The ^{3}H-Leucine Tracer: Its Use in Kinetic Studies of Plasma Lipoproteins

Waldo R. Fisher, Vaidehi Venkatakrishnan, Eric S. Fisher, Peter W. Stacpoole, and Loren A. Zech

^{3}H-leucine administered as a bolus has been widely used as a tracer in kinetic investigations of protein synthesis and secretion. After intravenous injection, plasma specific radioactivity decays over several orders of magnitude during the first half-day, followed by a slow decay lasting a number of weeks that results from recycling of the leucine tracer as proteins are degraded and ^{3}H-leucine reenters the plasma pool. In studies in which kinetic data are analyzed by mathematical compartmental modeling, plasma leucine activity is generally used as a forcing function to drive the input of ^{3}H-leucine into the protein synthesis pathway. ^{3}H-leucine is an excellent tracer during the initial hours of rapidly decreasing plasma activity; thereafter, reincorporation of recycled tracer into new protein synthesis obscures the tracer data from proteins with slower turnover rates. Thus, for proteins such as plasma albumin and apolipoprotein (apo) A-I, this tracer is unsatisfactory for measuring fractional catabolic (FCR) and turnover rates. By contrast, the kinetics of plasma very–low-density lipoprotein (VLDL)-apoB, a protein with a residence time of approximately 5 hours, are readily measured, since kinetic parameters of this protein can be determined by the time plasma leucine recycling becomes established. However, measurement of VLDL-apoB specific radioactivity extending up to 2 weeks provides further data on the kinetic tail of VLDL-apoB. Were plasma leucine a direct precursor for the leucine in VLDL-apoB, the kinetics of the plasma tracer should determine the kinetics of the protein. However, this is not the case, and the deviations from linearity are interpreted in terms of (1) the dilution of plasma leucine in the liver by unlabeled dietary leucine; (2) the recycling of hepatocellular leucine from proteins within the liver, where recycled cellular leucine does not equilibrate with plasma leucine; and (3) a "hump" in the kinetic data of VLDL-apoB, which we interpret to reflect recycling or retention of a portion of the apoB protein within the hepatocyte, with its subsequent secretion. Because hepatocellular tRNA is the immediate precursor for synthesis of these secretory proteins, its kinetics should be used as the forcing function to drive the modeling of this system. The VLDL-apoB tail contains the information needed to modify the plasma leucine data, to provide an appropriate forcing function when using ^{3}H-leucine as a tracer of apolipoprotein metabolism. This correction is essential when using ^{3}H-leucine as a tracer for measuring low-density lipoprotein (LDL)-apoB kinetics. The ^{3}H-leucine tracer also highlights the importance of recognizing the difference between plasma and system residence times, the latter including the time the tracer resides within exchanging extravascular pools. The inability to determine these fractional exchange coefficients for apoA-I and albumin explains the failure of this tracer in kinetic studies of these proteins. For apoB-containing lipoproteins, plasma residence times are generally determined, and these measurements can be made satisfactorily with ^{3}H-leucine.

AMINO ACIDS have been used for many years as biosynthetically incorporated or endogenous tracers in the study of protein metabolism, and leucine, an essential amino acid, has been frequently selected.[1,2] For this purpose, it may be labeled with either stable or radioactive nuclides, and its use in studies of lipoprotein metabolism, when labeled with stable isotopes, is a subject of increasing interest.[3-5] Alternately, ^{3}H-leucine has been used over many years as an apolipoprotein tracer,[6] and a comparative study has examined very–low-density lipoprotein (VLDL) metabolism using ^{3}H-leucine and exogenously labeled ^{125}I-apolipoprotein B tracers.[7] The present report focuses on the ^{3}H-leucine tracer administered as a bolus to investigate the metabolism of secretory proteins in the plasma of human subjects.

SUBJECTS AND METHODS

Clinical Protocol

The subjects were normal volunteers or patients with metabolic disorders of plasma lipoproteins who have been studied over 25 years on various protocols as specified, where appropriate, in the text that follows. All were housed on the Clinical Research Center, where they were fed a weight-maintenance diet with a constant leucine content of approximately 5 g/d that was divided into four equal feedings. Fat and carbohydrate contents were adjusted depending on the protocol for the particular study. After 1 week of metabolic stabilization, the fat content of the diet was reduced to 20 g/d beginning 12 hours before injection of tracer and continuing for 36 hours postinjection, to minimize chylomicron production during the period of frequent blood sampling. In 14 studies, during this 48-hour interval, the 20-g fat diet was prepared as a liquid feeding and administered continuously by a nasogastric feeding tube, thus obviating dietary fluctuations in nutrient intake. Alternately, the feeding was administered by mouth at 4-hour intervals. Thereafter, blood samples were drawn after an overnight fast. In all studies, the tracer was ^{3}H-4,5-L-leucine obtained in sterile solution from New England Nuclear (Boston, MA); subjects received 5 μCi/kg administered as an intravenous bolus in the early morning. To examine apolipoprotein metabolism, blood samples of 37 mL each were drawn following tracer injection, and the phlebotomy schedule varied with the metabolic study.[8,9] Five milliliters of blood for determination of plasma leucine was centrifuged at 4°C immediately, before coagulation, and this plasma was processed as described later. The remaining 32 mL blood was placed in tubes containing 6 mg sodium azide, 6 mg soybean trypsin inhibitor, 3 mg merthiolate, 45 mg EDTA, and 39 mg L-leucine; the plasma was separated by centrifugation at 4°C and then frozen in the presence of 20% sucrose to inhibit lipoprotein denaturation.[10]

From the Department of Medicine, University of Florida, Gainesville, FL; and the Laboratory of Mathematical Biology, National Institutes of Health, Bethesda, MD.

Submitted September 19, 1996; accepted September 23, 1996.

Supported by Grant No. NIH HL-29394 NHLBI, and Grant No. M01RR00082 to the Clinical Research Center, Shands Hospital, Gainesville, FL.

Presented at the National Institutes of Health Symposium, "In Vivo Tracer Kinetics and Modeling," May 3, 1996, National Institutes of Health, Bethesda, MD.

Address reprint requests to Waldo R. Fisher, MD, PhD, University of Florida, Box 100226, JHMHSC, Gainesville, FL 32610-0226.

0026-0495/97/4603-0017$03.00/0

Methods

Plasma leucine concentration and specific radioactivity were measured on plasma isolated from each timed sample. Forty nanomoles of norleucine was added to 2 mL plasma as a recovery standard, followed by 2 mL 6% sulfosalicylic acid. The deproteinized sample was centrifuged, and the supernatant was frozen at −20°C. After thawing, the samples were filtered (Whatman no. 1 filter paper) and applied to ion-exchange columns containing 5 mL Dowex 50W-8X (100 to 200 mesh) resin equilibrated with 0.5N HCl. The columns were washed with water until the effluent was neutral, and then the amino acids were eluted with two 7.5-mL washes of 6N HCl and the eluates were lyophilized. Thus, the protonated amino acids bound to the resin were washed free of sulfosalicylic acid, and α-ketoisocaproic acid was also removed. The retained amino acids were then eluted at high ionic strength with 6N HCl, a volatile solvent system described by Stein and Moore.[11] Each sample was resolubilized in 5 mL 0.5N HCl, and an aliquot was removed to quantify leucine and norleucine by amino acid analysis. Based on recovery of norleucine, the plasma concentration of free leucine was calculated. The remaining sample was transferred to a scintillation vial and relyophilized and then incubated for 18 hours at 25°C in the presence of 50 μL water. Then, 0.5 mL Solulene-350 (Packard, Meridien, CT) and 15 μL glacial acetic acid were added followed by 9 mL scintillation fluid (Permablend III; Packard) in toluene before scintillation counting. Previously, with the use of a stream-splitter to collect samples from the amino acid analyzer, we have demonstrated that virtually all of the radioactivity coelutes with leucine.

Plasma samples for isolation of albumin and lipoproteins were processed according to specific protocols, as previously reported, that varied depending on the objectives of the study.[8,9,12] In general, plasma lipoproteins were isolated ultracentrifugally by differential density flotation, followed by fractionation and recovery of the isolated proteins by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[12] The proteins were quantified by fluorescamine assay, and scintillation counting of the protein permitted determination of specific radioactivity.[12] All data were corrected for isotopic decay of the tracer between the date of the experiment and the time of the radioactivity determination.

Kinetic data were analyzed by mathematical compartmental modeling using the SAAM computer program.[13]

RESULTS AND DISCUSSION

Plasma Leucine

Plasma free-leucine kinetics were measured in 42 studies. Figure 1 shows total plasma leucine radioactivity, obtained by multiplying the specific radioactivity by the total plasma leucine content, that has been fit to a sum of four exponentials. Because the plasma leucine was used as a forcing function, it was desirable to use four rather than three exponentials, as discussed subsequently. This mathematical solution enables the plasma leucine precursor to drive the modeling of the system in the absence of an adequate mathematical model of whole-body leucine metabolism, which would be required for direct kinetic measurement of protein synthesis. Figure 1 demonstrates the rapid decay of the leucine tracer during the first day, followed by a subsequent slow decay that extends over many days, due to the recycling of leucine released from protein turnover.[14] The initial rapid decay in specific activity of almost three orders of magnitude constitutes an interval when leucine is an effective tracer for protein biosynthesis. Thereafter, recycling of the tracer into the plasma leucine pool and its continuing reincorporation into newly synthesized proteins obscures the kinetics of the more slowly turning-over proteins and thus limits the utility of this amino acid tracer for studying protein metabolism. In comparing kinetic studies in which subjects were provided equivalent feedings of a liquid diet at 4-hour intervals, spanning the time of tracer injection (28 subjects), with studies in which a constant liquid gastric feeding was provided (14 subjects), we observed no apparent differences in fluctuations of plasma leucine radioactivity.

Leucine has many advantages as a protein tracer. It is an essential amino acid, and the by-products of its degradation are not reincorporated into other amino acids. The leucine tracer used in our studies contains tritium in the 4,5 position. The initial step in leucine catabolism is transamination and formation of α-ketoisocaproic acid. This reaction is readily reversible, and leucine and the keto acid achieve equilibrium in plasma.[15] A subsequent series of oxidative reactions generate hydroxy-

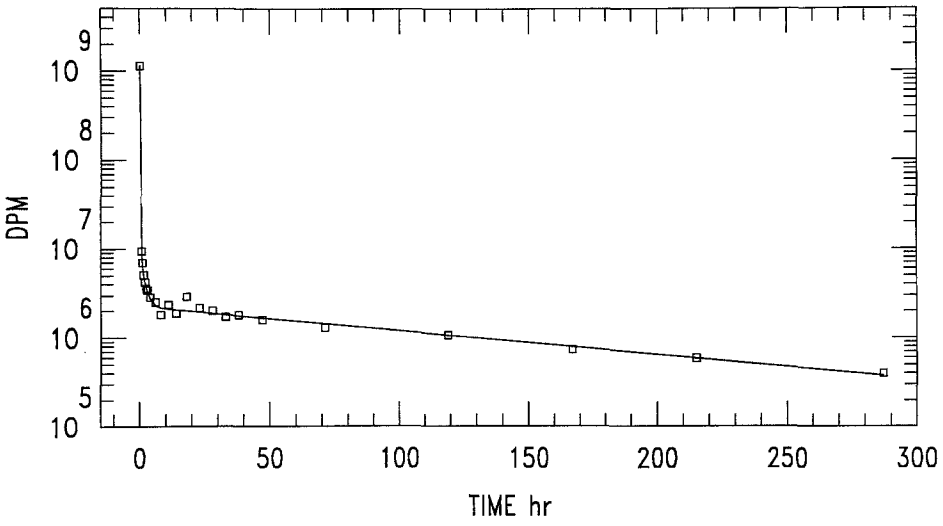

Fig 1. Plasma ^{3}H-leucine activity. Total plasma ^{3}H-leucine radioactivity (specific radioactivity × concentration × plasma volume) fit to the sum of 4 exponentials.

methyl glutaryl coenzyme A and acetate. In the oxidation of one of the intermediates, isovaleryl coenzyme A, the tritium on C-4 is lost, but the C-5 tritium can potentially appear in steroids or fatty acids. However, the tracer which is incorporated into lipid is greatly diluted. To measure this incorporation in a subject who had been administered ^{3}H-leucine, plasma triglyceride and free and esterified cholesterol were isolated from plasma VLDL and low-density lipoprotein (LDL) by solvent extraction and separation on thin-layer silicic acid plates. The ratio of radioactivity in these lipids compared with that in apolipoprotein (apo)B, with both recovered from the same lipoprotein fractions, is shown in Table 1. Thus, the amount of tritium incorporated into the lipid as compared with that in apoB is small and poses no problem in the determination of specific radioactivity of the lipoprotein.

Plasma Albumin

Recycling of the leucine tracer into the amino acid pool and its reincorporation into newly synthesized proteins causes problems in kinetic studies of slowly turning-over proteins that are well demonstrated by plasma albumin. In investigating the kinetics of albumin with the leucine tracer, we fitted our data to a modified version of the plasma albumin model developed by Gill et al[16] in studies with radioiodinated albumin. The model contains a single intravascular compartment in equilibrium with two extravascular exchange compartments. Although the data could be readily fit with the model, we discovered that the system residence time of albumin could be varied over a wide range by simply adjusting the fractional rate constants of the exchange compartments, but without perturbing the computer-generated fit for the experimental data. Clearly, kinetic parameters of the exchange compartments could not be determined with the ^{3}H-leucine tracer. Human albumin has a fractional catabolic rate (FCR) of approximately 0.006 h^{-1} when measured with a radioiodined albumin tracer.[16] For a protein with such a slow turnover, leucine recycling becomes the determining feature of the kinetic data, and the kinetics of the protein cannot be resolved with ^{3}H-leucine.

Plasma ApoA-I

Plasma apoA-I kinetics have been most carefully examined using radioiodinated tracers.[17] This protein exists in plasma in different states of association with various apolipoproteins and lipids, and it is also present in the extravascular space. Because of its rapid exchange, it has been difficult to examine the kinetics of any single class of apoA-I particles in vivo.[18,19] Instead, most studies have reported kinetic data on apoA-I that provide a holistic view of its kinetics without concern for that of the high-density lipoprotein subspecies. Thus, the current

Table 1. Incorporation of Tritium From ^{3}H-Leucine Into Plasma Lipids

Lipid Fraction	1 h	10 h	70 h	165 h
TG/VLDL-apoB	0.0025	0.0025	0.0013	—
FC/LDL-apoB	0.01	0.003	0.006	0.008
CE/LDL-apoB	0.02	0.008	0.004	0.01

NOTE. Data are dpm/mg lipid (triglycerides [TG], free cholesterol [FC], and cholesterol esters [CE]) divided by dpm/mg apoB recovered from either VLDL or LDL as indicated at the specified times following injection of ^{3}H-leucine tracer.

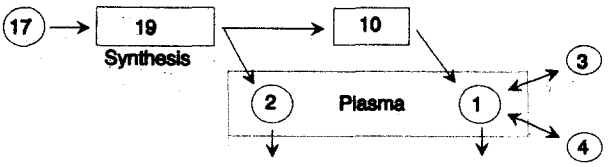

Fig 2. One of 2 models of human apoA-I metabolism used in analyzing kinetic data generated with ^{3}H-leucine. C(17) is the plasma leucine forcing function. C(2) is a rapidly turning-over plasma pool, and C(1) is a larger slowly turning-over pool into which tracer enters after a delay, C(10), of about 8 hours.[20]

apoA-I model consists of two plasma compartments: C(2) is small and turns over rapidly, and C(1) is a large slowly turning-over pool that is in equilibrium with two extravascular compartments that can be resolved with radioiodinated apoA-I tracers.[17]

We have recently reported studies on apoA-I kinetics in humans, using the ^{3}H-leucine tracer, that enabled us to examine the kinetics of C(2) in greater detail. The data were fit to either of two models that cannot be resolved, and model A is shown in Fig 2.[20] In either case, the flux through C(2) is threefold or greater than that through C(1), and the turnover of C(2) is much faster, but C(1) constitutes the major apoA-I pool in plasma. However, the residence time of C(1) and thus of apoA-I could not be determined.

Table 2 incorporates and expands previously published data on apoA-I kinetics.[20] The tabulated residence times are system residence times, that is, the time the tracer resides within the kinetically defined system, which includes the exchange compartments. These parameters can be measured with radioiodinated apoA-I, and a system residence time of approximately 120 hours is reported for normal subjects.[17] However, rate coeffi-

Table 2. Demonstration of Nondeterminability of Kinetic Parameters of ApoA-I Using a ^{3}H-Leucine Tracer

Parameter	A	B	C	D
Residence time (h)	120	96	60	28
L(0,1)	8.5 E-03	8.5 E-03	8.6 E-03	1.05 E-02
L(0,2)	0.1	0.1	0.1	0.1
L(1,3)	1.55	2.0	10.0	0
L(3,1)	2.24 E-02	2.24 E-02	2.24 E-02	0
L(1,4)	5.70 E-04	7.88 E-04	1.76 E-03	0
L(4,1)	1.96 E-03	1.96 E-03	1.96 E-03	0
M(1), mg	2,256	2,255	2,242	2,137
M(2), mg	704	704	718	822
M(3) + M(4), mg	7,807	5,649	2,508	0
1/FCR (h^{-1})	33	33	33	28

NOTE. Data are fit by 4 computer solutions, A-D. Each fits the apoA-I specific activity data as demonstrated in Fisher et al.[20] Values of L(3,1), L(4,1), L(0,1), and L(0,2) were fixed in solutions A to C at the values given in Zech et al.[17] Thus, the changes in system residence time were achieved by altering only the fractional exchange coefficients, L(1,3) and L(1,4), but when the exchange compartments were excluded, solution D, L(0,1) had to be adjusted. By excluding the exchange compartments, the residence time becomes a plasma residence time, and only in solution D could the value of L(0,1) be determined with this tracer. When the exchange parameters were freed, L(0,1) could no longer be determined. Thus, with ^{3}H-leucine, a system residence time for apoA-I could not be measured.

cients for the exchange of C(1) with C(3) and C(4) could not be determined from our data. Thus, by fixing the input to C(3) and C(4) at the rates reported in the literature[17] and adjusting their FCR [L(1,3) and L(1,4)], the system residence time could be varied over the range of 60 to 120 hours while maintaining equally good fits of the kinetic data. However, the time that an apoA-I particle remained in plasma (1/FCR) was unchanged, since L(0,1) and L(0,2) were arbitrarily fixed. In the final example, the exchange compartments are eliminated from the system and the data could still be fit, but now only by adjusting L(0,1) with a resultant change in the plasma residence time. Thus, the residence time and therefore the production rates of apoA-I cannot be determined with a ^{3}H-leucine tracer administered as a bolus.*

VLDL-apoB

VLDL-apoB kinetics have been examined with the ^{3}H-leucine tracer in normal, hypertriglyceridemic, and hypercholesterolemic subjects.[6-9] The kinetic response consists of an initial plasma peak as tracer appears in newly secreted apoB, followed by a relatively flat tail that is established by 30 hours and extends for over 2 weeks (Fig 3). A comparison with the kinetics of plasma leucine (Fig 1) shows that the tail becomes established at about the time that leucine kinetics become dominated by recycling. The inset to Fig 3 shows the model of VLDL-apoB used to fit these data, and the computer-generated fit is shown by the solid line. The pathway representing incorporation of plasma leucine into apoB is depicted as a chain of compartments by which a portion of plasma leucine traverses the protein biosynthetic pathway and emerges from the hepatocyte as apoB. C(17) is the plasma leucine forcing function that is the precursor, and the delay within this pathway is the residence time of the tracer within the hepatocyte. A synthesis time of 1.3 hours (standard deviation, 0.42) is measured in vivo for apoB, which agrees closely with that measured in hepatocyte cultures.[21,22] However, that this chain of compartments can be shown to be an oversimplification of the biosynthesis pathway will become apparent from the discussion that follows.

ApoB is released into plasma by two pathways (Fig 3). A fast pathway enters and exits C(8). It contributes to the initial rapid increase in apoB specific activity and is thought to represent a pool of VLDL rapidly converted to intermediate-density lipoprotein (IDL) and LDL.[9] As observed by most investigators, a delipidation chain is required to provide the rounded contour of the VLDL peak. Particles entering that pathway may traverse the length of the delipidation chain before entering IDL, or they may disappear from plasma at points along the chain, to reappear as IDL in a pathway thought to result from margination of VLDL particles on endothelial-bound lipase.[23,24] Kinetic curves calculated from the model for the fast pathway, through C(8), and the delipidation pathway are shown by dashed and dotted lines, respectively.

*Data from our apoA-I kinetic study were collected over 14 days (although in our report, results were truncated at 10 days to show the earlier time points with greater clarity). Perhaps if the data were collected over a longer time, it would be possible to resolve the exchange compartment; however, this is limited by the decreasing tracer content of the protein.

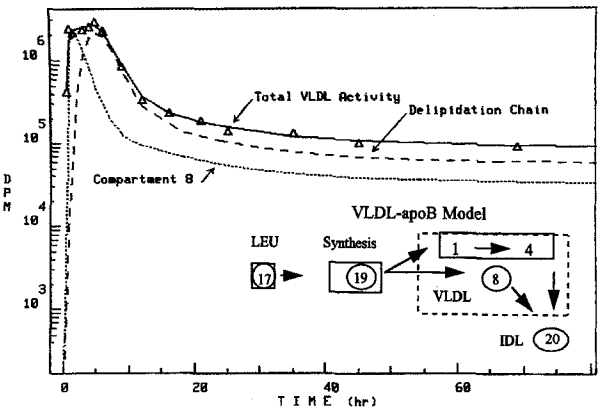

Fig 3. VLDL-apoB kinetic data fitted to an apoB model that incorporates a rapidly turning-over pool, C(8), and the delipidation cascade, C(1-4). Computer simulation of the fast pathway is shown by the dashed line, and that of the delipidation pathway by the dotted line. The data have been truncated at 80 hours to expand the early portion. C(17) is the plasma leucine forcing function, and C(19) is the final compartment in the hepatocellular synthesis pathway.

To analyze the effects of leucine recycling on the kinetics of VLDL, we turned to simulation. Initially, the leucine forcing function had to be replaced by a compartment having the kinetics of the hepatic apoB precursor, and C(19), representing the final step in cellular synthesis of apoB, was selected. After fitting the kinetic data for plasma leucine and plasma VLDL-apoB to the apoB model, the kinetics of C(19) were captured. In our simulation, this activity was entered as data in C(18) (Fig 4). The leucine tracer was then fed through a single-compartment delay and used to fit the VLDL-apoB data according to the model shown in the inset to Fig 4A. The pathways for VLDL-apoB catabolism are routed to C(25), a delay, and by directing this same amount of leucine tracer that was secreted as VLDL-apoB from C(18) back to the same compartment, a model depicting the recycling of VLDL-apoB was developed that retained the original kinetic parameters of VLDL. Alternately, in the nonrecycling model (Fig 4B), the output from C(25) was irreversibly lost from the system. Whereas the recycling model provides a reasonable fit of the data, in the nonrecycling model C(8) decays monoexponentially, and the delipidation pathway also has an accelerated decay, albeit with a predictably more complex function. Moreover, the tail on VLDL is eliminated in the nonrecycling model. The one constant feature of both solutions is the shape of the VLDL peak, and it is the fitting of this peak that is crucial in analyzing VLDL kinetics using a leucine tracer. The rapidly turning-over VLDL component determines the ascent of the peak, and the kinetics of the delipidation pathway shape the peak and its descending limb. By about 15 hours, or three residence times, the kinetics of VLDL-apoB become obscured by those of the recycling leucine. However, the fitting of these early times permits calculation of the FCR and transport parameters of VLDL-apoB, for these parameters remain the same in both the recycling and nonrecycling steady-state simulations. The residence time of VLDL is on the order of 5 hours, and thus by the end of the first day, VLDL has been largely metabolized; however, we have kinetic data on the VLDL tail that extend for another 13 days.

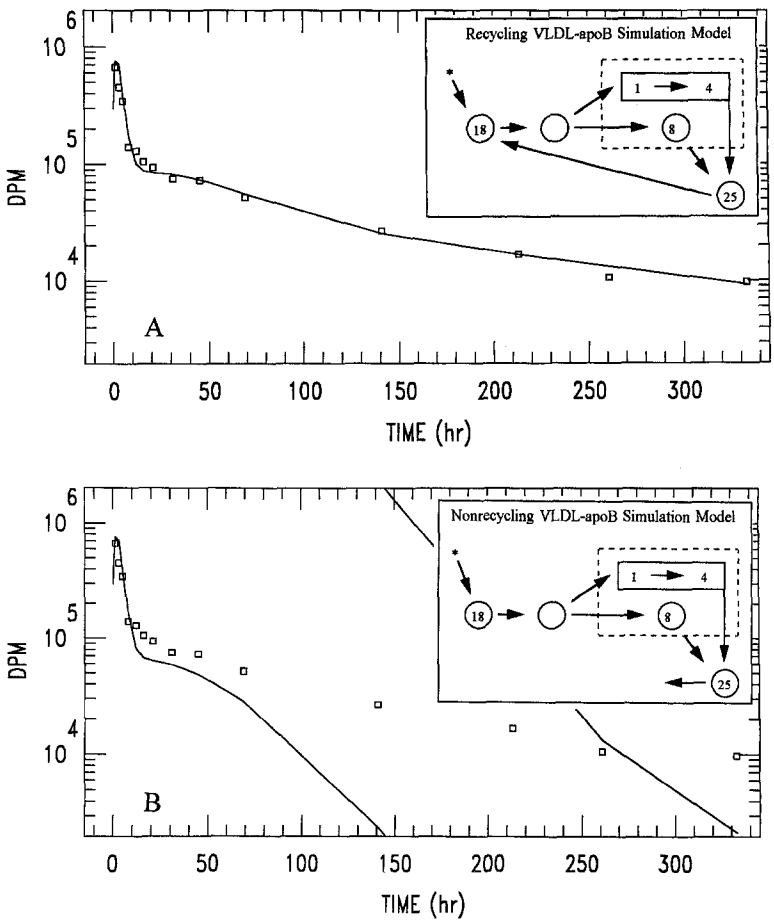

Fig 4. Simulation of VLDL-apoB kinetics (A) with recycling of leucine and (B) with a nonrecycling model. Insets show the models used in the simulations. C(17) contains simulated kinetic data for the cellular VLDL-apoB precursor generated using the model shown in Fig 3, although with data from a different subject, and the VLDL data are from that same subject.

The tail of VLDL is an interesting region of the kinetic curve. The primary determinant of its shape is the activity of the plasma leucine tracer that is expressed in the kinetic calculations by a forcing function, and our apoB model (Fig 3) assumes a simple linear relationship. However, the plasma leucine forcing function often fails to predict kinetic behavior of the VLDL tail. Thus, there appeared to be important physiologic information to be gained by a further investigation of the VLDL kinetic tail. Two aberrant features of the leucine tail have been consistently observed: (1) a displacement of the observed VLDL data above or below that predicted from the plasma leucine data, which becomes apparent after 15 to 20 hours; and (2) a hump appearing at 15 to 30 hours that is most prominent in hypertriglyceridemic subjects.

Displacement of the VLDL tail. In 18 of 29 studies in normal, hypercholesterolemia, or hypertriglyceridemic subjects, activity of the tracer in the VLDL-apoB tail decreased to a level below that predicted by the plasma leucine forcing function (Fig 5A). The kinetic tail of VLDL-apoB is determined primarily by the specific radioactivity of the hepatic leucyl tRNA tracer. Because leucine is an essential amino acid, the decay of the tail of plasma leucine activity is a consequence of the disappearance of leucine from the plasma pool, its return following the catabolism of proteins, and its replacement by dietary leucine. Since VLDL-apoB is synthesized in the hepatocyte, the accelerated decay rate, which must reflect the specific radioactivity of the tracer in the liver, suggests that the hepatic leucine precursor pool is being continually diluted compared with that of plasma leucine. Whether the intracellular pool from which protein synthesis occurs has the same specific activity as extracellular leucine is controversial.[25,26] In vivo, this concern is further confounded by the fact that there is a daily influx of dietary leucine, unlabeled, which enters the liver directly via the portal vein, diluting plasma leucine that is supplied via the hepatic artery. During the first day, the rapid decrease in plasma leucine tracer activity is largely the result of the redistribution of tracer into the extravascular space and the sequence of metabolic reactions that follows. The additional isotopic dilution from enteral leucine is difficult to appreciate until a more nearly steady rate of decline in the leucine tail becomes established. Thus, one might anticipate that after the initial bolus of leucine tracer has dissipated during the first day, the continuing input of dietary leucine would reduce the specific activity of the leucine tracer in liver as compared with that in plasma. Therefore, the rate of decline for hepatic leucine activity would be more rapid than that for plasma leucine and would give rise to the downward displacement of the tail in these 18 studies. Dilution of plasma leucine by enteral leucine can also be demonstrated in short-term, primed constant infusion experiments.[27,28]

However, this explanation begs the question of why the plasma and VLDL leucine tails decayed at nearly the same rate in 10 subjects. Differences in dietary leucine are not the explanation, since all studies were performed with subjects maintained on a constant leucine diet that began 1 week before

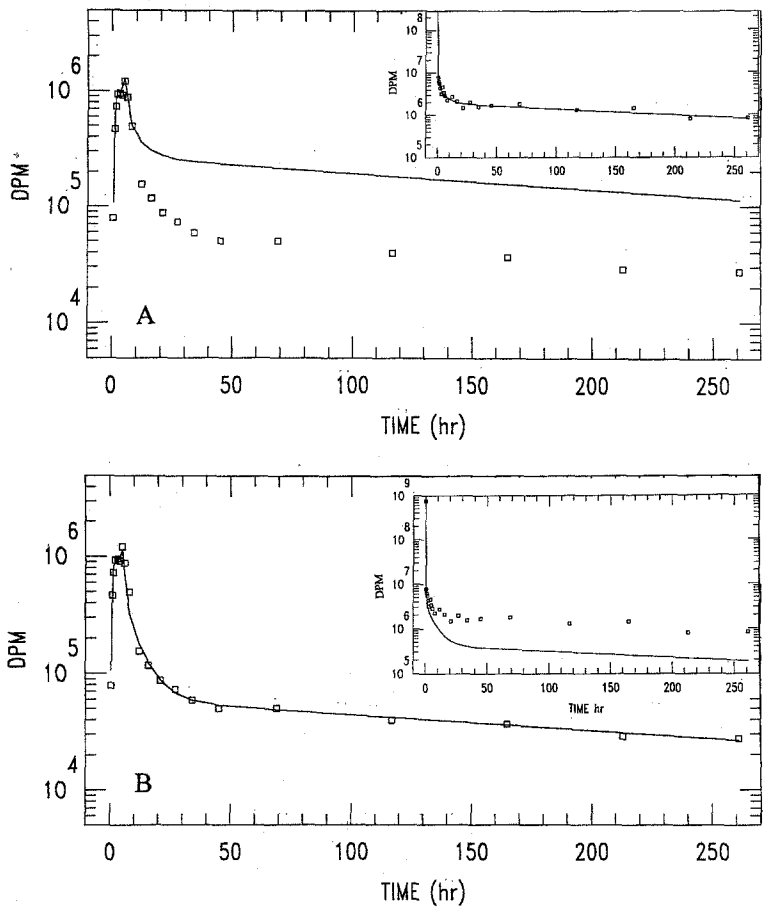

Fig 5. (A) VLDL-apoB kinetic data and best computer fit using the apoB model from Fig 3. The data decay at an accelerated rate compared with the decay of the tail as predicted from the model. Inset, plasma leucine fit to a sum of exponentials and used as forcing function. (B) Same data and apoB model, but with the leucine forcing function adjusted to enable the model to fit the observed VLDL tail. Inset, forcing function adjusted in accordance with the data for the VLDL tail.

tracer injection and continued throughout the investigation. The daily leucine content of the diet varied from 4 to 6 g depending on the subject's weight, and did not relate to the displacement of the leucine tail.

A plausible answer to this question is provided by one subject in whom the model-predicted VLDL tail declined at a faster rate than the observed values (Fig 6A). Undoubtedly, a considerable portion of leucine that enters the hepatocyte is converted into cellular proteins, some of which have a rapid turnover and will continue to enrich the intrahepatic leucine pool with tracer in a cycle that is independent of plasma leucine. Thus, leucine in newly synthesized proteins has a higher specific activity than would be predicted from free leucine in the plasma, and accordingly, the VLDL tail is displaced upward. By introducing such a recycling loop that diverts 50% of the leucine that enters the synthesis pathway and then delaying the transit time of a leucine molecule through this loop by an average of 7 days, one can fit the tail of VLDL in this subject (Fig 6B). In two of these 10 subjects, this pathway was also observed, but less than 10% of the total flux of leucine into apoB was diverted through it. Of interest, rat liver proteins have been reported to have half-life values of 2 to 8 days.[29] We thus appear to be observing two levels of leucine recycling: (1) at the whole-body level, where leucine liberated by protein catabolism reenters the plasma leucine pool; and (2) recycling of proteins catabolized within the hepatocyte in which leucine is reused without escaping into plasma. Both a cellular protein recycling pathway and dilution of hepatic leucine by dietary leucine probably occur, and specific radioactivity of hepatic leucyl tRNA is determined by the relative magnitude of these processes. Thus, in a given subject, the observed VLDL tail may be diverted above or below that predicted by the plasma leucine forcing function, but if the processes are in balance, it will coincide with the VLDL tail predicted by the model.

Thus, the extended VLDL kinetic tail provides an opportunity to monitor the in vivo intrahepatic leucine pool from which proteins are synthesized. During the half-day after intravenous bolus injection of the leucine tracer, the plasma tracer activity is high, the decay of activity is very rapid and essentially monoexponential, and the dilution by dietary leucine is not apparent. During this time, the kinetics of VLDL are largely determined, and the properties of the VLDL tail minimally influence determination of the FCR and transport calculations for VLDL-apoB. However, for proteins with a slower turnover, such as LDL, the kinetics of the leucine tail impact calculation of the FCR and transports. Accordingly, one must decide whether to use the plasma leucine data to set the tail of the forcing function or whether to reset the tail of the forcing function by adjusting the model-generated solution for the VLDL-apoB tail so that it fits the experimental data, assuming that the VLDL data provide a better measure of the specific activity of the hepatic leucine precursor than do plasma leucine. In our studies, we assumed the latter, and so adjusted the values of the third and fourth exponentials for the leucine forcing

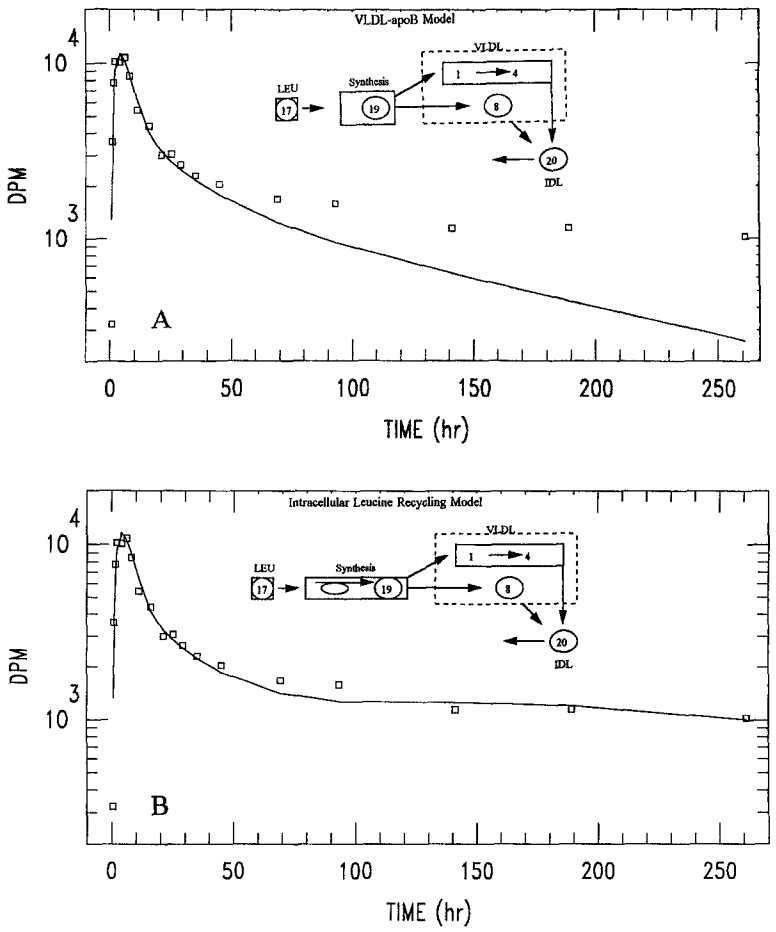

Fig 6. (A) Hypertriglyceridemic subject whose apoB model-predicted VLDL tail decayed at a faster rate than the observed data. Insert, apoB model. (B) Same data, but fit with a model that incorporates a recycling loop in the cellular synthesis pathway, inset.

function to bring the model-determined VLDL-apoB kinetic tail into concordance with the experimentally measured data (Fig 5B). This action does not affect determination of the FCR of VLDL. However, the kinetic parameters of LDL are decidedly altered, since in fitting LDL to the apoB model, the two crucial sets of data are the specific activities of LDL and its precursor. If the activity of the assumed precursor is wrong, the model solution will be incorrect. The importance of this decision is demonstrated by examining the difference in the calculated residence time of LDL-apoB when determined according to the two different assumptions for the subject whose VLDL data are shown in Fig 5. The residence time of LDL was about 9 hours when calculated using plasma leucine radioactivity as the forcing function. When the forcing function was reset according to the VLDL tail (Fig 5B), the residence time of LDL was 70 hours. The size of the correction clearly depends on the magnitude of displacement of the VLDL tail, and this subject presents an unusually striking example of the potential error. Accordingly, in analyzing our data, this correction is made routinely. In our study of LDL kinetics in heterozygous familial hypercholesterolemic subjects, we found that the FCR (mean ± SD) for LDL of seven subjects was 118 ± 68 hours.[9] The FCR calculated by averaging data reported from nine studies using ^{125}I-LDL was 114 ± 33 hours.[30] Although the ^{3}H-leucine tracer is not ideal for delineating the intricacies of LDL metabolism, it does provide a reliable measure of the FCR of LDL.

The hump at 15 to 30 hours. A common finding when examining VLDL-apoB kinetics with the leucine tracer in hypertriglyceridemic individuals is the occurrence of a hump that appears in the descending limb of the VLDL peak or shortly thereafter, as seen in six of 10 studies (Fig 7). A similar hump has been observed in VLDL-triglyceride kinetics, and is thought to arise from recycling of the triglyceride-glycerol tracer within the glycerol-containing lipids of the hepatocyte.[31] In the case of apoB, the fact that this hump is not seen in plasma leucine places its origin within the hepatocyte, and it is evident only during that relatively brief interval of 1 day to 1.5 days when VLDL-apoB metabolism can be traced. The process producing the hump involves apoB, but more general processes of hepatocellular protein recycling appear not to be involved, since the extended VLDL leucine tail is unaffected. Studies at the cellular level have established that a portion of newly synthesized apoB is not secreted, but instead is degraded.[32] Since intrahepatocyte apoB recycling provides a physiological explanation for the hump, we have incorporated a rapid cellular recycling pathway, and Fig 7C and D demonstrates the ability of this pathway to fit the hump in these two subjects. The data suggest several hypotheses: (1) a portion of apoB is retained within the hepatocyte and subsequently secreted, or (2) secretion and reuptake of apoB by the hepatocyte occurs with subsequent release into the circulation, as observed in cultures with hepatoma cells.[33,34] A less likely scenario is that amino acids released upon apoB catabolism are directly channeled into

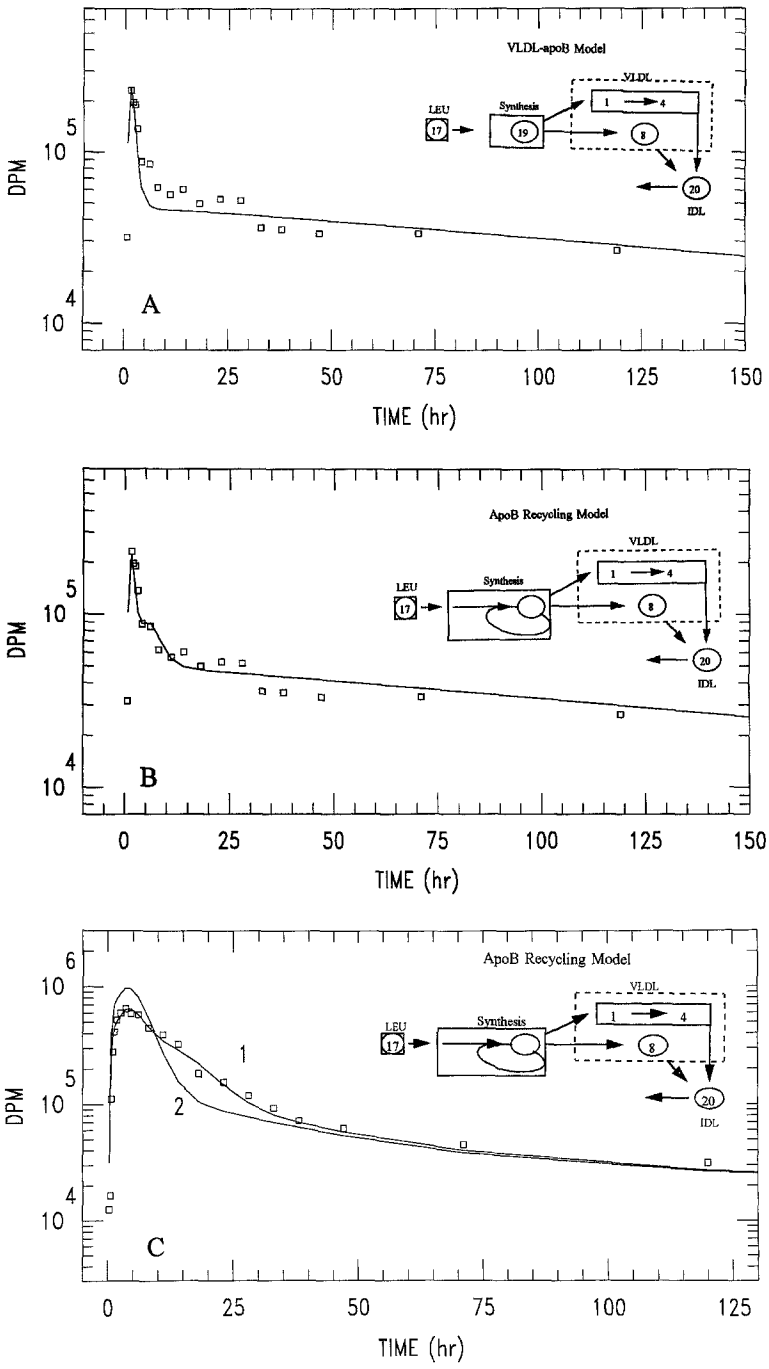

Fig 7. The hump in the descending limb of the VLDL peak. (A) Data from 1 subject in whom values between 5 and 15 hours were unable to be fit using the model, inset. (B) The fit to the same data following introduction of a rapid recycling loop, inset. (C) Model-generated fit from a second subject, in which the recycling model fit the data between 10 and 40 hours (curve 1) but a nonrecycling model failed (curve 2).

the synthesis of new apoB without equilibrating within the hepatic free–amino acid pool. Based on our kinetic data, about 20% of the leucine tracer that is secreted into plasma as apoB was initially retained or recycled through this pathway in these six subjects. That we see this pathway only in hypertriglyceridemic subjects possibly reflects our ability to resolve it only in the presence of higher concentrations of VLDL-apoB.

Within what limits, then, is leucine a useful in vivo tracer? One use that has been validated is in studying the synthesis and metabolism of proteins that occur before recycling of leucine in plasma becomes established. Thus, it is an excellent tracer for proteins such as VLDL- and IDL-apoB, but it cannot be used to measure the turnover of slowly metabolized proteins such as apoA-I and albumin. However, it can be an excellent tracer to examine the secretion and early events in the metabolism of more slowly turning-over proteins, as we have demonstrated with apoA-I and A-II.[20] LDL-apoB provides an interesting insight into the limitations of this tracer. In the normal subject, this protein has a residence time of about 2 days, which can be determined with ^{3}H-leucine as with radioiodinated LDL. In familial hypercholesterolemic heterozygotes, a comparison of reported values for the prolonged residence time of LDL-apoB measured with ^{3}H-leucine and ^{125}I-LDL gave virtually the same values, as noted earlier. Why is it that we can measure the

residence time of the hypercholesterolemic LDL-apoB with reasonable accuracy but we cannot measure that of apoA-I when the values are so nearly the same, 120 versus 114 hours[9,30]? Both lipoproteins have extravascular exchanging pools; however, fractional exchange coefficients for the transfer of apoA-I between the intravascular and extravascular pools are of an order of magnitude such that they can be measured with a ^{125}I-apoA-I tracer, and the residence times reported for apoA-I and apoA-II are system residence times, which include the time the tracer spends within the exchange compartments. But these exchange coefficients cannot be resolved with ^{3}H-leucine.[1]

The situation with apoB is different. VLDL-apoB and IDL-apoB are not thought to have significant extravascular pools; hence, only their time of residence within plasma is calculated. LDL-apoB appears to exist within the extravascular space[35]; however, the exchange between plasma and extravascular pools is either too fast or too slow to resolve with either radioiodinated or tritiated LDL tracers. Thus, in determining the residence time of apoB, whether in VLDL, IDL, or LDL, the contribution of extravascular apoprotein is generally ignored, and the reported residence times for apoB are plasma residence times, ie, the time the tracer resides within the plasma compartment or the reciprocal of the FCR, in contrast to system residence times that are generally reported for apoA-I and plasma albumin, which include the extravascular pools. Because it is not possible to determine extravascular exchange coefficients for apoA particles or albumin with ^{3}H-leucine, this tracer fails when measuring their kinetics. However, since with LDL-apoB neither ^{3}H-leucine nor ^{125}I-LDL determine the extravascular exchanges satisfactorily, investigators report only plasma residence times. Under these circumstances, values measured by the two tracers are not materially different. Nonetheless, the iodinated tracer should be more useful in studying the details of intravascular metabolism and catabolism of LDL, whereas ^{3}H-leucine serves better for examining reactions involved in the production of LDL.[7]

In conclusion, our experience with ^{3}H-leucine has confirmed its value as an in vivo tracer to study protein synthesis and secretion. However, data generated by the kinetic tail of VLDL must be used to determine the specific radioactivity of the hepatocellular leucine precursor in kinetic calculations. Reeds et al[5] have also recognized the importance of using the enrichment of leucine in the VLDL tail during stable isotopic infusion studies to determine fractional synthesis rates. In both bolus and infusion protocols, it is crucial to know the activity of the leucine precursor, and the assumption that plasma leucine and hepatocellular leucine have the same kinetics appears invalid. The primary limitation in the use of the ^{3}H-leucine tracer is in studies of protein catabolism with slowly turning-over proteins, for which measuring the kinetics of exchanging metabolic pools is crucial to understanding the kinetics of the protein.

REFERENCES

1. Waterlow JC, Garlick PJ, Millward DJ: Protein Turnover in Mammalian Tissues and in the Whole Body. New York, NY, North-Holland, 1978

2. Bier DM, Matthews DE: Stable isotope tracer methods for in vivo investigations. Fed Proc 41:2679-2685, 1982

3. Lichenstein AH, Cohn JS, Hachey DL, et al: Comparison of deuterated leucine, valine, and lysine in the measurement of human apolipoprotein A-I and B-100 kinetics. J Lipid Res 31:1693-1701, 1990

4. Parhofer KG, Barrett PHR, Bier DM, et al: Determination of kinetic parameters of apolipoprotein B metabolism using amino acids labeled with stable isotopes. J Lipid Res 32:1311-1323, 1991

5. Reeds PJ, Hachey DL, Patterson BW, et al: VLDL apolipoprotein B-100, a potential indicator of the isotopic labeling of the hepatic protein synthetic precursor pool in humans: Studies with multiple stable isotopically labeled amino acids. J Nutr 122:457-466, 1992

6. Phair RD, Hammond MG, Bowden JA, et al: A preliminary model for human lipoprotein metabolism in hyperlipoproteinemia. Fed Proc 34:2263-2270, 1975

7. Beltz WF, Kesaniemi A, Miller NH, et al: Studies on the metabolism of apolipoprotein B in hypertriglyceridemic subjects using simultaneous administration of tritiated leucine and radioiodinated very low density lipoprotein. J Lipid Res 31:361-374, 1990

8. Fisher WR, Zech LA, Bardalaye P, et al: The metabolism of apolipoprotein B in subjects with hypertriglyceridemia and polydisperse LDL. J Lipid Res 21:760-774, 1980

9. Fisher WR, Zech LA, Kilgore LL, et al: Metabolic pathways of apolipoprotein B in heterozygous familial hypercholesterolemia: Studies with a [^{3}H]leucine tracer. J Lipid Res 32:1823-1836, 1991

10. Fisher WR, Schumaker VN: Isolation and characterization of apolipoprotein B-100, in Segrest JP, Albers JJ (eds): Plasma Lipoproteins, vol 128. New York, NY, Academic, 1986, pp 247-262

11. Stein WH, Moore S: Chromatographic determination of the amino acid composition of proteins. Cold Spring Harb Symp Quant Biol 14:179-190, 1950

12. Kilgore LL, Rogers JL, Patterson BW, et al: The determination of specific radioactivity of proteins eluted intact from polyacrylamide gels, utilizing a fluorescamine assay. Anal Biochem 145:113-119, 1985

13. Berman M, Weiss M: SAAM Manual. Washington, DC, US Government Printing Office, Department of Health, Education, and Welfare Publication No. 78-180, National Institutes of Health, 1977, p 200

14. Poole B: The kinetics of disappearance of labeled leucine from the free leucine pool of rat liver and its effect on the apparent turnover of catalase and other hepatic proteins. J Biol Chem 246:6587-6591, 1971

15. Cobelli C, Saccomani MP, Tessari P, et al: Compartmental model of leucine kinetics in humans. Am J Physiol 261:E539-E550, 1991

16. Gill JR Jr, Waldman TA, Bartter FC: Idiopathic edema. I. The occurrence of hypoalbuminemia and abnormal albumin metabolism in women with unexplained edema. Am J Med 52:444-451, 1972

17. Zech LA, Schaefer EJ, Bronzert TJ, et al: Metabolism of human apolipoproteins A-I and A-II: Compartmental models. J Lipid Res 24:60-71, 1983

18. Grow TE, Fried M: Interchange of apoprotein components between the human plasma high density lipoprotein subclasses HDL_2 and HDL_3 in vitro. J Biol Chem 253:8034-8041, 1978

19. Rader DJ, Castro G, Zech LA, et al: In vivo metabolism of apolipoprotein A-I on high density lipoprotein particles LpA-I and LpA-I,A-II. J Lipid Res 32:1849-1859, 1991

20. Fisher WR, Venkatakrishnan V, Zech LA, et al: Kinetic evidence for both a fast and a slow secretory pathway for apolipoprotein A-I in humans. J Lipid Res 36:1618-1628, 1995

21. Borchardt RA, Davis RA: Intrahepatic assembly of very low density lipoproteins. J Biol Chem 262:16394-16402, 1987

22. Bamberger MJ, Lane MD: Assembly of very low density lipoprotein in the hepatocyte: Differential transport of apoproteins through the secretory pathway. J Biol Chem 263:11868-11878, 1988

23. Barter PJ, Nestel PJ: Precursor-product relationship between

pools of very low density lipoprotein triglycerides. J Clin Invest 51:174-180, 1972

24. Higgins J, Fielding CJ: Lipoprotein lipase. Mechanism of formation of triglyceride-rich remnant particles from very low density lipoproteins and chylomicrons. Biochemistry 14:2288-2292, 1975

25. Low RB, Stirewalt WS, Rittling SR, et al: Amino acid pools in cultured muscle cells. J Cell Biochem 25:123-129, 1984

26. Watt PW, Lindsay Y, Scrimgeour CM, et al: Isolation of aminoacyl-tRNA and its labeling with stable-isotope tracers: Use in studies of human tissue protein synthesis. Proc Natl Acad Sci USA 88:5892-5896, 1991

27. Matthews DE, Marano MA, Campbell RG: Splanchnic bed utilization of leucine and phenylalanine in humans. Am J Physiol 264:E109-E118, 1993

28. Bennet WM, O'Keefe SJD, Haymond MW: Comparison of precursor pool with leucine, α-ketoisocaproate, and phenylalanine tracers used to measure splanchnic protein synthesis in man. Metabolism 42:691-695, 1993

29. Glass RD, Doyle D: On the measurement of protein turnover in animal cells. J Biol Chem 247:5234-5242, 1972

30. Kesaniemi YA, Vega GL, Grundy SM: Kinetics of apolipoprotein B in normal and hyperlipidemic man: Review of current data, in Berman M, Grundy SM, Howard BV (eds): Lipoprotein Kinetics and Modeling. New York, NY, Academic, 1982, p 181

31. Zech LA, Grundy SM, Steinberg D, et al: Kinetic model for production and metabolism of very low density lipoprotein triglycerides. Evidence for a slow production pathway and results for normolipidemic subjects. J Clin Invest 63:1262-1273, 1979

32. Ginsberg H: Synthesis and secretion of apolipoprotein B from cultured liver cells. Curr Opin Lipidol 6:275-280, 1995

33. Williams KJ, Petrie KA, Brocia RW, et al: Lipoprotein lipase modulates net secretory output of apolipoprotein B in vitro. A possible pathophysiologic explanation for familial combined hyperlipidemia. J Clin Invest 88:1300-1306, 1991

34. Ji JS, Fazio S, Lee YL, et al: Secretion-capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. J Biol Chem 269:2764-2772, 1994

35. Sloop CH, Dory L, Roheim PS: Interstitial fluid lipoproteins. J Lipid Res 28:225-237, 1987