Comparison of Serine and Hippurate as Precursor Equivalents During Infusion of [^{15}N]Glycine for Measurement of Fractional Synthetic Rates of Apolipoprotein B of Very–Low-Density Lipoprotein

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Enrichment in hippurate has been measured to indicate precursor enrichment during glycine tracer infusion studies to estimate fractional synthetic rates of individual hepatic export proteins. However, hippurate tends to overestimate precursor enrichment. Since glycine is rapidly converted to serine by liver cells, we compared tracer enrichment in hippurate and serine with that of glycine incorporated into apolipoprotein (apo) B-100. Ten healthy control subjects were studied in the postabsorptive state during an 8-hour primed-constant infusion of [^{15}N]glycine (10 μmol · kg^{-1} · h^{-1}). Apo B of very–low-density lipoprotein (VLDL) was isolated by standard ultracentrifugation and isopropanol precipitation. Glycine and serine were isolated from plasma and hydrolyzed apo B, hippurate was isolated from plasma, and [^{15}N]enrichment was determined by gas chromatography–mass spectrometry. Enrichment in serine and glycine isolated from apo B was identical at all time points, and their enrichment in apo B increased asymptotically, approaching an apparent plateau (mean ± SD: 91% ± 10% of calculated plateau at 8 hours) that was taken to represent hepatic protein precursor enrichment. Enrichment in both plasma serine and hippurate followed a biphasic pattern and continued to increase until the end of the study, raising the possibility that precursor enrichment had not reached a steady state during the study. The apo B plateau was lower (factor 0.76 ± 0.27) than the final enrichment in hippurate and higher (factor 1.38 ± 0.36) than that in plasma serine; however, predictions of protein precursor enrichment based on either metabolite were flawed by a large coefficient of variation (35% *v* 26%). We conclude that glycine enrichment in the hepatic protein precursor pool may not be constant during an 8-hour infusion study, and that only a rough approximation of this level may be obtained using either enrichment in plasma hippurate or plasma serine.

IN VIVO SYNTHETIC RATES of individual human plasma proteins may be determined by observing tracer incorporation rates during infusion of amino acid tracers. Increasingly, amino acids labeled with stable isotopes are being used for this purpose, and several methods have been developed.[1-4] Apolipoprotein (apo) B has been a focus of attention, because of both its clinical importance in atherogenesis and its relatively rapid turnover.[5-9]

Since interpretation of tracer incorporation data requires knowledge of the precursor enrichment,[10] ie, enrichment of the appropriate amino acyl-transfer RNA (tRNA), different approaches have been used to estimate precursor enrichment in vivo. Isolation of tRNA is cumbersome[11] and not generally possible. As an alternative, it has been assumed that amino acid metabolites are being synthesized from the same intracellular pool that charges tRNA. Therefore, enrichment has been measured in amino acid metabolites isolated from plasma or urine.[3,12-17]

Among different amino acid tracers, [^{15}N]glycine is readily available and has been used frequently to estimate synthetic rates of hepatic export proteins.[1-5,12,14,18] Glycyl-tRNA appears to be synthesized mainly from an intracellular pool.[19-21] To judge intrahepatic dilution of an infused glycine tracer, enrichment has been monitored in urinary[1-4,12,18] and plasma[5,16,17] hippurate. However, hippurate enrichment has been shown consistently to be higher than the true precursor enrichment if this was taken to be the final plateau enrichment in apo B in very–low-density lipoprotein (VLDL).[4,5,16,17] In addition, equilibration of enrichment in hippurate is slow.[4,5]

Glycine is rapidly and reversibly converted to serine by L-serine hydroxymethyl transferase (EC 2.1.2.1.).[20,22] This enzyme is widely distributed and found both in the cytosol and mitochondria of various organs.[22,23] In fact, infusion of [^{15}N]glycine leads to identical incorporation of [^{15}N]glycine and [^{15}N]serine into serum albumin[24] or mixed liver proteins.[19,20] It thus appeared possible that during infusion of a glycine tracer, enrichment in plasma serine might approximate intrahepatic glycine enrichment. We therefore monitored the transfer of [^{15}N] from infused glycine to serine in plasma and apo B and compared this with the enrichment in plasma hippurate.

SUBJECTS AND METHODS

Subjects

The study protocol was reviewed by the Ethics Committee of the University of Göttingen. Ten healthy non-obese male subjects participated in this study after providing informed consent. Control subjects ranged in age from 19 to 30 years and were of normal height (175 to 187 cm) and weight (62 to 86 kg). None of the subjects were taking any medication.

Tracer Material

[^{15}N]glycine was obtained from MSD Isotopes (Merck Chemical Division, St Louis, MO). Isotopic purity and chemical purity as determined by conventional mass spectrometric methods were 99.5% [^{15}N] and 100% glycine. For infusion, the tracer was dissolved in 0.15 mol/L NaCl and sterile-filtered (0.22 μm; Millipore, Milford, MA). The tracer material was shown to be pyrogen-

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free by a licensed commercial firm (Scientific Associates, St Louis, MO) using the rabbit body-temperature assay.

Experimental Design

The studies were performed in the Metabolic Research Unit at the Medical Center of the University of Göttingen. All tracer infusion studies were performed after an overnight fast in the postabsorptive state. No food was given during tracer infusions, but subjects were allowed free access to drinking water. Subjects remained recumbent throughout the infusion studies. At 7 AM, two intravenous lines were placed in cubital veins of both arms; one served for tracer infusion and the other for sampling. The sampling line was kept patent with a slow infusion of 0.15 mol/L NaCl. Tracer infusion was begun at 8 AM and involved a priming dose (8.7 $\mu mol \cdot kg^{-1}$) followed by a constant infusion (10 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of [$^{15}N$]glycine for 8 hours. Blood samples (10 mL) were collected at −30, 0, 30, and 60 minutes and hourly thereafter until 480 minutes. Samples were drawn into tubes containing EDTA (final concentration, 0.1%), and plasma was separated by centrifugation at 4°C. Plasma was either processed immediately for isolation of lipoproteins or stored at −20°C until further analysis.

Isolation of Lipoproteins

Lipoproteins were isolated from plasma by sequential ultracentrifugation. VLDLs were prepared from 4 mL plasma after overlayering with 6 mL 0.196-mol/L NaCl (containing 2 mol/L EDTA) by a single ultracentrifugation at a density of 1.006 $g \cdot mL^{-1}$ (84,000 × g for 24 hours at 12°C) in a Type 40 fixed-angle rotor (Beckman Instruments, Palo Alto, CA). After centrifugation, VLDLs were removed in the top 1.5 mL of each tube by tube slicing.

Isolation of Apo B and Hydrolysis

Apo B was isolated from VLDL by isopropanol extraction as described by Egusa et al.[25] This method has been reported to result in preparation of almost pure apo B in normocholesterolemic subjects. In our hands, when subjected to polyacrylamide gel electrophoresis and densitometric scanning, isopropanol precipitates were free of apos C and B-48 and contained only minimal amounts of apo E. The precipitated protein was hydrolyzed in 6N HCl for 16 hours at 110°C,[26] and the hydrochloric acid was evaporated at 110°C under a stream of nitrogen.

Measurement of [^{15}N] Enrichment

Measurement of [^{15}N] enrichment in glycine and serine was performed as described previously.[17] Briefly, amino acids were isolated from 0.5-mL aliquots of plasma by cation-exchange chromatography (AG-50W-X8 resin; Bio-Rad Laboratories, Richmond, CA). Amino acids obtained from plasma or apo B samples were derivatized to yield the N-acetyl-1-propanol ester, and [^{15}N] enrichment was determined by gas chromatography–mass spectrometry using a Finnigan 3300 quadrupole mass spectrometer (San Jose, CA) and methane positive chemical ionization.

Calibration standards containing known amounts of [^{15}N]glycine or [^{15}N]serine in the range of 0% to 10% enrichment were analyzed by gas chromatography–mass spectrometry. For each standard, the observed ion current ratios of MH^+ ions 161/160 for glycine or 233/232 for serine were plotted over isotope mole ratios (R) of $^{15}N/^{14}N$. Standard curves were fitted by linear regression and were used to obtain isotope ratios from observed ion current ratio data of unknowns. Isotope ratios (R) were converted to enrichment values (E) by calculating fractional abundances as $q = R/(R + 1)$ and subtracting the contribution of natural material (q_n) to yield $E = q - q_n$.[27] Measurement of enrichment in hippurate was performed as described previously.[16] All enrichment results were multiplied by 100 and expressed as mole percent excess. Assay precision was 1.2% or less relative standard deviation for measured ion current ratios.[17]

Plasma Glycine Flux

Plasma glycine flux was calculated as $R_a = i \cdot (E_{tr}/E_{pl} - 1)$,[28] where R_a is endogenous plasma glycine flux, i is [^{15}N]glycine infusion rate, E_{tr} is [^{15}N] enrichment of the infused tracer, and E_{pl} is [^{15}N] enrichment of plasma glycine at plateau. For each experiment, E_{pl} was taken to be the mean enrichment measured from 300 to 480 minutes.

Model for VLDL Apo B Metabolism

A single-compartment model was used to describe the dynamic aspects of apo B metabolism in VLDL, where input is from hepatic synthesis and output is catabolic conversion to low-density lipoprotein or receptor-mediated removal by the liver. For each experiment, amino acid enrichment in apo B was modeled by nonlinear regression according to the equation[5] $E_{VLDL} = E_p \cdot [1 - e^{-k\cdot(t-d)}]$, where E_p is VLDL apo B enrichment plateau, k is fractional turnover rate for VLDL apo B (measured in pools per day), and d is intrahepatic delay time between the beginning of apo B synthesis and VLDL secretion. The enrichment plateau E_p is identical to the enrichment in newly synthesized apo B and should thus be identical to intrahepatic glycine enrichment. Adding a delay time d to the model was necessary, since enrichment in VLDL apo B was undetectable for 20 to 30 minutes after beginning tracer infusions. Nonlinear regression was performed on an IBM personal computer (Amoug, NY) by an iterative least-squares procedure using the Quasi-Newton method (Systat, Evanston, IL).

Statistical Analysis

Unless stated otherwise, all data are presented as the mean ± SE. Significance of linear and nonlinear regressions was tested by calculating F values by dividing the regression mean squares by residual mean squares.[29]

RESULTS

[^{15}N]glycine infusion labeled approximately 5% of plasma glycine (Fig 1A), plateau enrichment was reached rapidly, and glycine flux was calculated to be 176.5 ± 5.5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. [$^{15}N$] enrichment in both plasma hippurate and plasma serine increased rapidly within 30 minutes and then continued to increase slowly after that time without reaching a definite plateau within 8 hours. Enrichment in hippurate was higher than enrichment in plasma serine and approached 50% of plasma glycine enrichment; plasma serine enrichment reached roughly 25% of plasma glycine enrichment.

[^{15}N] enrichment in both glycine and serine of apo B was almost undetectable at 30 minutes, but increased rapidly after that time. The time course of enrichment appeared to follow an asymptotic curve approaching a plateau value. Enrichment in apo B glycine and serine was virtually identical throughout the infusion study (Fig 1A). The mean serine to glycine enrichment ratio of all measurements (1.03 ± 0.02) did not differ significantly from unity (Fig 2A). Nonlinear regression yielded highly significant solutions (Table 1): average plateau enrichment was 1.95 ± 0.15 mole percent excess, fractional turnover rate for apo B was

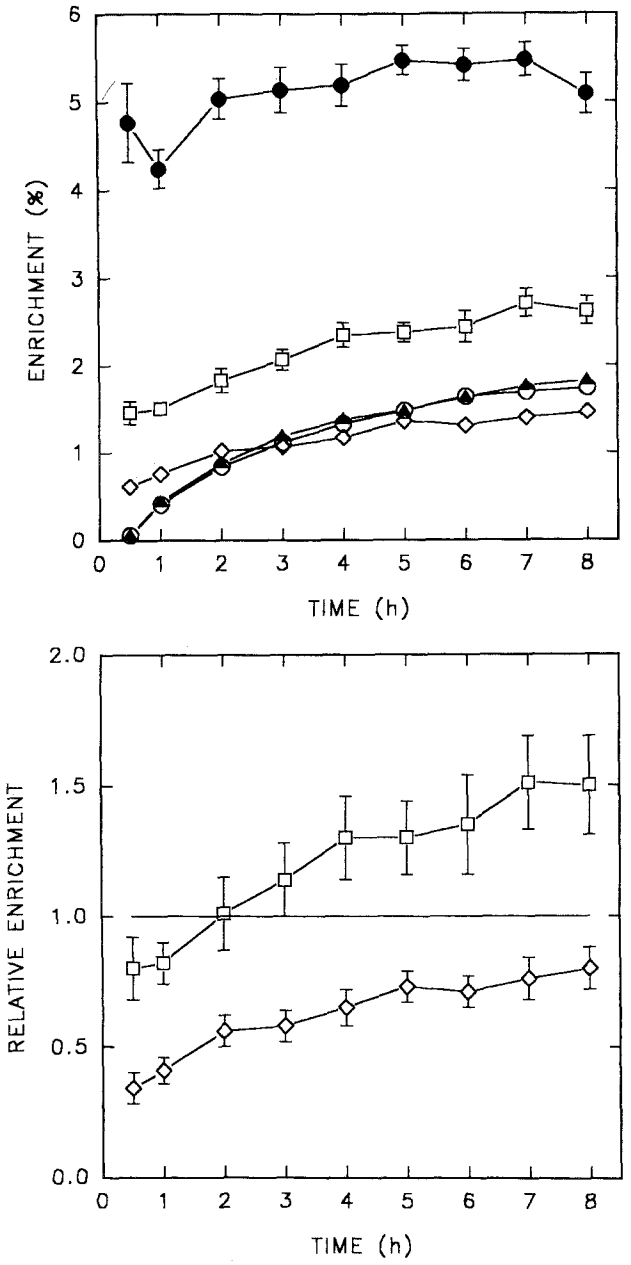

Fig 1. (A) Enrichment in plasma glycine (●), plasma hippurate (□), plasma serine (◇), apo B glycine (○), and apo B serine (▲) in 10 healthy volunteers during infusion of [15N]glycine. (B) Relative enrichment versus calculated apo B glycine plateau in plasma hippurate (□) and plasma serine (◇). Mean ± SE. Where no error bars are shown, error bars are within symbols.

9.7 ± 1.4 pools · d^{-1}, and computed delay time was 25 ± 2 minutes. At the end of the study, 91% ± 3% of the calculated plateau enrichment had been reached in apo B.

Enrichment in plasma hippurate reached the calculated apo B plateau enrichment at 2 hours and 149% ± 18% of the apo B plateau at 8 hours. Serine enrichment was always less than the apo B plateau value, and while still increasing it reached 80% ± 8% of the plateau at the end of the study (Fig 1B). The increase in enrichment of plasma serine and hippurate could not be explained by changes in plasma glycine enrichment, since throughout the study enrichment in both metabolites increased at a similar rate relative to plasma glycine enrichment (Fig 2B). Linear regression of 1- to 8-hour data yielded positive slopes for the hippurate to glycine (R^2 = .932, $P < .001$) and serine to glycine ratios (R^2 = .892, $P < .001$), while the serine to hippurate ratio remained constant (R^2 = .257, NS).

Absolute and relative final enrichment data are listed in Table 2. The apo B enrichment plateau could be predicted from 8-hour enrichment data by multiplying plasma glycine

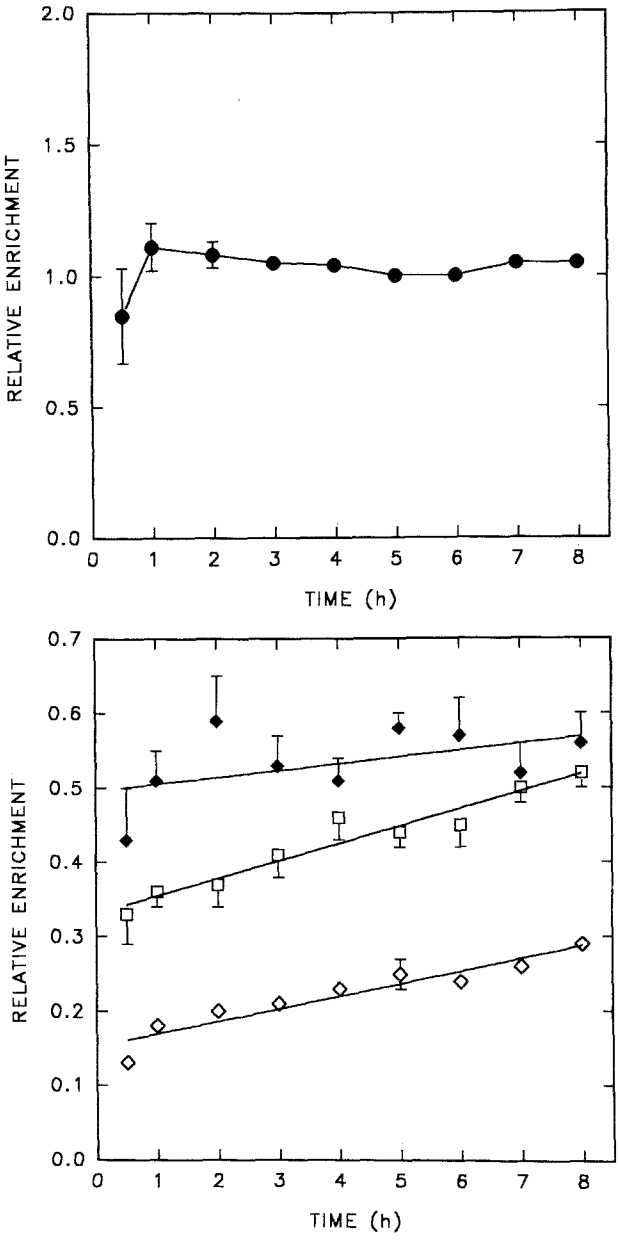

Fig 2. (A) Enrichment in apo B serine relative to apo B glycine in 10 healthy volunteers during infusion of [15N]glycine. (B) Plasma enrichment in serine (◇) and hippurate (□) relative to glycine and to each other (◆) during infusion of [15N]glycine. Corresponding linear regression lines were fitted to 1- to 8-hour data. Mean ± SE. Where no error bars are shown, error bars are within symbols.

Table 1. Modeling of Glycine Enrichment Data in VLDL Apo B

Subject No.	Plateau (M%E)	FSR (per day)	Delay (hours)	F	% of Plateau at 8 Hours
1	1.790	0.233	0.302	454	0.83
2	1.708	0.555	0.584	341	0.98
3	1.501	0.513	0.389	657	0.98
4	2.333	0.220	0.517	536	0.81
5	1.782	0.353	0.435	230	0.93
6	2.914	0.162	0.472	448	0.70
7	2.269	0.407	0.232	327	0.96
8	1.243	0.788	0.366	537	1.00
9	1.871	0.361	0.342	1,003	0.94
10	2.040	0.446	0.476	6,637	0.97
Mean	1.945	0.404	0.412		0.91
SD	0.472	0.186	0.106		0.10
SE	0.149	0.059	0.033		0.03

Abbreviations: M%E. mole percent excess; FSR, fractional synthetic rate of apo B; F, regression mean squares/residual mean squares.

enrichment by 0.37, plasma serine enrichment by 1.38, and plasma hippurate enrichment by 0.76. Corresponding coefficients of variation (relative standard deviation) were 29.7%, 26.0%, and 35.5%.

DISCUSSION

Determining in vivo protein synthetic rates using an amino acid tracer requires knowledge of the magnitude of intracellular tracer dilution. In our subjects, tracer appearance in apo B of VLDL during infusion of [^{15}N]glycine could be well approximated by a one-compartment model. Although such a model is an oversimplification due to the known heterogeneity of VLDL,[30] its advantage in the present case is the integration of tracer behavior in VLDL.[8] Since glycine enrichment in VLDL apo B had nearly reached a plateau at the end of the study, the apparent hepatic protein precursor enrichment could be predicted with high accuracy. This analysis can indicate final hepatic precursor enrichment, but does not establish that precursor enrichment was stable throughout the study. However, overestimation of the average precursor enrichment will result in underestimation of protein turnover rates.

Infusion of [^{15}N]glycine led to the immediate appearance of labeled serine in newly synthesized hepatic apo B. Since enrichment of incorporated serine was identical to that of glycine, hepatic interconversion of glycine and serine had to be extremely rapid. This is in agreement with in vitro and in vivo data in rats[19,20] and confirms earlier human studies.[24] In rats, L-serine hydroxymethyl transferase is present in large amounts in the liver, whereas the kidney contains less and other tissues only small amounts.[20,23]

Whereas enrichment in plasma glycine was constant throughout the study, enrichment in plasma serine and hippurate never reached a steady state. The capacity for rapid intrahepatic glycine-serine equilibration may have been responsible for the rapid early increase in plasma serine enrichment, indicating an intense exchange between hepatic and plasma serine. Plasma serine enrichment was always less than the plateau enrichment in apo B, ie, the presumed intrahepatic precursor enrichment, reaching 80% of the apo B plateau after 8 hours. Similar results have been observed in animal studies[20] indicating contributions to plasma serine by other organs with low enrichment.

Due to the low availability of the renal transferase, tracer equilibration in the kidney is slow.[20,31] Serine contributed to the circulation by the kidneys[31-33] will thus display a gradual increase in enrichment. This process might explain the slow secondary increase in plasma serine enrichment. As a consequence, serine exchange between plasma and liver would tend to produce a slow increase of hepatic serine and subsequently hepatic glycine enrichment. Thus, assuming constant precursor enrichment may not be valid in studies using an infusion of [^{15}N]glycine.

Hippurate is mainly synthesized in liver mitochondria from glycine and benzoic acid.[34] Plasma hippurate enrichment increased rapidly after the start of the glycine infusion, indicating rapid glycine tracer appearance within liver mitochondria. This early phase was followed by a slow and prolonged increase in hippurate enrichment similar to the

Table 2. Absolute and Relative Final Enrichment Values

	M%E									
Subject No.	PL-GLY	PL-SER	PL-HIP	VLDL-GLY	PL-SER/PL-GLY	PL-HIP/PL-GLY	PL-SER/PL-HIP	VLDL-GLY/PL-GLY	VLDL-GLY/PL-SER	VLDL-GLY/PL-HIP
1	4.96	1.33	2.02	1.79	0.27	0.41	0.66	0.36	1.35	0.89
2	5.52	1.57	2.38	1.71	0.28	0.43	0.66	0.31	1.09	0.72
3	5.82	1.55	3.55	1.50	0.27	0.61	0.44	0.26	0.97	0.42
4	5.11	1.36	2.87	2.33	0.27	0.56	0.47	0.46	1.72	0.81
5	4.29	1.22	2.34	1.78	0.28	0.55	0.52	0.42	1.46	0.76
6	4.94	1.47	2.14	2.91	0.30	0.43	0.68	0.59	1.99	1.36
7	5.45	1.47	2.65	2.27	0.27	0.49	0.55	0.42	1.55	0.86
8	6.18	1.64	3.01	1.24	0.26	0.49	0.54	0.20	0.76	0.41
9	5.17	1.33	2.87	1.87	0.26	0.55	0.46	0.36	1.41	0.65
10	5.84	1.37	2.82	2.04	0.23	0.48	0.49	0.35	1.49	0.72
Mean	5.33	1.43	2.66	1.95	0.27	0.50	0.55	0.37	1.38	0.76
SD	0.55	0.13	0.46	0.47	0.02	0.07	0.09	0.11	0.36	0.27
SE	0.17	0.04	0.14	0.15	0.01	0.02	0.03	0.03	0.11	0.08

Abbreviations: PL, plasma; GLY, glycine; SER, serine; HIP, hippurate; M%E, mole percent excess.

course of serine enrichment. This observation supports our assumption that despite a constant plasma glycine enrichment level, glycine enrichment was increasing in tissues synthesizing hippurate and serine, possibly driven by a gradual increase in plasma serine enrichment. We cannot prove it directly, but we suspect that the intrahepatic protein precursor pool was affected similarly. In support of this hypothesis, higher turnover rates have been calculated for the same protein using a leucine as compared with a glycine tracer.[35] However, if protein precursor enrichment is not constant, then its time course needs to be known.

Obviously, glycine distribution within hepatocytes is not homogeneous,[36] and different pools are responsible for hippuric acid synthesis (mitochondria), loading of tRNA, and possibly serine-glycine interconversion (cytosol). In our subjects, enrichment in the apo B precursor pool was less than 40% of plasma glycine enrichment; thus, tracer in this pool was diluted substantially by intracellular sources. Hippurate enrichment at 2 hours reached the plateau enrichment in apo B and by the end of the study always surpassed it. Thus, in healthy controls the hippurate precursor pool was labeled more intensely than that used for protein synthesis. This agrees with data from rat liver-perfusion experiments[37,38] and confirms previous human data.[4,5,16,17] However, even though hepatic glycine pools were not labeled similarly, the enrichment in different pools might have been closely related.

Hippurate enrichment has been used by several investigators as a precursor equivalent during [^{15}N]glycine infusion.[1-5,16-17] However, the validity of this assumption was only tested in a few studies comparing direct labeling in hippurate and individual proteins close to steady state. Consistently, labeling in hippurate was 20% to 30% higher,[4,5,16,17] and a similar difference has been observed between rat hepatic glycyl-tRNA and newly synthesized hippurate.[37,38] From this, use of a factor has been proposed to convert hippurate enrichment to precursor enrichment.[37] Our data show that correction factors to obtain plateau enrichment in apo B could be applied equally well to enrichment in plasma glycine, serine, or hippurate. However, in all cases predictions were inaccurate and handicapped by coefficients of variation of approximately 30%, indicating only modest correlations between the respective tracer pools.

In conclusion, from our data it appears improbable that during a primed-constant glycine tracer infusion an early steady state may be reached in the hepatic protein precursor pool. Although calculation of protein synthetic rates then requires knowledge of the time course of precursor enrichment, our data do not support a sufficiently tight relationship between the protein precursor pool and either plasma glycine, serine, or hippurate. However, it may be argued that at least enrichment in hippurate and serine reflected the pattern of change in the precursor pool. These difficulties are probably related to the complexity of glycine metabolism, with intracellular compartmentation and rapid hepatic but slow renal glycine-serine interconversion. It thus has to be questioned whether glycine is an ideal tracer for determining rates of hepatic protein synthesis.

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