Measurement of apolipoprotein B synthesis in perfused rat liver using stable isotopes: [^{15}N]hippurate as a measure of the intracellular [^{15}N]glycine precursor enrichment

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Abstract Rat livers were perfused by the nonrecirculating technique with medium containing [^{15}N]glycine and sodium benzoate. At various times, the isotopic enrichment of hepatic free glycine, hepatic glycyl-tRNA, and perfusate hippurate was measured by GLC-MS. After 60 min, these parameters had reached approximately maximal values. At 90 min, the perfusate hippurate had a 30% greater enrichment of ^{15}N than the intracellular glycine or glycyl-tRNA. Hippurate enrichment was half that of the medium glycine. The rat livers secreted apolipoprotein B (B-100 plus B-48) at a rate of 22 μg/g per h. From the ^{15}N enrichment and the secretion rate, an intrahepatic pool size of 86 μg/g of apoB was calculated. From the minimal intracellular transit time of 30 min, an apoB fractional synthetic rate (FSR) of 2 pools/h was indicated, whereas the FSR estimated from the ^{15}N-enrichment was 0.26/h. A possible explanation for the discrepancy is that apoB may recycle within the hepatocyte. On the basis of the present experiments, when hippurate enrichment is used as a measure of the enrichment of intrahepatic glycine in in vivo studies with ^{15}N-labeled glycine, a correction should be applied, under normal metabolic circumstances, of approximately 20-30%.—**Matsushima, T., D. R. Cryer, K. E. Winkler, J. B. Marsh, and J. A. Cortner.** Measurement of apolipoprotein B synthesis in perfused rat liver using stable isotopes: [^{15}N]hippurate as a measure of the intracellular [^{15}N]glycine precursor enrichment. *J. Lipid Res.* 1989. **30:** 841-846.

Supplementary key words glycyl-tRNA • fractional synthetic rate • VLDL-apoB

The use of stable isotopes for the measurement of metabolic processes has increased during the past several years owing to improvements in mass spectroscopic instrumentation (1) and a desire to avoid radioactive tracers in human subjects. We have previously reported the use of [^{15}N]glycine as a precursor for the measurement of VLDL-apolipoprotein B (apoB) synthesis in human subjects (2). The [^{15}N]glycine was administered by constant intravenous infusion following a priming dose. Benzyl alcohol was present during the infusion and the ^{15}N-enrichment of urinary hippurate was employed as a measure of the enrichment of the intracellular glycine pool, which is necessary to calculate the fractional synthetic rate (FSR). However, a problem arose in this work stemming from the observation that the isotope enrichment of the hippurate was approximately 20% greater than that of the VLDL-apoB at a time when VLDL-apoB-enrichment had reached what appeared to be a plateau value in the five control subjects (2). Theoretically, with this technique, the plateau isotope enrichment value for any secretory protein should be equal to that of the precursor amino acid. This discrepancy suggested that the use of the value for hippurate enrichment may have introduced a systematic error in the calculation. We have therefore carried out the present experiments in a nonrecirculating rat liver perfusion system in which the ^{15}N-enrichment of hepatic glycyl-tRNA could be directly measured and compared with that of hepatic free glycine and the perfusate hippurate under conditions where apoB synthesis could be simultaneously measured from the secretion rate. The results confirm the observation that hippurate enrichment overestimates that of the hepatic glycine precursor pool and they suggest that a simple correction factor should be used (under normal metabolic circumstances) to make hippurate enrichment a more accurate measure of the

Abbreviations: EDTA, ethylene diamine tetraacetate; GLC-MS, gas-liquid chromatography-mass spectrometry; PMSF, phenyl methyl sulfonyl fluoride; TBDMS, tertiary butyl dimethyl silyl; TCA, trichloroacetic acid; FSR, fractional synthetic rate.

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glycine precursor enrichment in vivo. In addition, the present experiments provide the first estimate of the size of the intrahepatic pool of apoB in the intact liver.

MATERIALS AND METHODS

Liver perfusion

Male Sprague-Dawley rats weighing between 230 and 303 g and fed a high carbohydrate diet (3) were used. The livers were perfused in situ in the nonrecirculating mode as previously described (4), except that the animals were killed by cervical dislocation without prior anesthetization. Provided that vessel cannulation was achieved within 2 min, no clotting problems were encountered. The perfusion medium was Krebs-Ringer-bicarbonate buffer containing 0.1% glucose and 2% of Eagle's essential amino acid mixture (Whitaker Microbiological Associates, Walkersville, MD). Livers were flushed with perfusion medium without [^{15}N]glycine for 3 min, after which time 0.3 mM glycine (0.21 mM unlabeled glycine and 0.09 mM [^{15}N]glycine), and 0.014 mM sodium benzoate were present in the medium. The perfusion flow rate was approximately 3 ml/g liver per min. The perfusion medium was collected from 0–15, 0–30, 30–60, and 60–90 min for the measurement of ^{15}N-enrichment of medium hippurate and apoB. After 15, 30, or 90 min, the perfusion medium was changed to the glycine-free medium and the liver was flushed for 1.5 min, frozen with dry ice, and stored at −70° until analyzed. The perfusion medium, collected on ice, was concentrated by ultrafiltration prior to lipoprotein isolation using an Amicon PM-10 membrane as previously described (4). Aliquots of the ultrafiltrate were used to isolate perfusate glycine and hippurate for the measurement of ^{15}N-enrichment as described below.

Measurement of ^{15}N-enrichment of liver glycine, medium glycine, and medium hippurate

Samples of liver were extracted with 10% trichloroacetic acid at 0°C. After removal of the acid by ether extraction, the solution was lyophilized and the residue was partially purified by ion exchange chromatography on AG50W-X8 columns (2). The amino acids were derivatized by the TBDMS procedure and subjected to GLC–MS analysis as previously described (2). Hippurate was partially purified by methanol elution from octadecyl columns (Baker-10 SPE) and GLC–MS analysis was carried out on the TBDMS derivative (2).

Measurement of ^{15}N-enrichment of liver glycyl-tRNA

Frozen livers were homogenized with a Polytron at 0°C in 10 vol of buffer A and 10 vol of 90% phenol according to the procedure of Flaim et al. (5). RNA was precipitated with ethanol as described (5) and the final precipitate was extracted three times with cold 5% trichloroacetic acid to remove glycogen and any traces of amino acids. After washing with cold ethanol, the precipitate was dissolved in 0.05 N ammonium hydroxide and incubated for 1 h at 37°C. The solution was then ultrafiltered through an Amicon PM-10 membrane. After lyophilization, the amino acids were derivatized and the ^{15}N content was determined as indicated above.

Apolipoprotein B isolation

The $d < 1.006$ and the $1.006 < d < 1.063$ g/ml fractions were isolated by sequential density ultracentrifugation as previously described (4) after the addition of 10 mM EDTA and 10 μM PMSF. Apolipoprotein B was isolated by the isopropanol method (6) and extracted with isopropanol to remove lipids. The dried protein was hydrolyzed in 6 N HCl for 24 h at 110°C under nitrogen. After removal of the HCl, the TBDMS derivative was made and analyzed for the content of ^{15}N. An aliquot of the hydrolysate was reacted with fluorescamine (7) and the amount of apoB was measured using as a secondary standard a purified sample of human LDL subjected to the hydrolysis procedure. The protein content of the human LDL was measured by the method of Lowry et al. (8) using bovine serum albumin as the primary protein standard.

Other methods

The purity of the isolated RNA was checked by measuring the 260/280 nm absorbance ratio, which ranged from 1.92 to 1.97, indicating less than 10% contamination with protein (9). The purity of the human LDL standard was checked by SDS-gel electrophoresis, and appeared to contain only apoB. In some experiments, the TCA-insoluble fraction of the liver was treated with hot 10% TCA and extracted with chloroform–methanol 2:1 (v/v) to remove lipid. The dried protein was weighed and hydrolyzed as described above for apoB to obtain values for the [^{15}N]glycine enrichment of total liver protein.

RESULTS

The ^{15}N-enrichment of hepatic free glycine and of glycyl-tRNA, shown in **Fig. 1,** reached essentially plateau values after 30 min of flow-through perfusion. By contrast, hippurate enrichment increased between 30 and 60 min, with no further increase between 60 and 90 min. At 15 and 30 min, the enrichment of hepatic free glycine was significantly less than that of glycyl-tRNA (-14.8 ± 2.04%, as shown in **Table 1**). Hippurate enrichment was

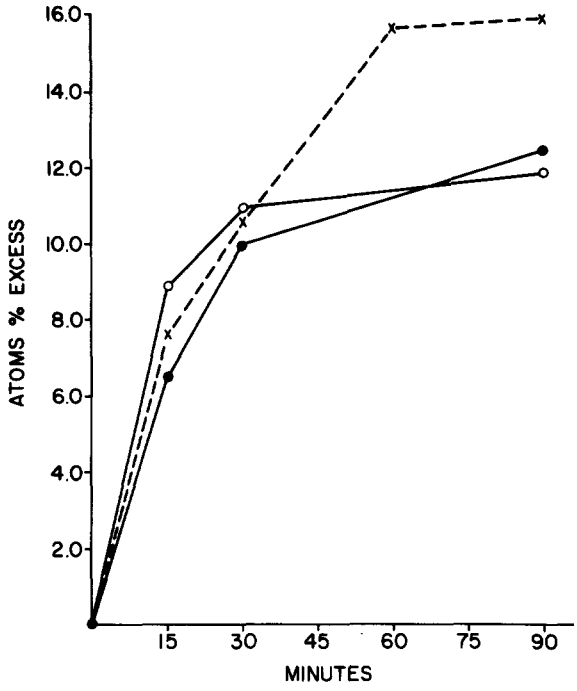

Fig. 1. Time course of ^{15}N-enrichment in hepatic free glycine (●—●), hepatic glycyl-tRNA (○—○), and perfusate hippurate (X---X) during single-pass perfusion of rat livers with [^{15}N]glycine.

not significantly different from that of free hepatic glycine at 15 and 30 min. At 90 min, when hepatic glycine and glycyl-tRNA enrichment had reached a maximum, hippurate enrichment was significantly greater (+30.6 ± 9.46%). The medium glycine enrichment was 30% of [^{15}N]glycine by weight and 33.1% ± 0.456% (SEM, n = 31) by analysis. No significant differences were observed between hepatic inflow and outflow medium[^{15}N]-glycine enrichment.

The output of apoB into the perfusion medium was linear over the 90-min perfusion period (**Fig. 2**). ^{15}N incorporation into apoB was negligible during the first 30 min and increased linearly between 30 and 90 min. From the enrichment of secreted apoB during 30–60 min of 1.36 atoms % excess and the approximate mean enrichment of hepatic glycyl-tRNA at the midpoint of the collection period, the FSR was 1.36 × 2/11.2 or 0.243/h. From the 60–90-min period, the value was 1.54 × 2/11.6 or 0.266/h, yielding a mean value of 0.255/h. The mean secretion rate for apoB was 22 μg/g per h (Fig. 2); therefore, the total intrahepatic pool size of apoB can be estimated as 22/0.255 or 86 μg/g.

At 90 min, the [^{15}N]glycine enrichment of the total liver protein averaged 0.26 ± 0.0066 atoms % excess (n = 3). From Fig. 1, the average enrichment of precursor glycyl-tRNA over the 90 min period was estimated at 10.5 atoms % excess. Assuming a linear increase in [^{15}N]glycine enrichment of total liver protein, the FSR can be calculated as 0.173/10.5 = 0.0165/h. From the dry weight of TCA-insoluble liver protein, which averaged 190 ± 7.41 mg/g wet wt. (n = 4), the absolute synthetic rate of the total liver protein during the perfusion period can be calculated as 190 × 0.015, or 3.14 mg/g per h.

DISCUSSION

The present experiments were designed to determine whether or not the isotopic enrichment of the intrahepatic glycine precursor pool could be estimated by measuring the enrichment of the glycine conjugate, hippuric acid, in a rat liver perfusion system. In human studies, using a priming dose of [^{15}N]glycine followed by intravenous infusion, we initially observed (2) that urinary hippurate enrichment was about 20% higher than the enrichment of plasma VLDL-apoB when the latter reached, or appeared to reach, a plateau value in five normolipemic subjects. The advantage of using hippurate for this purpose is that

TABLE 1. ^{15}N-Enrichment of hepatic free glycine, hepatic glycyl-tRNA, and perfusate hippurate during perfusion with [^{15}N]glycine

Time	Glycine	Glycyl-tRNA	Hippurate
min		*atoms % excess ± SEM*	
15	7.31 ± 0.837 (3)[a]	8.46 ± 0.826 (3)	7.55 ± 1.11 (3)
30	9.81 ± 0.782 (3)[b]	11.0 ± 0.779 (3)	10.6 ± 0.593 (7)
60	n.d.	n.d.	15.7 ± 1.07 (4)[c]
90	12.4 ± 0.962 (4)	11.8 ± 0.765 (4)	15.9 ± 1.33 (4)[d]

[a]Significantly different from the value for glycyl-tRNA, based on individual perfusions (−14.8% ± 2.04%, n = 3, $P < 0.02$). The numbers in parentheses represent the number of experiments.
[b]Significantly different from the value for glycyl-tRNA, based on individual perfusions (−10.4% ± 2.28%, n = 3, $P < 0.05$).
[c]Significantly different from the average value for 30 min ($P < 0.01$).
[d]Significantly different from the value for glycyl-tRNA at 90 min based on individual perfusions (+37.7% ± 6.04%, n = 4, $P < 0.01$). Also, the mean value for glycine and glycyl-tRNA at 90 min (12.1 ± 0.580, n = 8) is significantly different from the mean value for hippurate at 90 min ($P < 0.05$).

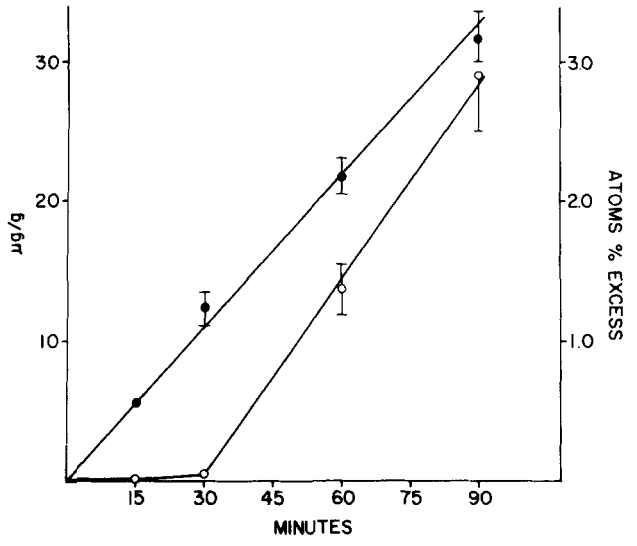

Fig. 2. Secretion rate of d < 1.063 g/ml lipoprotein apoB (●—●) and ^{15}N-enrichment (○—○) in perfused livers. The error bars represent one standard deviation (n = 2, 4, 3, and 3, respectively, at 15, 30, 60, and 90 min).

the plateau value for a given protein may not be reached within a reasonable time period if the plasma concentration is high, or the turnover rate is slow. Such a situation exists with respect to LDL-apoB in comparison with VLDL-apoB, and in individuals with hypertriglyceridemia in which the plasma VLDL concentration is increased (2). Therefore, it would be advantageous to use a readily excreted amino acid conjugate, such as hippurate, to measure the enrichment of the intracellular precursor pool. The discrepancy between the two approaches—hippurate versus plateau enrichment of the protein—led to the present experiments using the isolated single-pass perfused liver in which the hippurate secreted into the perfusate and the intracellular glycine precursor pool (glycyl-tRNA) enrichment could be directly compared.

The nonrecirculating (single-pass) perfusion technique has been used in this laboratory to measure lipoprotein secretion rates and the performance of this perfusion system compared favorably with recirculating systems using more complex media containing red cells (10). In the present circumstance, the advantage of using this method is that there is minimal opportunity for dilution of the isotopic glycine in the perfusion medium with intracellular glycine, given the rapid flow rate, and the enrichment remains constant throughout the experiment. Furthermore, one can measure the secretory rate of a given protein without the complication of hepatic reuptake. The disadvantage is that large volumes of medium must be concentrated, and there may be adsorptive losses of lipoproteins or apolipoproteins in the process. However, the use of a single, preconditioned filter in the concentrating apparatus for all of the experiments minimized this problem. In the present experiments, we did not use anesthetic agents prior to surgery in order to avoid changes in permeability in any intrahepatic compartment, especially mitochondrial, which can be caused by these compounds (11). This was considered important because the glycine conjugation system is intramitochondrial (12) in contrast to cytoplasmic tRNA synthesis (5). The liver is deprived of its oxygen supply during the 1–2 min required to cannulate the portal vein prior to perfusion with an oxygenated buffer, but we found no evidence that the livers performed any differently than in previous studies where rats anesthetized with nembutal were used (10).

The hepatic glycyl-tRNA enrichment was slightly greater than that of the TCA-soluble glycine during the first 30 min. This observation agrees with previous reports (13) and the extent of the increased enrichment of glycyl-tRNA is close to that predicted from the equation of Vidrich et al. (13). By 90 min, however, these enrichments were not significantly different from one another. Hippurate, on the other hand, was less enriched in the early times but did not reach a plateau value until 60 min of perfusion, at which point it was 30% higher than hepatic glycine. At its plateau value, the hippurate in the perfusion medium had an average enrichment of 15.8 ± 0.861 (n = 8, Table 1). This value is 48% of that of the glycine in the perfusion medium. In the study with human subjects (2) the average enrichment of the hippurate was 40–50% of that of the plasma glycine between 30 min and 8 h (2). Therefore, we suggest that glycine metabolism in this respect probably does not differ greatly between human and rat liver, and that if one were to apply a 30% correction, the isotopic enrichment of urinary hippurate would be equal to that of VLDL-apoB at its plateau value and would be a reasonable measure of the intrahepatic free glycine (or glycyl-tRNA) pool. However, the extent of the correction required could vary under different circumstances, such as dietary or hormonal perturbations. When a correction value of 30% is applied to the data (2) for the calculation of the FSR of human VLDL-apoB, a value of 13.1 $days^{-1}$ is obtained in normal subjects, which is close to the value of 13.9 $days^{-1}$ reported by Eaton, Allen and Schade (14) using ^{75}Se-labeled methionine as an endogenous label. In preliminary studies in human subjects, the correction may be closer to 20%, based on comparisons of hippurate enrichment with plateau values for VLDL-apoB.

The explanation for the difference in average enrichment of the two intrahepatic glycine pools after 90 min of perfusion is not evident. Glycine has many metabolic pathways within the liver cell, and it may be assumed that the dilution of isotopic glycyl-tRNA from unlabeled glycine occurs without complete mixing with the glycine newly transported into the cell and subsequently actively

transported into the mitochondria prior to conjugation. A greater enrichment of the glycine of hippurate compared to hepatic free glycine has been observed by Garfinkel and Lajtha (15). A kinetic computer model was developed by Garfinkel (16) in which the existence of diffusion gradients was assumed because portal blood flow occurs from the periphery to the center of the lobule. In this model, if peripheral hepatocytes synthesize more hippurate as a consequence of greater availability of benzoate or glycine, a higher isotopic enrichment of hippurate glycine would be predicted. It is also possible that mitochondria that bump up against the cell membrane have access to extracellular glycine before it is diluted with cytoplasmic glycine.

The secretion of apoB (including both apoB-100 and apoB-48) during the 90-min perfusion period averaged 22 μg/g per h in these experiments, a value in agreement with that of 17 μg/g per h found in our previous study (17). Our estimate of the intracellular pool size of apoB of 86 μg/g is close to the value of 102 μg/g obtained by Patsch, Franz, and Schonfeld (18) and of 85 μg/g by Sparks et al. (19) with immunochemical methods in cultured rat hepatocytes. Much of this pool may be in Golgi vesicles.

The time delay in secreted ^{15}N-labeled apoB represents the minimum intracellular transit time of about 30 min. If one assumes a unidirectional secretory pathway and an intracellular pool of apoB that is turning over, then under steady state conditions the FSR = 1/residence time, or 2 pools/h. This is far higher than the FSR of 0.26/h calculated from the isotope enrichment data. ApoB can be compared to plasma albumin. An albumin transit time of 30 min can be estimated from the data of Feldhoff, Taylor, and Jefferson (20). Peters and Peters (21) found that albumin synthesis as measured by leucine incorporation over 16 min into microsomal albumin was 0.35 mg/g per h and this was equal to the synthesis rate estimated from the loss of microsomal albumin in the presence of cycloheximide. This is evidence that the steady state assumption is correct for albumin and therefore the secretion rate can be equated to the synthesis rate. Based on measurements of the intracellular pool of albumin, 0.443 mg/g, the FSR can be calculated as 0.443/0.35 or 1.3 pools/h which is closer to a 1/transit time of 2. In the case of apoB, the discrepancy between the transit time and the FSR could be explained by assuming more than one pathway for apoB within the cell. The most likely explanation would be intracellular recycling of apoB, in a manner analogous to that of receptor recycling. The possibility of a qualitatively different intracellular processing of apoB, in contrast to albumin, is supported by the work of Borchardt and Davis (22) who reported evidence for the intracellular degradation of newly synthesized apoB. Alternatively, the kinetic data in the present study may simply reflect a difference between apoB-100 and apoB-48, which were not separated in the present work. This seems less probable since the transit times of both apoB-100 and apoB-48 have been measured by Borchardt and Davis (22) in cultured rat hepatocytes. The longest transit time, approximately 45 min for apoB-100, would give a calculated FSR of 1.3/h, which is still five times higher than expected.

The synthetic rate of the total liver protein as calculated from the present experiments, 3.14 mg/g per h, is close to previous estimates of 2.8 mg/g per h in a similar nonrecirculating liver perfusion system using ^{14}C-labeled leucine at its plasma concentration (5). We believe the present work supports the conclusion that the [^{15}N]glycine infusion technique using hippurate as a measure of the intracellular glycine pool enrichment is a useful approach to the study of apolipoprotein biosynthesis in vivo, both in human and animal studies.

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