Direct measurement of apolipoprotein B synthesis in human very low density lipoprotein using stable isotopes and mass spectrometry[1]

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Abstract Stable isotope methodology has been adapted to the study of lipoprotein turnover in human subjects. Using endogenous [^{15}N]glycine labeling and gas–liquid chromatographic–mass spectrometric analysis, synthesis of apolipoprotein B in very low density lipoprotein (VLDL) was measured directly in five normal and two hyperlipidemic subjects. An isotopic precursor steady state was achieved during the studies by utilizing a priming dose and constant infusion containing [^{15}N]glycine. Measurement of the plateau in ^{15}N enrichment in the urinary hippurate produced during each study was used to estimate the ^{15}N enrichment of the hepatic glycine precursor pool. The range of values for the fractional synthetic rate of VLDL apoB in the normal subjects obtained by this method was 5.9 to 11.5 day^{-1}, with a mean of 9.2 ± 2.4 (SD). This value agrees with the results of previous investigations which have utilized other methods. The method was also tested in two hypertriglyceridemic subjects and gave fractional synthetic rates of VLDL apoB that were significantly lower than in normals (1.5 and 2.8 day^{-1}). This stable isotope method allows calculation of the fractional synthetic rate of VLDL apoB by maintaining an isotopic steady state throughout the study. It makes possible repeated studies in the same individual since no risk of exposure to radioisotopes is involved. —**Cryer, D. R., T. Matsushima, J. B. Marsh, M. Yudkoff, P. M. Coates, and J. A. Cortner.** Direct measurement of apolipoprotein B synthesis in human very low density lipoprotein using stable isotopes and mass spectrometry. *J. Lipid Res.* 1986. **27:** 508–516.

Supplementary key words gas–liquid chromatography–mass spectrometry • fractional synthetic rate • hepatic secretory protein • [^{15}N]glycine • hippurate • protein turnover • hypertriglyceridemia • atherosclerosis

Apolipoprotein B (apoB) plays an important role in lipoprotein metabolism (1). This protein (sometimes designated apoB-100 to distinguish it from the related but smaller, intestinally derived apoB-48) is synthesized in the liver, packaged with lipids and other apolipoproteins, and secreted into the plasma as very low density lipoprotein (VLDL) (2). Hydrolysis of VLDL triglycerides by lipoprotein lipase at the capillary endothelial wall results in a reduction in size and an increase in density of the VLDL particle, which then can be taken up by the liver. Alternatively, this VLDL remnant can undergo further delipidation, lose the non-B apolipoproteins and evolve into low density lipoprotein (LDL), which is then cleared from the plasma through the LDL receptor mechanism (3). Primary or secondary alterations in the production, metabolism, and clearance of VLDL and related B-100-containing lipoproteins can be associated with an acceleration of the atherosclerotic process (4). For example, increased synthetic rates for VLDL apoB have been reported in subjects with familial combined hyperlipidemia (5). In addition, increased turnover rates for apoB have been reported in some normocholesterolemic patients with premature coronary artery disease (6).

The study of apoB metabolism has been approached in a variety of ways in normal and hyperlipidemic states. The most widely used technique to measure clearance employs radioiodination of purified lipoproteins obtained by plasmapheresis which are reinjected into the subject; the radioactive label is then followed in lipoproteins from sequential blood samples (7). Another approach has utilized the injection of a radioactively labeled amino acid precursor (such as ^{75}Se-labeled methionine or [^{3}H]leucine) with subsequent observation of its appearance in and disappearance from the lipoproteins of interest (8, 9). Both of these approaches use radioactive isotopes and

Abbreviations: apoB, apolipoprotein B; EDTA, ethylene diamine tetraacetic acid; FSR, fractional synthetic rate; GLC–MS, gas–liquid chromatography–mass spectrometry; HDL, high density lipoprotein; LDL, low density lipoprotein; TAB, *n*-butyl-N-trifluoroacetyl;TBDMS, *t*-butyl-dimethylsilyl; TG, triglycerides; TMS, trimethylsilyl; VLDL, very low density lipoprotein.

[1]Portions of this work were presented at the Third Clinical Research Centre International Symposium, Harrow, UK, 1984, and at the 57th Scientific Sessions of the American Heart Association, Miami Beach, FL, 1984, and were published in abstract form (Cryer, D. R., et al. 1984. 3rd CRC International Symposium "Substrate and Energy Metabolism in Man," Harrow, UK; Cryer, D. R., et al. 1984. *Circulation.* **70:** II-313).

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measure the catabolism of the lipoprotein utilizing the plasma decay curves observed as the radioactivity moves out of the lipoprotein pool of interest. Such methods require computerized modeling of the data for a proper interpretation of the lipoprotein kinetics. The present work utilizes a new approach to the study of the kinetics of VLDL apoB. The method involves the administration of stable isotope-labeled amino acids by a constant intravenous infusion, following a priming dose, that achieves and maintains an isotopic steady state. After isolation of the VLDL apoB, the isotopic enrichment of a constituent amino acid is measured by gas–liquid chromatography–mass spectrometry (GLC–MS) and the change in enrichment over time is used to calculate synthetic rates for the protein.

METHODS

Subjects

Five adult control subjects were selected for this study (subjects 1–5 in **Table 1**) based on normal plasma lipid levels as determined by the methods outlined below. All were in good health without evidence of hepatic, renal, or thyroid dysfunction. None had diabetes nor were any receiving drugs known to affect lipid metabolism. None had family histories of significant premature atherosclerosis.

Two hyperlipidemic subjects were selected for study (subjects 6 and 7 in Table 1). Both had significant family histories for early heart disease, both had plasma triglyceride elevations at the time of study, and the second had established coronary artery disease. Both were otherwise in good health and neither was currently receiving any drug known to affect lipid metabolism.

Study protocol

Subjects were studied in the Lipid-Heart Research Center or the General Clinical Research Center after giving informed consent; the protocol was approved by the Committee for the Protection of Human Subjects of The Children's Hospital of Philadelphia. Each study was begun in the morning following a 12-hr fast and subjects were allowed only clear, noncaloric liquids during the course of the study. No specific dietary restrictions were imposed prior to the studies. An intravenous line was inserted into one forearm and a heparin lock was placed on the dorsal surface of the opposite hand for blood sampling. The hand containing the heparin lock was warmed to promote arterialization of blood flow and minimize local tissue effects prior to each sampling. Blood and urine samples were obtained prior to the start of each protocol. At time zero, a priming dose of 9.3 μmol/kg of [^{15}N]glycine (99% ^{15}N, KOR Isotopes, Cambridge, MA) was given intravenously over 2 to 3 min. The [^{15}N]glycine was administered as a 3% (w/v) solution in normal saline containing 0.9% (v/v) benzyl alcohol as preservative. This was followed immediately by a continuous intravenous infusion of [^{15}N]glycine (0.328 μmol/kg per min) delivered by a Harvard syringe pump over the 8- to 10-hr duration of the study. The fixed pump settings required that the infused solution be diluted slightly with additional saline to compensate for variations in weight of subjects. Blood samples of 7 to 35 ml were drawn at various intervals after a steady state level of [^{15}N]glycine in plasma was reached, which occurred within 15 min under these conditions. Drinking of clear liquids was encouraged and urine samples were collected at frequent intervals during the studies.

TABLE 1. Plasma lipid values for five controls and two patients

Subject	Age/Sex	Weight	Cholesterol		Total TG
			Total	HDL	
	yr	*kg*		*mg/dl*	
Controls					
1	31/M	62	177	47	65
2	35/F	53	128	59	60
3	36/M	60	138	69	51
4	52/M	73	220	61	67
5	40/M	75	192	71	76
Patients					
6	36/M	73	212	19	338
7	54/M	87	211	26	492

Glycine analysis

Blood samples (7 ml) for free plasma [^{15}N]glycine analysis were drawn into standard heparinized blood tubes (Vacutainer; Becton, Dickinson and Co., Rutherford, NJ), immediately placed on ice, and kept cold until plasma could be separated by centrifugation. Heparinized plasma samples were processed immediately or stored at −70°C. Disposable columns were prepared for the partial purification of plasma amino acids using Pasteur pipettes containing 2 ml of AG 50W-X8 resin (H^+ form; Bio-Rad Laboratories, Richmond, CA) supported in the pipette by a GF/D glass fiber filter (Whatman; Fisher Scientific Co., Pittsburgh, PA). The resin was prewashed with distilled deionized water and equilibrated with a 2-ml wash of buffered saline, pH 7.4. One ml of plasma was acidified with 3 ml of 1 N acetic acid and applied to the column. This was followed by a 5-ml wash with distilled, deionized water. Amino acids were eluted with 6 ml of 4 M NH_4OH, collected in tubes, and lyophilized. The dry residue was transferred to a tapered reaction vial (Pierce Chemical Co., Rockford, IL) and volatile derivatives of glycine and other amino acids were then produced in a two-step procedure in which the amino acids were first heated at 120°C for 30 min with 3 N HCl in butanol, followed by drying under nitrogen. The tubes were then heated at 70°C for 15 min with 25% (v/v) trifluoroacetic

anhydride in methylene chloride to form the *n*-butyl-N-trifluoroacetyl (TAB) derivatives (10).

Blood samples for isolation of apoB were drawn into EDTA tubes (Vacutainer) to which had been added a mixture of inhibitors of enzymes and bacterial growth (10 mM phenylmethylsulfonyl fluoride; 0.01% (w/v) sodium azide; 2 U/ml aprotinin) (11). The tubes were placed immediately on ice. VLDL was prepared by standard ultracentrifugal methods (12) using a Type 50Ti fixed angle rotor (Beckman Instruments, Palo Alto, CA) at 35,000 rpm (81,000 *g*) for 18 hr at 10°C. Six-ml plasma samples were overlaid with 2 ml of 0.195 M NaCl containing 2 mM EDTA (density 1.006 g/ml) prior to centrifugation and the VLDL was floated into the NaCl solution to free it of contaminating proteins. The VLDL was removed in the top 1.5 ml and the sample was divided into aliquots for VLDL apoB measurement or isolation.

The apoB of VLDL was isolated and purified using the isopropanol precipitation method of Egusa et al. (13). None of the other VLDL apolipoproteins or other plasma proteins was detectable when the apoB precipitate was solubilized and subjected to SDS polyacrylamide gel electrophoresis and Coomassie blue staining (14); the apoB migrated as a single band of approximately 400,000–500,000 daltons.

The precipitated VLDL apoB was dried under a nitrogen stream and then quantitatively hydrolyzed to amino acids using 6 N HCl at 120°C for 16 hr under nitrogen. The HCl was evaporated and the TAB derivatives of glycine and other amino acids were produced as described above. The VLDL apoB amino acid derivatives thus obtained from 5 ml of plasma are adequate for at least 20 GLC–MS analyses (below).

GLC–MS analysis was performed using a Finnigan 4121 low resolution quadrupole mass spectrometer (Finnigan Instruments, San Jose, CA) equipped with a Finnigan 9610 gas chromatograph and capillary column (30 m × 0.32 mm, DB-5 bonded phase; Alltech Associates, Inc., Deerfield, IL) or a quadrupole design Hewlett-Packard 5970B Mass Selective Detector (Hewlett-Packard, Palo Alto, CA) equipped with an HP 5890A gas chromatograph and capillary column (25 m × 0.20 mm, cross-linked 5% phenylmethyl silicone bonded phase; Hewlett-Packard). Both instruments were computer controlled. Helium was used as carrier gas; flow rates and temperature program were optimized for the clean separation of the glycine TAB ester. The column effluent was ionized in the electron impact mode and selected ion monitoring of the glycine peak was performed for ^{15}N enrichment using the ions of m/z = 172 and 173 (15).

Enrichment calculations were made using a computer program based on the method of Biemann (16). Enrichment, in terms of atom percent excess, is the percentage of stable isotope measured in a sample above natural abundance and is equivalent to the term "specific activity" used in radioisotope tracer studies. Natural abundance is taken to be the level of the stable isotope measured in the sample obtained at time zero; it is equivalent to "background" in radioisotope studies and is assumed to be constant.

Hippurate analysis

A 1-ml urine sample was used for each hippurate extraction. The pH was adjusted to approximately 2.5 by dropwise addition of 6 N HCl. The sample was applied to a disposable 3-ml octadecyl (C_{18}) column (Baker-10 SPE®; J. T. Baker Chemical Co., Phillipsburg, NJ) which had been conditioned by sequential addition of one column volume of methanol and one column volume of 0.1 N HCl. After sample addition, the column was washed twice with 1-ml aliquots of 0.1 N HCl, air dried, then washed with 1 ml of hexane followed by a final drying for 1 min. The hippurate was eluted from the column by adding 0.25 ml of methanol to the column and allowing it to stand for 1 min, followed by two additional 0.5-ml aliquots of methanol (17). The partially purified hippurate was dried under a nitrogen stream at room temperature. Two methods were used to make volatile derivatives of hippurate for GLC–MS analysis. In the first, the residue was dissolved in 160 μl of bis-trimethylsilyltrifluoroacetamide plus 40 μl of trimethylchlorosilane, then heated at 60°C for 30 min under nitrogen to form the trimethylsilyl (TMS) derivatives. The sample was then cooled, dried under a nitrogen stream, and redissolved in ethyl acetate for injection into the gas chromatograph. The second method involved dissolving the residue in an equal volume mixture of acetonitrile and N-methyl-N-*t*-butyldimethylsilyltrifluoroacetamide containing 1% *t*-butyldimethylchlorosilane and heating at 60°C for 60 min under nitrogen to yield the *t*-butyldimethylsilyl (TBDMS) derivative. Again the sample was cooled, dried, and redissolved in ethyl acetate for analysis.

Hippurate derivatives were analyzed by GLC–MS using the same columns as used for glycine analysis, with temperature programming optimized for separation of the less volatile hippurate derivatives. ^{15}N enrichment was determined using electron impact and selected ion monitoring modes with measurements of the m/z = 206 and 207 fragments for the TMS derivative of hippurate and the m/z = 236 and 237 fragments for the TBDMS derivative. Standard curves of known ^{15}N enrichment for hippurate were developed using mixtures of labeled and unlabeled derivatives of hippurate prepared by both methods, and these curves were used in the enrichment calculation.

Other laboratory methods

Plasma cholesterol and triglycerides were measured using reagents available in kit form from BMC Diag-

nostics, Indianapolis, IN. HDL-cholesterol was measured following precipitation of apoB-containing lipoproteins with phosphotungstic acid/magnesium chloride, also obtained from BMC Diagnostics. Determinations of lipid levels on over 500 control subjects by this laboratory gave normal ranges for these lipids that were not significantly different from those reported by the Lipid Research Clinics (18).

Labeled hippurate for the generation of mass spectrometer standard curves was synthesized from [^{15}N]glycine and benzoyl chloride using a modification of the method of Ingersoll and Babcock (19).

Mathematical methods

Calculation of the fractional synthetic rate (FSR) for VLDL apoB was performed using the relationship:

$$FSR = \frac{(\text{change in product enrichment})/\text{time}}{\text{precursor enrichment}}$$

where the product was VLDL apoB, the precursor enrichment was assumed to be equivalent to the enrichment of hippurate at plateau, and the change in product enrichment was determined for the early period of rapid enrichment in protein where the data approximate a straight line (20). Linear regression analysis was used to determine the slope of the line used for the numerator. Calculation of the plateau values for ^{15}N enrichment in hippurate was based on the solution of an exponential model assuming a single compartment. A Hewlett-Packard HP-85 computer was used for these calculations. Preliminary calculations of fractional synthetic rates in the five normal subjects were performed using the same equation but, instead, calculating a plateau value for the ^{15}N enrichment of VLDL apoB glycine, assuming this to be a good approximation of the precursor pool enrichment, and using this value as the denominator of the equation.

Results are expressed as mean ± SD.

RESULTS

^{15}N enrichment of VLDL apoB glycine and urinary hippurate

The data from a typical [^{15}N]glycine infusion study on subject 4 are presented in **Fig. 1.** With a primed constant infusion, the second plasma sample (taken 15 min after beginning the study) already showed [^{15}N]glycine enrichment of the plasma glycine pool (upper symbols) at a level that remained relatively stable throughout the course of the study and was assumed to be effectively at steady state during the first 3 hr of the study on which the calculations were based.

The measured incorporation of [^{15}N]glycine into VLDL apoB during the 8-hr infusion is also shown in Fig. 1. The level of ^{15}N enrichment in VLDL apoB glycine at time zero, prior to isotope administration, is defined as zero atom percent excess. There was a slow initial appearance of labeled glycine in the plasma pool of VLDL apoB (lower curve). This was followed by a 90-min period of rapid increase in ^{15}N enrichment, which then approached a plateau at 4 to 5 atom percent excess by 6 to 7 hr.

The ^{15}N enrichment of hippurate extracted from sequential urine samples was determined in order to obtain an indirect measure of the ^{15}N enrichment of the hepatic precursor of VLDL apoB, since both VLDL apoB and hippurate are synthesized from hepatic glycine (see Discussion). The results of these measurements for subject 4 are shown by the middle curve in Fig. 1. In this subject, the appearance of labeled hippurate in the urine occurred very rapidly, such that by the time the second urine sample was collected in this study, the ^{15}N enrichment in hippurate had increased sharply relative to the zero time value and was already close to the final ^{15}N enrichment level determined at the end of the study.

Table 2 lists the VLDL apoB glycine enrichment data from all seven studies reported here. The five normal subjects (1 through 5) display rapid incorporation of [^{15}N]glycine into VLDL apoB between about 0.5 and 3 hr, followed by a progressively slowing rate of increase in enrichment to the end of the study. The mean VLDL apoB enrichment data for the studies on these subjects are

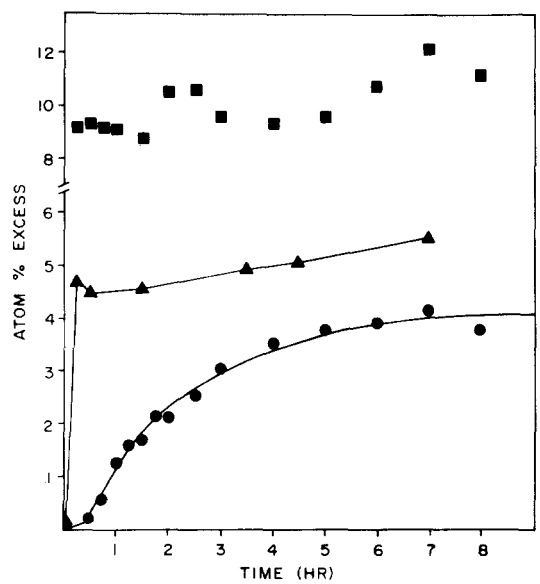

Fig. 1. Incorporation of [^{15}N]glycine into VLDL apoB, plasma glycine, and urinary hippurate of a normal subject (#4) during a primed constant [^{15}N]glycine infusion; (■) concentration of free [^{15}N]glycine in plasma; (▲) ^{15}N enrichment of urinary hippurate; (●) [^{15}N]glycine incorporated into VLDL apoB.

TABLE 2. Incorporation of [^{15}N]glycine into VLDL apoB of subjects (1-7) during intravenous [^{15}N]glycine infusion

	Enrichment in Subjects						
Time	1	2	3	4	5	6	7
hr							
0	0[a]	0	0	0	0	0	0
0.5	0.03		0.06	0.20	0	0	0.05
0.75		0.13	0.45	0.54	0.01		0.09
1.0	1.31	0.55	0.90	1.25	0.29	0.07	0.23
1.25		0.86	1.35	1.59	0.59	0.09	0.33
1.5	2.33	1.11	1.74	1.68		0.44	0.31
1.75				2.15			
2.0	2.71		2.33	2.14	1.57	0.54	0.42
2.5	2.75		2.72	2.58			0.46
3.0	3.26	2.50	3.11	3.04	2.24	0.73	0.72
3.5	3.11						
4.0	3.73	2.28	3.30	3.53	2.53	0.52	0.97
5.0	3.93	2.88	4.13	3.80	3.39	1.78	1.34
6.0	4.13	2.75	3.33	3.93		2.25	1.92
7.0	4.91	4.08		4.19	3.59	1.39	1.94
8.0	4.52	3.80		3.83		2.40	2.22
9.0	5.01						

[a]Enrichment of VLDL apoB in [^{15}N]glycine at time zero, before isotope administration, is defined as zero. Enrichment is expressed as atom % excess.

shown in **Fig. 2** with the standard deviations for each time point depicted by the vertical brackets. The curve of the mean values is seen to be very similar to that shown for subject 4 in Fig. 1.

The ^{15}N enrichment in VLDL apoB glycine for the two hyperlipidemic subjects (6 and 7) is also contained in Table 2. It is noted that incorporation into the VLDL apoB pool proceeds more slowly in these subjects than in the control subjects.

The values of ^{15}N enrichment in urinary hippurate for all subjects are presented in **Table 3.** Urine samples were collected during the time periods for which their enrichments are listed, although not at precisely the same times in each case. The ^{15}N enrichment in the urine sample obtained immediately prior to beginning each study was defined as zero atom percent excess. This sample was used as the standard against which subsequent urine samples were compared. For subjects who voided more than once during a time period, the enrichment levels are listed sequentially. While there was some variation in the rate of appearance of labeled urinary hippurate from subject to subject, all seven subjects reached similar plateau values by the end of their studies. The mean values for ^{15}N enrichment in urinary hippurate, also in Table 3, show relatively little deviation, particularly late in the studies.

^{15}N enrichment of VLDL apoB glycine and urinary hippurate in a hyperlipidemic subject

The data for ^{15}N enrichment of VLDL apoB glycine and urinary hippurate in hypertriglyceridemic subject 7 are shown in **Fig. 3.** The conditions of the study were the same as those used for subject 4 in Fig. 1, as described in Methods. In this subject, although ^{15}N enrichment in hippurate (upper curve) achieved a plateau similar to that observed in normolipidemic subjects, the incorporation of [^{15}N]glycine into VLDL apoB (lower curve) proceeded at a significantly slower rate and did not approach a plateau during the course of the study.

Calculation of fractional synthetic rates for VLDL apoB

The FSR was calculated using the equation described in Methods. Between 30 min and 2 hr the increase in enrichment was essentially linear, so that a simple linear slope was determined for each study in this time period and defined as the change in enrichment of VLDL apoB for use in the numerator of this equation. The calculated plateau of ^{15}N enrichment in urinary hippurate was used as the denominator of the equation. The individual FSR values and the means for the five normal studies are given in **Table 4.** The range of values obtained was 5.9 day^{-1} to 11.5 day^{-1} with a mean of 9.2 day^{-1}.

The fractional synthetic rates for the two hyperlipidemic subjects were calculated in the same manner as used for the normal subjects. A linear slope was determined for VLDL apoB between 30 min and 3 hr and defined as the change in ^{15}N enrichment for the protein. Again, the calculated plateau in hippurate enrichment was used as an estimate of hepatic precursor (glycine) ^{15}N enrichment. Calculated fractional synthetic rates for subjects 6 and 7 were 2.8 and 1.5 day^{-1}, respectively, as noted in Table 4.

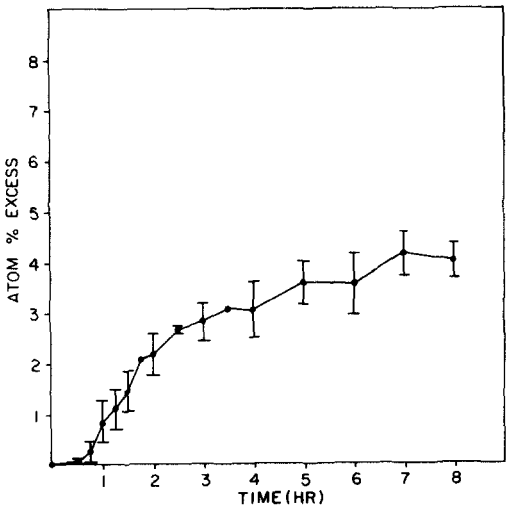

Fig. 2. Mean enrichment values (± SD) of [^{15}N]glycine in VLDL apoB for all five normal subjects. Points at 1.75 and 3.5 hr are data from single experiments.

TABLE 3. ^{15}N enrichment in urinary hippurate from seven subjects

Time[a]	Enrichment in Subjects 1	2	3	4	5	6	7	Mean	± SD
hr									
0-1	4.85[b]	2.10	2.00	4.70	1.80	3.15	4.10	3.42	1.36
	5.05			4.50	1.95				
1-2	4.90	3.65	2.50	4.60	2.80	4.15	4.80	3.91	0.97
2-3	4.20	4.30			3.35	4.70	4.30	4.17	0.50
3-4			4.10	5.00	3.60	4.45	4.75	4.38	0.55
4-5		5.00		5.10	4.00	4.80	5.10	4.80	0.46
5-6	4.90	4.95	4.65					4.83	0.16
6-8	4.80	5.10		5.60	4.20	4.95	4.90	4.93	0.45

[a]Urine samples were obtained during the time periods indicated. Subjects voided immediately prior to each study. In time periods during which a subject voided more than once, the measured enrichments are listed sequentially.

[b]Enrichment values are expressed as atom % excess; values are rounded off to nearest 0.05 atom % excess. Enrichment of hippurate from the urine specimen obtained immediately before each study was defined as zero atom % excess and was used as the control value against which subsequent hippurate samples were compared.

DISCUSSION

The stable isotope incorporation method that we have developed to study VLDL apoB synthesis in human subjects has yielded mean values for the fractional synthetic rate for five fasted normolipidemic individuals of 9.2 ± 2.4 day^{-1}. This is close to the value of 13.9 day^{-1} reported by Eaton, Allen, and Schade (21) for VLDL apoB using endogenous ^{75}Se-labeled methionine. It is also well within the range of 4.3 to 13.9 day^{-1} obtained when ^{125}I-labeled VLDL is reinjected into the subject (reviewed in 5). The stable isotope method reported here gives FSR values that have a small variance and compares very favorably to other studies thus far reported using five or more subjects. In subjects such as those studied, who maintain constant VLDL levels, it is assumed that the fractional synthetic rates are equivalent to the fractional catabolic rates measured for VLDL apoB by others.

Stable isotopes have important advantages for the study of plasma lipoprotein synthetic rates. The primary advantage is their relative safety, since no radioactivity is involved. This allows their use in children and pregnant or lactating women and permits repeated studies in the same individual. Furthermore, these stable isotope incorporation methods do not require the metabolic steady-state assumption necessary to estimate synthetic rates from measurements of catabolic rates. The use of a priming dose of labeled precursor followed by an intravenous infusion allows the rapid establishment of an isotopic steady state in plasma glycine, and more importantly in hepatic glycine (22) (as reflected in urinary hippurate measurements, discussed below). This hepatic steady state is maintained over the relatively short period of measurement (8 to 10 hr) and thus allows a simple calculation of the synthetic rate (23). Significantly, Fern and Garlick (22) have demonstrated rapid equilibration of radioactive glycine between free plasma and liver glycine pools in continuous infusion experiments on rats. The constant plasma levels they observed correlated with constant, though lower, intracellular liver levels.

Another substantial advantage of stable isotope methodology is the capability of the GLC-MS technique to measure an internal ratio of labeled (^{15}N) to unlabeled (^{14}N) apoB glycine in a sample of any size from an apoB pool. Thus quantitation of the protein is unnecessary for accurate calculation of its fractional synthetic rate. Other considerations that apply to endogenous labeling methods in general have been reviewed by Peters (24).

The present method requires some assumptions. The fractional synthetic rate calculation depends on knowledge of the isotopic enrichment of the precursor pool, hepatic glycine. To estimate this, we initially used the plateau value for VLDL apoB ^{15}N enrichment since in an isotopic steady state the enrichment of a product should ultimately approach the enrichment of its precursor. This gave estimated values for hepatic glycine enrichment which were 40-50% of the level of plasma glycine enrichment; these data were consistent with data obtained from [^{14}C]glycine or [^{14}C]lysine infusions in rats (22, 25).

We subsequently replaced this method of estimation with measurement of the ^{15}N enrichment of urinary hippurate, reasoning that hepatic proteins and hippurate

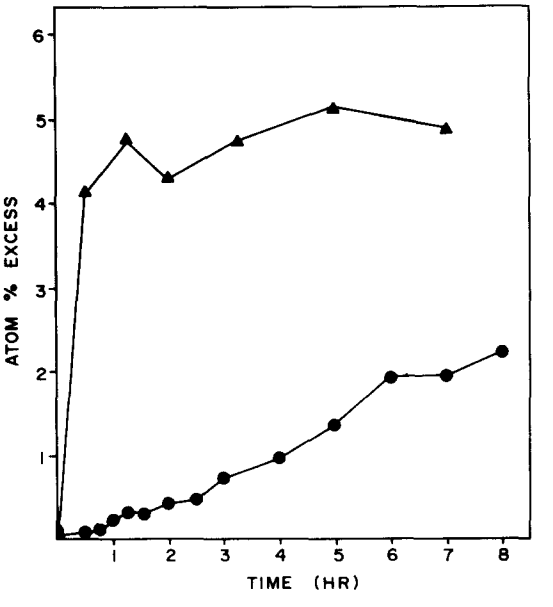

Fig. 3. Incorporation of [^{15}N]glycine into hippurate and VLDL apoB in a hypertriglyceridemic subject (#7) during a primed [^{15}N]glycine infusion; (▲) ^{15}N enrichment in hippurate; (●) [^{15}N]glycine enrichment in VLDL apoB.

are both synthesized from hepatic glycine and that a plateau in enrichment of either should be the same and would be a valid measure of precursor pool enrichment. This was necessary since it was expected that subjects with expanded VLDL apoB pools (e.g., hypertriglyceridemics) would not approach a plateau of ^{15}N enrichment in apoB during the course of a study and thus precursor levels could not be estimated using the apoB measurements in such patients.

The use of hippurate measurement in these studies involves practical considerations as well as assumptions. The benzyl alcohol in the [^{15}N]glycine infusate is rapidly oxidized to benzoate ($t_{1/2}$ = 90 min) and, in these studies, adds to the endogenous substrate pool which is conjugated with glycine to form hippurate in the liver. Rapid renal clearance of hippurate is known to occur (26), so that long-term retention of hippurate in a plasma pool does not represent a potential problem, but it must be assumed that the glycine of hippurate has the same ^{15}N enrichment as the glycyl-tRNA from which apoB is formed. This assumption could be incorrect, since hippurate is synthesized in the mitochondria and apoB in the cytoplasm of the hepatocyte. However, there is good experimental evidence for rapid equilibration between the cytosolic and mitochondrial glycine; thus any systematic error would probably be small (27). Furthermore, careful studies on albumin turnover by Weissman et al. (28) and on fibrinogen turnover by Stein, Leskiw, and Wallace (29) indicate that specific activity of urinary hippurate is a good measure of free liver glycine specific activity. Although the kinetics of the appearance of labeled urinary hippurate are under the influence of some factors that are totally independent of apoB synthesis (e.g., additional sources of benzoate such as gut flora, body hippurate pool size, fluid balance, renal clearance rates, urinary retention, etc.), the references cited support the assumption that, by the end of these studies, the enrichment of hippurate appearing in freshly voided urine should have attained plateau.

Similarly, another possible source of error would be dilution of the mitochondrial [^{15}N]glycyl-tRNA pool by mitochondrial conversion of unlabeled serine to glycine, which might not simultaneously dilute the cytoplasmic glycine destined to be incorporated into VLDL apoB. Again, we assume rapid equilibration between cytoplasmic and mitochondrial glycine pools. In addition, if labeled glycine is converted to serine and then the serine is reconverted to glycine, an isotope recycling error would occur. Although such pathways for interconversion do exist (30, 31), this is not likely to be an important source of error since we were unable to detect significant isotopic enrichment of apoB with [^{15}N]serine after hydrolysis of apoB at the conclusion of a study.

When we calculate FSR values in normal subjects, we also assume that the initial appearance of label in VLDL apoB approximates a straight line. Linear incorporation kinetics for hepatic proteins have been observed using constant infusions of labeled amino acids in rats (22, 32). This straight line interpretation of our data is consistent with a simple model of VLDL apoB synthesis with the following features. There is an initial delay in appearance of label in VLDL apoB during which time plasma [^{15}N]glycine equilibrates with hepatic glycine and newly synthesized labeled apoB is assembled into intact VLDL particles. These particles are subsequently secreted into the plasma where most undergo delipidation before removal from the plasma. Thus, during the early time points, we are observing pure appearance of label in the VLDL apoB plasma pool without disappearance. Sixty to 90 min after label appears in VLDL apoB, partially delipidated labeled VLDL begins to be taken out of the plasma. At this point, the ^{15}N enrichment curve reflects disappearance as well as appearance of labeled VLDL apoB, and the slope of the curve begins to change toward plateau. When we applied the exponential equation used for hippurate plateau determination to the apoB ^{15}N enrichment data for control subjects, similar plateau values were obtained (4.1 ± 0.2 for apoB vs. 4.7 ± 0.2 atom percent excess for hippurate). Alternatively, using the initial rates for apoB ^{15}N enrichment generated by the exponential model to estimate FSR also gave a mean value quite close to that determined assuming a linear slope for the early period of rapid incorporation (mean = 11.3 ± 3.8 day^{-1}). Therefore, with this method, we feel the use of a straight line gives a good approximation of the FSR.

The measurements of FSR in the hypertriglyceridemic subjects have been included here primarily to illustrate an important potential extension of this methodology. They demonstrate that the stable isotope method can be applied successfully to studies of hyperlipidemic subjects with

TABLE 4. Synthesis of VLDL apoB by five controls and two patients

Subject	Fractional Synthetic Rate[a]
	day^{-1}
Controls	
1	11.5
2	5.9
3	11.3
4	7.8
5	9.7
Mean ± SD	9.2 ± 2.4
Patients	
6	2.8
7	1.5

[a]Fractional synthetic rate (FSR) is calculated as described in Methods using the plateau enrichment value in urinary hippurate as an estimate of precursor enrichment.

expanded VLDL apoB pools provided that the expansion is not so large as to preclude measurable isotope enrichment of apoB within the period of study. The GLC-MS instruments we have employed are capable of accurate detection of isotope enrichment at the 0.1 atom percent excess level and were adequate to measure isotope incorporation into apoB of the subjects studied under our conditions. Isotope dilution in a larger VLDL pool might prevent accurate enrichment measurements and preclude the estimate of synthetic rates in severely hypertriglyceridemic subjects. It should be noted that isotope ratio mass spectrometers are capable of far greater sensitivity (33) and thus offer a technically feasible alternative approach to the study of apoB or other apolipoproteins in larger pools (such as LDL apoB or HDL apoA-I) using stable isotopes. As predicted on the basis of their larger VLDL pools, incorporation of label into VLDL apoB in hypertriglyceridemic subjects was lower than that observed in normal subjects. This gave lower calculated fractional synthetic rates for VLDL apoB in these subjects, which is in accord with the existing literature (34), and gave further evidence of the usefulness of hippurate as a measure of precursor ^{15}N enrichment in such studies. Attainment of VLDL apoB plateau is not seen to occur in studies on hypertriglyceridemic subjects; however, the use of hippurate as a measure of precursor enrichment obviates the need to obtain a plateau value of apoB enrichment in order to calculate FSR.

We are currently developing methods of isotope ratio mass spectrometry and multicompartmental computer modeling in order to extend the present work to studies of the rate of conversion of VLDL to LDL and the effects of perturbations on VLDL apoB synthesis. The present method should also be readily adaptable to studying the synthesis of other liver secretory proteins.

The authors wish to thank Sydell Lewis and Marie C. Ragni for expert technical assistance and Randi Plotnick for secretarial assistance. We also thank Dr. Itzhak Nissim for helpful discussions in the early phases of this work and Dr. Stanton Segal for his encouragement and support of this project. This work was supported by NIH grants HL-18723, HL-22633, HD-08536, HD-07107, and RR-00240, a grant from Merck and Co., Inc., and a grant from the W. W. Smith Charitable Trust.

Manuscript received 29 July 1985.

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