Measurement of human lipogenesis using deuterium incorporation

Catherine A. Leitch and Peter J. H. Jones[1]

Division of Human Nutrition, School of Family and Nutritional Sciences, University of British Columbia, 2205 East Mall, Vancouver, B. C., Canada V6T 1Z4

Abstract In order to determine human triglyceride fatty acid (TG–FA) synthesis rates, the incorporation of deuterium from the plasma water pool in vivo into TG–FA was measured over 48 h in six healthy males under steady state conditions. Subjects consumed a nutritionally complete liquid diet as six evenly spaced small meals per day for 3 days and drank 0.7 g D_2O/kg estimated body water, (99.8 APE) at 0800 h on day 2. D_2O was added to the liquid diets to maintain plasma deuterium enrichment at plateau over days 2 and 3. Blood samples were obtained prior to dosing and every 4 h thereafter. Total TG–FA were obtained from plasma at each timepoint and combusted. The resultant water was vacuum-distilled into Zn-containing Pyrex tubes, reduced to hydrogen gas, and analyzed for deuterium abundance by isotope ratio mass spectrometry. TG–FA deuterium enrichment reached a plateau at approximately 12 h post-dose. To establish the amount of newly synthesized TG–FA in the total plasma TG–FA pool, two models were developed relating the maximum observed deuterium enrichment to the calculated theoretical maximum enrichment. In model A, it was assumed that TG–FA come solely from chylomicrons. This yields a value of 8.8 ± 4.6 g/day (mean ± SD) de novo synthesized TG–FA. On the opposite extreme in model B, it was assumed that all the TG–FA are derived from recycled very low density lipoprotein (VLDL); the amount of newly synthesized TG–FA calculated by this model was 1.7 ± 0.8 g/day. Given rapid postprandial clearance of chylomicrons, model B is considered to most closely approximate the in vivo lipogenesis rate. ▮▮ These results suggest that in vivo TG–FA synthesis is minor in these subjects under steady state dietary conditions.—Leitch, C. A., and P. J. H. Jones. Measurement of human lipogenesis using deuterium incorporation. *J. Lipid Res.* 1993. **34:** 157–163.

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It is difficult to demonstrate net de novo lipid synthesis in humans. Using respiratory gas exchange, Acheson et al. reported that the conversion of dietary carbohydrate to fat is not responsible for the accumulation of body fat (1) and subsequently that humans convert large quantities of dietary carbohydrate to glycogen stores rather than manufacturing fat (2). They later showed that substantial de novo lipogenesis occurs accompanied by increased carbohydrate oxidation rates only after glycogen stores are saturated (3). Other studies have linked high carbohydrate diets to elevated triglyceride levels (4–6) and to body fat accumulation (7). Whether increased synthesis or decreased catabolism is responsible remains unknown. These apparently conflicting observations have led to controversy over whether lipogenesis occurs in humans, and, if so, to what extent.

In an attempt to determine human lipogenic capacity, Hellerstein et al. (8) investigated de novo hepatic TG–FA production using ^{13}C-labeled acetate in normal men. After an overnight fast, subjects were refed with intravenous glucose, oral Ensure, or a high-carbohydrate meal. Respiratory quotients (RQ) remained below unity during each refeeding protocol. The authors concluded that while de novo FA synthesis does occur to a minor degree (<500 mg/day), this amount is quantitatively insignificant and that only a small fraction of non-fat caloric intake is converted into TG–FA.

In a previous study, we demonstrated the feasibility of using the incorporation of deuterium, a stable isotope, from the plasma water pool into newly formed circulating TG to measure TG–FA production rate (9). Our present aim was to extend the deuterium uptake method to measure short-term in vivo TG–FA synthesis under steady-state conditions and develop a model to permit calculation of the contribution of newly synthesized TG–FA to the total plasma TG pool. Such a technique has potential use in investigating the relationship between dietary TG intake and total plasma FA level as well as elucidating the relative importance of in vivo lipid synthesis and catabolism in lipid disorders.

METHODS

Model development

TG-FA synthesis was determined from the incorporation rate of deuterium using two model systems. Each model used the final plateau of deuterium enrichment relative to the theoretical maximum. We originally used a model where the initial near-linear exponential component of the deuterium uptake curve was taken to represent synthetic rate for TG-FA (9). However, the turnover rate obtained by this approach represents overall pool turnover, not only that of synthesis. Given that entry of unlabeled TG-FA continually occurs, synthesis would be overestimated. Moreover, the rapid tendency of uptake towards plateau requires very early sampling timepoints, during the period when the D_2O dose may not have fully reached equilibrium. Use of enrichment plateau relative to theoretical, as used presently, avoids these problems.

Using the present system, if there were no unlabeled TG-FA source, TG-FA deuterium enrichment will increase exponentially and asymptotically approach the maximum theoretical enrichment given by the following equation (9):

$$\text{del } (^0/00)\text{TG max} = \text{del } (^0/00)_{\text{plasma}} \times$$
$$0.87 \text{ D/C} \times \frac{51C}{93H} \qquad \qquad Eq. \ 1)$$

where isotopic enrichments are expressed in per mil $(^0/00)$ and del$_{\text{plasma}}$ is the mean plasma deuterium enrichment over the study period. The correction factor of 0.87 D/C was observed by Jungas (10), who found 0.87 g-atoms ^3H per g-atom C incorporated into adipose fatty acids. In that study, comparative incorporation of radioactivity into fatty acid was monitored during synthesis in an adipose tissue preparation incubated with ^3H_2O and [U-^{14}C]glucose. Tritium was found only in those fatty acids that incorporated ^{14}C. Along the fatty acid carbon chain, even-numbered carbon positions were found to contain 0.96 $^3H/^{14}C$ whereas odd-numbered positions contained 0.71 $^3H/^{14}C$. The ratio 51C/93H represents three monounsaturated 17-carbon fatty acids within a typical triglyceride (9). An assumption is made that the five additional protons of the TG-glycerol moiety are deuterated at identical enrichments as the three fatty acids.

The exponential model outlined above is an extension of a linear model for cholesterol synthesis originally proposed by Dietschy and Spady (11) involving several assumptions. The first is that cell membranes are permeable to D_2O and that plasma deuterium enrichment is identical to that of the intracellular pool from which TG-FA incorporates deuterium. Second, it is assumed that a constant fraction of deuterium atoms is incorporated into precursor FA under different metabolic conditions. Third,

the TG-FA pool size must remain constant over the study period. The last was approximated by the use of six evenly spaced liquid meals of identical composition per day.

Van Heyningen, Rittenberg, and Schoenheimer (12) have demonstrated that the C-H bonds of FA are stable to proton exchange. Also, we have incubated isolated plasma TG-FA in D_2O for 48 h and analyzed the TG-FA for deuterium enrichment. No deuterium was incorporated into the pre-formed TG-FA (C. A. Leitch and P. J. H. Jones, unpublished data). Thus the incorporation of hydrogen isotopes into FA can occur only during synthesis or chain elongation.

Our models were based on the following assumptions. If there were no unlabeled component to plasma TG-FA, deuterium would be incorporated at the rate given by equation 1 and asymptotically approach the theoretical maximum enrichment. At the opposite extreme, if there were complete absence of synthesis, there would be no deuterium uptake into TG-FA and the observed enrichment would remain at pre-dose levels.

The calculated value for the amount of de novo synthesized TG-FA is strongly dependent on the assumptions made about the unlabeled TG-FA pool. There are three major contributors to the total plasma TF-FA pool: de novo synthesized TG-FA that incorporates deuterium; chylomicron TG-FA that is unlabeled; and recycled VLDL TG-FA that will incorporate deuterium over time. The proportions of these components in the total plasma TG-FA pool are not constant, but reflect individual differences in TG-FA metabolism and feeding state. In developing an approach to determining net synthesis, we initially established two models, A and B.

In model A, we assume that the recycled VLDL TG-FA component is small compared to the chylomicron TG-FA component. Because the subjects were fed a liquid diet of uniform composition, total dietary fat intake is known. Of this amount, 98% is assumed to be absorbed (13). The observed plateau value, X, was calculated as the average TG-FA deuterium enrichment over the last 24 h of the study period. Theoretical enrichment maxima were calculated using equation 1 and individual plasma deuterium enrichments. Conversely (model B), we can assume that the dietary contribution to total plasma TG-FA is small compared to the unlabeled recycled VLDL TG-FA component. Because chylomicrons have an estimated plasma half-life of 4.5 min (14), whereas the VLDL turnover rate is much slower ($t_{1/2} > 1$ h (15)), chylomicrons would be virtually absent in the fasted state (16) and the majority of the TG-FA would be in the form of VLDL TG-FA. Published VLDL TG production rates for normal subjects range from 200 to 400 mg/kg per day (17-20). Furthermore, it cannot be ruled out that some variable collective contribution of chylomicron and VLDL TG-FA may occur.

Subjects

Six healthy male volunteers reporting no history of diabetes or lipid disorders and no current use of medication participated. The subjects' physical characteristics and energy requirements are shown in **Table 1.** Daily caloric requirement for each subject was calculated using a predictive equation for resting energy expenditure (REE) based on weight, height, age, and gender (21). This value was multiplied by the activity factor of 1.7 suggested for moderately active adult men (22) in order to estimate 24 h energy needs. Screening total cholesterol and plasma TG levels, analyzed by enzymatic techniques (23, 24), fell within normal ranges. The protocol was approved by the Ethical Review Committee of the University of British Columbia. Subjects gave informed consent prior to the investigation.

Protocol

The study was conducted over 72 h in a Metabolic Research Unit. Subjects were instructed to abstain from caffeine and alcohol for 3 days prior to and during the study. During the 72-h study, subjects consumed a nutritionally complete liquid diet (Ensure Plus TM, Ross Laboratories, Montreal, PQ, Canada) as six evenly spaced small meals in volumes sufficient to meet their total daily energy requirements. The liquid diet (355 kcal/235 ml) was composed of 14.7% protein, 53.3% carbohydrate, and 32% fat. The FQ of this diet was 0.88. In addition to providing ease of feeding, use of a liquid diet insured exact knowledge of energy content of each meal. Using evenly spaced meals minimized large variations in TG-FA deuterium uptake patterns caused by influx of dietary TG in the typical self-selected "normal" diet. At 0730 h on day 2 of the nibbling diet, a blood sample (28 ml) was drawn, and subjects drank 0.7 g D_2O/kg estimated total body water (99.8 atom percent excess (APE), ICN Biomedicals, Montreal, PQ, Canada) prior to the 0800 h meal. Total body water was estimated as 60% of body weight. Over the next 48-h period, blood samples

were drawn every 4 h, prior to each meal. Additional D_2O was added to the liquid meals (1.4 g D_2O/kg diet) to maintain body water deuterium enrichment at plateau by compensating for unlabeled water obtained through the diet and drinking water over the study period. It was estimated that the diet provided 50% of total water intake. Subjects were not restricted to the Metabolic Unit during this period, but were required to return every 4 h.

Analytical

Plasma was obtained by centrifugation at 1500 g and 4°C and frozen at −10°C. Lipids from each timepoint were extracted from 1.5 ml plasma using methanol and hexane–chloroform 4:1 (v/v) followed by heating to 55°C. Water was added and the mixture was shaken. Centrifugation at 1500 g for 10 min was followed by removal of the organic phase. This process was repeated, the organic phases were combined, and the solvent was removed under nitrogen. Extracts were redissolved in 200 μl chloroform and spotted onto thin-layer silica plates (Whatman Inc., Clifton, NJ). Plates were developed using petroleum ether–diethyl ether–acetic acid 135:15:1.5 (v/v/v) for 1 h (25). Identification of TG fractions was made by visualization in iodine vapor against a triolein standard. TG fractions were scraped from the plates, eluted with chloroform–methanol 2:1 (v/v), shaken for 15 min, and centrifuged at 1500 g for 15 min. Checks for purity were carried out by re-chromatographing TG fractions using the same solvent system. After removal of the silica, TG fractions were redissolved in chloroform and transferred into pre-annealed 20-cm Pyrex (Corning Glass Works, Corning N.Y.) combustion tubes containing 500 mg cupric oxide wire (BDH Chemicals, Poole, England) and a 2-cm length of silver wire, the latter pre-cleaned in ammonium hydroxide. After thorough evacuation to remove chloroform, samples were combusted at 520°C for 4 h and allowed to cool to room temperature. The combustion water was vacuum-distilled into pre-annealed 20-cm Pyrex tubes containing 60 ± 5 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington

TABLE 1. Physical characteristics of subjects

ID	Age	Wt	Ht	Energy Intake	Screening Plasma Levels	
					Cholesterol	Triglyceride
	yr	*kg*	*m*	*kcal/day*	*mmol/l*	
A	31	63.0	1.68	2604	5.10	0.83
B	26	77.3	1.83	3029	4.89	0.62
C	37	66.4	1.68	2612	4.19	0.99
D	34	70.0	1.80	2854	3.41	0.74
E	32	71.1	1.76	2806	4.03	1.02
F	28	84.0	1.85	3166	4.00	0.87
Avg.	31.3	72.0	1.77	2845	4.27	0.84
SD	4.0	7.6	0.08	224	0.62	0.15

IN). Tubes were flame-sealed under vacuum. TG samples were analyzed in triplicate.

Plasma samples from the 36- and 48-h post-dose time-points were diluted sixfold with tap water of known isotopic composition in order to bring plasma water deuterium enrichments to within the normal analytical range of the mass spectrometer; baseline plasma samples were not diluted. Triplicate 2-μl samples were vacuum-distilled into Zn-containing Pyrex tubes and sealed under vacuum.

Both TG and plasma water samples were reduced to hydrogen gas by heating at 520°C for 30 min. Deuterium enrichment of the hydrogen was analyzed by isotope ratio mass spectrometry (VG Isomass, 903D, Cheshire England) with an internal analytical error of 0.17 per mil (^{0}/00). The H_3^+ contribution was checked daily and appropriate correction factors were applied (26). The mass spectrometer was calibrated daily using three water standards. All samples for each subject were analyzed on the same day using a single set of standards.

Isotopic enrichments are expressed in per mil (^{0}/00) using the del notation defined as:

$$\text{del } (^0/00) = \frac{R_{sample}}{R_{standard}} - 1 \times 1000 \qquad Eq. \ 2)$$

where R is the ratio of the heavy to the light isotope. Standard Mean Ocean Water (SMOW) was the reference standard. In this scale, ^{0}/00 values represent part per thousand changes in enrichment of the sample relative to the deuterium abundance in SMOW. The maximum variation observed among replicate TG-FA deuterium analyses was $\pm 10^0$/00.
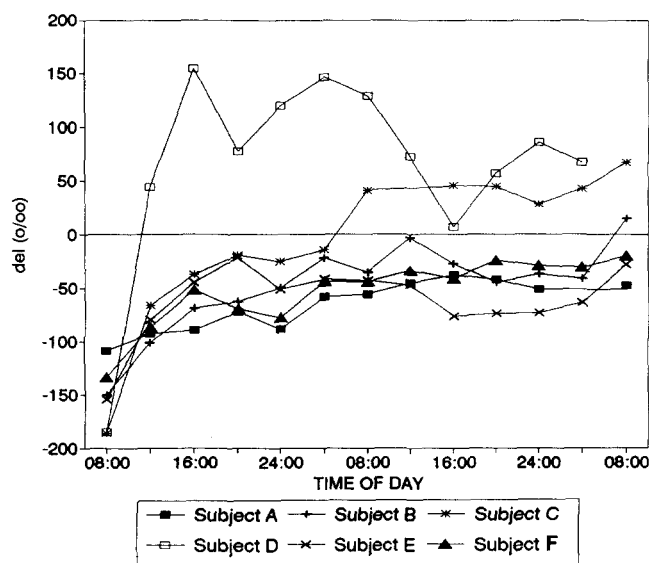

Fig. 1. Observed TG-FA D enrichments of subjects A through F over a 48-h measurement period.
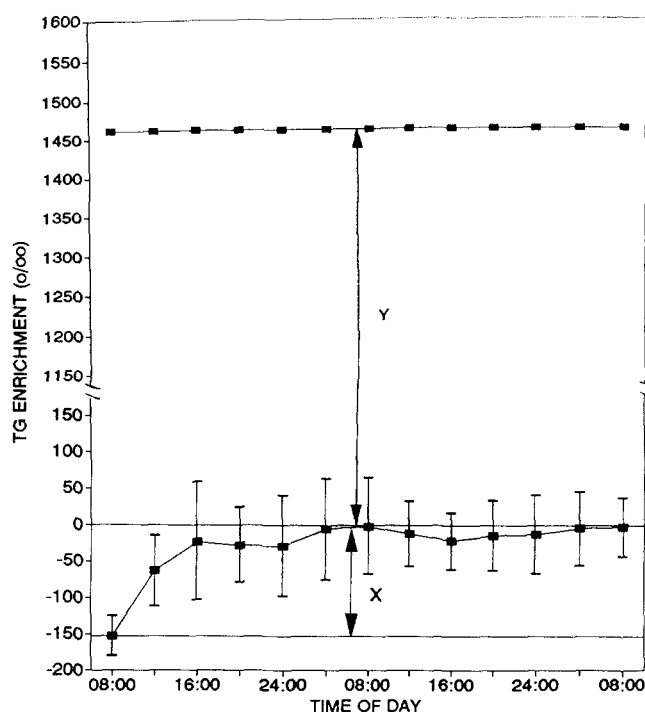

Fig. 2. Average observed TG-FA D uptake curve and theoretical maximum TG-FA D enrichment. X represents the proportion of newly synthesized TG-FA; Y represents the proportion of unlabeled TG-FA. Note the break in the vertical scale.

RESULTS

Mean plasma deuterium levels were not significantly different at 36 and 48 h post-dose, (3255 $\pm$ 339 ^{0}/00 and 3350 $\pm$ 694 ^{0}/00, respectively) indicating that plasma water deuterium enrichments attained plateau.

Fig. 1 shows the TG-FA deuterium enrichments for individual subjects over the study duration. TG-FA deuterium enrichment increased rapidly over the initial 12 h post-dose, followed by a period of minimal changes in TG-FA deuterium enrichment. Each subject also displayed short-term, nonperiodic variations in TG-FA deuterium enrichment although these variations were smaller than those reported previously (9).

Fig. 2 shows the average observed deuterium uptake curve for the six subjects. Also plotted is the theoretical maximum TG-FA deuterium enrichment calculated from equation 1 using the average plasma enrichment. The observed TG-FA deuterium uptake adhered to an exponential function but did not attain the theoretical maximum enrichment. Instead, the observed enrichment reached a plateau after about 12 h post-dose at a value well below the theoretical maximum enrichment. The relationship between observed and theoretical maxima was used to calculate the amount in vivo newly synthesized TG-FA.

This concept is illustrated diagramatically in Fig. 2. X is the observed plateau TG–FA deuterium enrichment and hence is a measure of in vivo TG–FA synthesis; whereas Y is the difference between the observed and theoretical maxima, a measure of the unlabeled TG–FA contribution to the total TG–FA pool. The ratio of the observed plateau enrichment (X) to the theoretical (X + Y) is thus a measure of the contribution of endogenous TG–FA production to the total TG–FA pool. As shown in **Table 2,** although all subjects consumed a constant percentage total calories as fat using model A, the interindividual variation in fat synthesized was considerable, ranging from 4.4 to 17.1 g/day with a mean of 8.8 ± 4.6 g/day.

The results of calculations using model B, assuming a value for VLDL TG–FA production of 300 mg/kg per day and the body weight and percentage of the total VLDL TG–FA pool shown to be labeled, are shown in Table 2. The amounts ranged from 0.87 to 3.09 g/day with a mean of 1.71 ± 0.80 g/day.

One data-set (subject D) shown in Fig. 1 displayed an unusually rapid and variable deuterium uptake pattern compared to the others. As this subject was otherwise normal, the data are included in further calculations. Without this subject, average synthesis rates using models A and B were 7.1 ± 2.5 g and 1.44 ± 0.47 g, respectively.

DISCUSSION

We have shown that deuterium from the plasma water pool undergoes incorporation into plasma TG–FA. While the magnitude of deuterium incorporation varied from individual to individual, a pattern of initial rapid deuterium uptake, followed by a period of relative plateau, emerged. Using this steady-state approach, we calculated the relative contributions of newly synthesized (labeled) and unlabeled TG–FA to the total plasma TG–FA pool. Therefore, the ratio between observed and theoretical maxima reflects the ratio between newly synthesized and unlabeled TG–FA.

Recently Hellerstein et al. (8) determined VLDL–FA synthesis rate in healthy males as less than 500 mg/day using an acetylated-xenobiotic probe technique in which sulfamethoxazole and [^{13}C]acetate were administered simultaneously. Sulfamethoxazole-acetate levels were used to measure the hepatic acetyl-CoA at the time of lipogenesis. Even assuming that the majority of the TG–FA sampled 4 h after a meal would be in the form of VLDL TG–FA, our results are higher than those reported by Hellerstein et al. (8). Moreover, in the present study, our subjects were fed multiple small meals that maintained low plasma insulin concentrations (data not shown) whereas Hellerstein et al. refed oral Ensure or high carbohydrate meals after an overnight fast. We would expect lipogenesis to be reduced in our nibbling paradigm compared with feeding of large meals. A possible explanation of this disparity stems from the labeling protocol used by Hellerstein et al. (8) where the [^{13}C]acetate infusion began at 0200 h yet TG–FA enrichment measures were not commenced until 6 h thereafter. It is possible that TG–FA enrichments were approaching or had already reached plateau enrichment. If so, the observed rate of TG–FA synthesis would be erroneously low.

This report represents the first reported use of deuterium to quantitate the relative proportions of dietary and newly synthesized TG–FA in humans. However, a recent paper by Haggerty et al. (27) describes an experiment in growing piglets examining the incorporation of deuterium from body water into FA in order to assess the magnitude of the effect of deuterium sequestration into

TABLE 2. Amount of fat synthesized in vivo

ID	Dietary Intake[a]	VLDL TG–FA Synthesized	X[b]	X + Y[c]	X/X + Y	TG–FA Synthesized	
						Model A	Model B
	g/day	g/day	$^0/oo$	$^0/oo$	%	g/day	
A	92.6	18.9	61.5	1338.3	4.6	4.4	0.87
B	108.4	23.2	125.2	1856.1	6.7	7.7	1.55
C	92.9	19.9	231.1	2153.7	10.7	10.9	2.13
D	101.4	21.0	254.5	1732.9	14.7	17.1	3.09
E	99.8	21.3	96.6	1782.3	5.4	5.6	1.15
F	112.6	25.2	101.7	1738.2	5.9	6.9	1.49
Avg	101.3	21.6	145.1	1766.9	8.0	8.8	1.71
SD	8.1	2.3	78.7	262.1	3.9	4.6	0.80

[a]Ninety-eight percent of fat intake is assumed to be absorbed.
[b]Average TG deuterium enrichment over the last 24 h of the study period.
[c]Calculated using Eqn. 2 and individual plasma deuterium enrichments.

FA on the results of doubly labeled water studies of energy expenditure and body composition. They observed a rapid rate of lipogenesis as measured through deuterium uptake in growing piglets fed a low fat diet.

Limitations to study

In addition to the uncertainty in the amount of TG–FA synthesis due to our lack of knowledge of the form of the unlabeled TG–FA component, several potential errors in the present approach would be anticipated to result in over- or underestimation of true TG–FA synthetic rate. Overestimation of TG–FA synthesis may result from selection of the H/C incorporation ratio of 0.87 for at least three reasons. First, the ratio used was derived using $^3H_2O/[^{14}C]$glucose incorporation comparisons (10). Swain et al. (28) have estimated that in the absence of isotope effects, the ratio becomes 1.44, compared with 1.28 observed using deuterium (10). The deuterium isotope effect, i.e., the difference between the estimated ratios obtained using deuterium or tritium, will be up to 12%.

Second, the H/C incorporation ratio used presently was obtained in rat adipose tissue. A potential source of error exists in that interorgan differences likely occur in the relative contribution of the pentose phosphate pathway and malic acid-derived NADPH. Because no exchange with cellular water takes place in the pentose cycle, unlabeled protons will contribute to the growing FA during synthesis. Conversely, NADPH produced via the malic enzyme pathway does exchange with cellular water, and thus contributes labeled protons to synthesized fat (29). In adipose tissue, the pentose cycle contribution was estimated at 60% of the total NADPH pool. Wadke et al. (30) suggest that the $^3H/^{14}C$ incorporation ratio in liver is nearly the same as in adipose tissue. However, if this value is lower in the liver, the resulting overestimation of synthesis is unlikely to exceed 20%.

A third potential source of error that may result in overestimation of actual synthesis is the uptake of deuterium label into de novo synthesized glycerol at a ratio lower than that occurring for FAs. A $^3H/^{14}C$ incorporation ratio for glycerol of 1.1 was previously reported (10), which differs from that observed for FA by about 20%. Given that only about 6% of hydrogen atoms are present on the glycerol moiety of TG, the magnitude of error would be small.

New questions

Several present and future questions arise from this experiment. Further examination of the source of the TG–FA pool is required, perhaps through concurrent stable isotope apolipoprotein kinetic studies. Hydrolysis of the glycerol fraction will improve the accuracy of the technique. Future potential applications of the deuterium uptake method include investigation of effects of macronutrient ratio and energy balance on de novo TG–FA synthesis.

In summary, we have monitored the uptake of deuterium into TG–FA in healthy males consuming liquid diets during a steady-state feeding experiment. We have developed a model that uses the relationship between the observed plateau TG–FA deuterium enrichment and the theoretical maximum TG–FA enrichment to calculate the relative contributions of newly synthesized and unlabeled TG–FA to the total pool. The calculated amount of newly synthesized TG–FA is strongly dependent on the source of the TG–FA. If the amount of VLDL TG–FA is small relative to the amount of chylomicron TG–FA, the calculated amount of de novo synthesized TG–FA varies considerably between individuals ranging from 4.4 to 17.1 g/day with a mean of 8.8 ± 4.6 g/day. If, on the other hand, the unlabeled recycled VLDL TG–FA is the major component of the unlabeled total TG–FA pool, the amount of de novo synthesized TG–FA is 1.1–2.2 g/day. Since the chylomicron TG–FA component is expected to be dominant for only a short period after a meal, the average amount of TG–FA synthesis in healthy humans under normal dietary conditions probably falls closer to the lower production estimate determined using model B. Further research is needed to quantitate the proportions of chylomicron and VLDL TG–FA present, the amount of inter-individual variability in TG–FA synthesis, and the changes in synthetic rate in individuals during differing dietary and physiological conditions. Techniques such as presently described should permit better understanding of factors that control human lipogenesis. ▮▮

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