 h	60 h
Free cholesterol	4.0 ± 0.9	4.7 ± 1.0	4.9 ± 0.3	8.1 ± 0.5[b]	10.0 ± 2.0[b]
TG palmitate	8.8 ± 0.4	11.8 ± 0.6	11.5 ± 3.6	12.8 ± 2.7	13.8 ± 4.8
TG VLDL palmitate	9.4 ± 1.9	9.7 ± 2.2	11.9 ± 4.8	10.4 ± 2.8	11.4 ± 4.4
	Study 2				
	12 h		22 h		
Free cholesterol	4.5 ± 0.7		6.3 ± 1.0[b]		
TG palmitate	7.1 ± 1.2		2.1 ± 0.4[b]		

a Results are averages ±SEM.
[b] $p < 0.05$ *vs*. values at 12 h.

0.25–0.40 h^{-1} and 1.7–3 h, respectively.[27–28] Therefore, 12 h is sufficient to achieve a near perfect isotopic steady state. The decrease in enrichment in TG palmitate during study 2 is also consistent with studies showing that *de novo* lipogenesis is lowered by carbohydrate and caloric restriction.[29] The kinetics of cholesterol are more complicated. Plasma cholesterol is considered to equilibrate rapidly with cholesterol in liver, blood cells and the intestine within a central, rapidly exchangeable compartment (pool 1) equilibrating slowly with two other slowly turning over pools of tissues cholesterol.[30–32] However the fractional turnover rate of this 'rapidly' exchangeable pool 1 is only 4–6% per day, i.e. much lower than for plasma TG, and several weeks would be needed before an equilibrium between endogenously produced labelled and ingested unlabelled cholesterol is obtained. This has been demonstrated in rats[14] and is evidenced in man by the fact that cholesterol enrichment is maximum only 40 days after a unique loading dose of deuterated water.[33] Theoretically an *FSR* could be calculated from this increase in cholesterol enrichment. However, there are diurnal variations in cholesterol synthesis rate[6,8] and precise calculations require a more frequent blood sampling schedule than that performed in this work.

We also found significant labelling of palmitate in the plasma FFA from 20 to 60 h, which was unexpected. An artefactual *in vitro* release from TG seems unlikely as no significant labelling was found at 12 h, despite identical sample processing. Moreover, control experiments in a previous study of post-prandial TG metabolism[34] showed no evidence for such an artefact. This labelling of plasma FFA palmitate could suggest that some fatty acids were synthesized in adipose tissue and quickly released in plasma. However, a more likely explanation is that some fatty acids released from circulating TG by lipoprotein lipase were not taken up by tissues and appeared in the circulation.[34,35] Whatever the exact mechanism, this labelling of the plasma FFA pool results in a recycling of the label through hepatic re-esterification of FFA. Therefore, it might seem surprising that plasma TG palmitate enrichment did not increase from 12 to 60 h. However, one should also take into account the simultaneous dilution by unlabelled palmitate from orally ingested TG. Thus, without the simultaneous measurement of hepatic re-esterification or intestinal absorption by dual tracer studies, a correct interpretation of this part of the study is difficult.

The *F* value obtained at 12 h for TG palmitate (8%) is consistent with previous studies using deuterated water and IRMS,[3,4] but higher than values found with ^{13}C-labelled acetate infusion (2–5%).[9,10] We have no precise explanation for this discrepancy apart from possible differences in dietary habits between the groups studied. The subsequent plateauing of *F* in study 1 reflects equilibrium in the plasma pool between endogenously produced TG palmitate (through either lipogenesis or FFA recycling) and ingested TG. Indeed, the *F* value observed is consistent with reported values of ingested (80–100 g per day) and synthesized (10–15 g per day) TG.[36] The *F* values obtained at 12 h for cholesterol agree with those reported in studies using either deuterated water and IRMS[5,6] or ^{13}C-labelled acetate;[10] the subsequent progressive increase in *F* reflects only the further entry of labelled cholesterol into the large pool of exchangeable cholesterol.

For these calculations of endogenous synthesis, we used *N* values of 22 for palmitate and 27 for cholesterol, on the basis of previous studies in rats.[15,16] These values are higher than those found *in vitro* (17 and 20, respectively). We suggested that these higher *in vivo* values were related to an additional entry of deuterium through the metabolism of labelled glucose and lactate leading to labelled acetyl-CoA.[16] Since we also found significant labelling of glucose and lactate in humans, we consider it appropriate to use the *N* values previously found in rats *in vivo*.

In conclusion, our results show that it is possible to study lipogenesis and cholesterol synthesis in humans with deuterated water and simple GC/MS techniques. We suggest that short-term studies (12 h overnight) could be sufficient to obtain reliable estimates of overnight synthetic rates. Longer studies could be useful for studying the equilibrium between endogenously produced and orally ingested TG and the diurnal periodicity of cholesterol synthesis.[6–8]

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