Dynamics of glutathione and ophthalmate traced with $^2$H-enriched body water in rats and humans


Departments of Nutrition, Surgery, Pharmacology, and Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio; and Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts

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Kombu RS, Zhang GF, Abbas R, Mieyal JJ, Anderson VE, Kelleher JK, Sanabria JR, Brunengraber H. Dynamics of glutathione and ophthalmate traced with $^2$H-enriched body water in rats and humans. Am J Physiol Endocrinol Metab 297: E260–E269, 2009. First published April 28, 2009; doi:10.1152/ajpendo.00080.2009.—We developed a LC-MS-MS assay of the $^3$H labeling of free glutathione (GSH) and bound glutathione [GSSR; which includes all DTT-reducible forms, primarily glutathione disulfide (GSSG) and mixed disulfides with proteins] and ophthalmate (an index of GSH depletion) labeled from $^2$H-enriched body water. In rats whose body water was 2.5% $^2$H enriched for up to 31 days, GSH labeling follows a complex pattern because of different rates of labeling of labeling of its constituent amino acids. In rats infused with $[^{14}C_2,^{15}N$-glycine]glutathione, the rate of appearance of plasma GSH was 2.1 μmol·min$^{-1}$·kg$^{-1}$, and the half-life of plasma GSH/GSSG was 6–8 min. In healthy humans whose body fluids were 0.5% $^2$H enriched, the $^2$H labeling of GSH/GSSR and ophthalmate can be precisely measured after 4 h, with GSH being more rapidly labeled than GSSR. Since plasma GSH/GSSR derives mostly from liver, this technique opens the way to 2) probe noninvasively the labeling pattern and redox status of the liver GSH system in humans and 2) assess the usefulness of ophthalmate as an index of GSH depletion.

mass isotomer analysis; oxidative stress; protein synthesis; peptide synthesis; deuterated water; liver

THE GLUTATHIONE REDOX SYSTEM includes the reduced and various oxidized forms of glutathione, abbreviated GSH, GSSG, and DTT-reducible forms of GSH (GSSR), where R represents the moiety of another thiol molecule. The GSH redox system is one of the main lines of defense of cell homeostasis against oxidative stress. Many studies have measured variations in the concentrations of GSH and GSSG in tissues under physiological and pathological conditions (12, 22, 23).

The biosynthesis of GSH has been investigated using amino acid tracers labeled with $^{35}$S, $^{13}$C, $^{14}$C, or $^2$H (6, 8, 13, 15, 24, 27, 36). One problem with these techniques is that the labeling of the amino acid at the site of GSH synthesis may not be the same as in body fluids, especially after bolus injection of label (27). In some studies, label administered as $[^{14}C]$glycine was reported as identified in the glutamate and cysteine moieties of GSH (8, 24). Jahoor et al. (15) infused $[^{13}C]$glycine in piglets and followed the enrichment of glycine in plasma and red blood cell GSH. They calculated a fractional synthetic rate of red blood cell GSH of 60%/day. Similar fractional synthesis rates of red blood cell GSH were reported during infusion of $[^{14}N]$glutamate in dogs (13) and during infusion of $[^{13}C]$glycine in humans (36). Note that erythrocytes synthesize their GSH, as shown by Elder and Mortensen (10) using $[^{14}$C]glycine.

The rate of synthesis of proteins and peptides in animals can be followed by enriching body water with $^2$H, and following the $^2$H enrichment of the product (4, 28, 33, 34). After administration of $^2$H$_2$O, the $^2$H enrichment of all body water equilibrates rapidly (29). Also, some free amino acids become labeled by enzyme-catalyzed isotope incorporation from the $^2$H of water as an inherent aspect of amino acid metabolism. The mechanisms of amino acid labeling involve reversible transaminations, reactions of the citric acid cycle (for aspartate, asparagine, glutamate, and glutamine), and the synthesis of alanine, serine, and glycine from glycolytic intermediates. Once incorporated into proteins or peptides, the $^2$H enrichment of the amino acid moieties of these compounds is stable even when the proteins are chemically hydrolyzed to amino acids (28).

Cabral et al. (5) recently reported a liquid chromatography-mass spectrometry (LC-MS) technique for measuring the $^2$H enrichment of GSH from body water. They reported that, during the synthesis of GSH in liver, six $^2$H atoms from water are incorporated into GSH on average. Since oxidized GSH, i.e., GSSG, yields a very weak LC-MS signal, it is difficult to precisely measure its concentration and labeling in plasma. Therefore, we decided to assay the concentration and labeling of all DTT-reducible “bound” forms of glutathione, i.e., GSSR, which includes GSSG. We recognize that GSSR comprises a very heterogeneous pool of substances. We expanded on the technique developed by Cabral et al. (5) by 1) converting the glutathione moieties of GSH and GSSR to two different stable thiourethanes and 2) conducting the analyses by LC-MS-MS, which allows measurement of the labeling of amino acid constituents of GSH. Using this new technique, we investigated the dynamics of labeling of the GSH/GSSR system in the liver, plasma, and erythrocytes of rats as well as in the plasma of healthy humans. In the same studies, we followed the dynamics of ophthalmate, a GSH analog (glutamate-2-aminobutyrate-glycine). This tripeptide, which is formed by the same enzymes as GSH (26), has recently been proposed as an index of oxidative stress since its concentration increases after depletion of the GSH pools by a load of acetaminophen (32).

METHODS

Materials and reagents. General chemicals, as well as $^2$H$_2$O (99%, glass distilled) and $[^{13}C_2,^{15}N$-glycine]glutathione (M3 GSH) were from Sigma-Aldrich (St. Louis, MO). Homoglutathione was from...
Animal experiments. All animal protocols were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Nineteen male Sprague-Dawley rats (200 ± 10 g; Charles River) received an intraperitoneal injection of normal saline in amounts calculated to achieve a 2% ²H enrichment of body water, assuming that total body water accounts for 66% of body weight. The rats were fed regular rat chow and provided drinking water 3.25% enriched in ²H to compensate for the production of unlabeled water from the oxidation of foodstuffs and endogenous substrates (20). One control rat was injected with unlabeled normal saline. At various times after ²H loading (2 h to 31 days), the rats were anesthetized with 2% isoflurane. After laparotomy, blood was sampled from the bifurcation of the aorta, using a large bore (18-gauge) needle attached to a syringe, and transferred to a heparinized Vacutainer tube (Gentest). Minimal pulling was exerted on the syringe piston to avoid hemolysis. Plasma samples with any visible coloration by hemoglobin were rejected. After the blood was collected, a large lobe of the liver was excised and immediately quick-frozen between steel plates precooled in liquid nitrogen, weighed, and stored in liquid nitrogen. The rest of the liver was weighed.

Nine other overnight-fasted rats (270 g), anesthetized with isoflurane and fitted with carotid and jugular catheters, were infused for 24 min with M3 GSH (1.71 μmol·min⁻¹·kg⁻¹ without bolus). The tracer was dissolved just before use in N₂-prebubulated saline. Arterial blood (150 μl) was sampled every 3 min until 40 min and analyzed for the concentration and labeling of GSH and GSSR.

Human investigations. Investigations on healthy humans were approved by the Institutional Review Board of University Hospitals-Case Medical Center. Subjects were 27–52 yr old (3 men, 3 women). The subjects were admitted to the Clinical Research Center after an overnight fast. After baseline blood sampling, they ingested ³H₂O in amounts calculated to enrich body fluids at 0.5%. The dose of ³H₂O, diluted with the same volume of tap water, was ingested in four aliquots over 1 h. Blood samples were taken at regular intervals for 8 h. After taking a meal, the subjects were discharged but returned to the center on the morning of days 2, 3, and 4, when additional blood samples were taken.

In vitro experiments. Blood was collected in a heparinized syringe from a healthy, overnight-fasted, adult man. After centrifugation, 5 ml of plasma was incubated at 37°C in a glass tube containing a small magnetic bar. After baseline sampling (t = −0.5 min), 100 nmol of M3 GSH was added to the plasma (t = 0). Samples of plasma (0.2 ml) were collected at various times from 0 to 60 min, transferred to tubes containing iodoacetate preservative solution (see below), and processed for the assay of the concentration and the mass isotopomer distribution of GSH and GSSR. An identical control experiment was conducted in 4% diazylized bovine serum albumin (fatty acid free; Intergen) dissolved in saline.

Sample processing. The heparinized blood, collected in glass tubes, was cooled by gentle repeated inversions in an ice + water slurry for 1 min, and centrifuged (1,800 g) at 4°C for 10 min (Fig. 1). To prevent its oxidation, GSH was immediately converted to a stable thioether by treating 100 μl of plasma with 100 μl of 50 mM iodoacetate in 10 mM ammonium bicarbonate, pH 10, adjusted with concentrated ammonia. After the buffy layer was removed, 50 μl of the red blood cell pellet was taken and treated with 450 μl of iodoacetate buffer. After vortexing to lyse the cells, the tubes were quick-frozen and stored at −80°C until analysis.

On the day of analysis, the protected plasma samples were spiked with 623 pmol of homoglutathione (20 μl of 10 μg/ml water) internal standard (13). For the assay in erythrocytes, 10 μl of the protected sample was diluted with 90 μl of water and spiked with the same amount of homoglutathione. Powdered frozen liver (100 mg) was homogenized in 1 ml of ice-cold iodoacetate buffer. Ten microliters of the slurry was diluted with 90 μl of water and spiked with the same amount of homoglutathione.

Samples treated with iodoacetate were kept at room temperature in the dark for 45 min to allow completion of the reaction (based on data from incubations for various lengths of time). To liberate the bound glutathione, 200 μl of DTT (100 mM, pH 10, in 10 mM ammonium bicarbonate) was added and allowed to react for 15 min at room temperature in the dark. Control experiments without GSH showed that all of the iodoacetate was removed by reaction with DTT. To this solution, 200 μl of iodoacetatitrol [200 mM, pH 10, in 10 mM ammonium bicarbonate containing 3.12 μM homo-GSH (623 pmol/200 μl)] was added to convert the liberated glutathione to a cyanomethyl thioether. After 30 min standing at room temperature in the dark, 1.5 ml of acetonitrile was added to precipitate the proteins. The sample was vortexed and centrifuged at 12,000 g for 15 min. The supernatant was collected and dried in a Turboprep (Caliper Life Sciences, Hopkinton, MA) at 50°C under air at 20 psi for 40 min. The residue was reconstituted in 200 μl of formic acid in water (0.1% vol/vol), and 25 μl was injected onto the LC-MS column.

Calibration curve standards were prepared in water by adding known amounts of GSH and GSSG. All calibration curves consisted of two blanks and eight calibration points. The curve ranges were as follows: GSH, 1.4–3,300 pmol; GSSG, 0.7–1,600 pmol; ophthalmic, 1.5–3,500 pmol. A weighting factor of 1/y² was applied over the calibration curves (17). The resulting peak area ratios of analyte/internal standard were plotted against the concentrations.

LC-MS-MS. An API 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with turbo electrospray ion source was operated under positive ionization mode. The LC system consisted of a Waters 2695 Separation Module (Waters, Milford, MA) and an API 4000 Q-Trap mass spectrometer (Applied Biosystems). The column used was a 5-μm Acquity UPLC HSS T3 column (2.1 × 150 mm, Waters). A flow rate of 0.2 ml/min and the total run time was 19 min. For elution, mobile phase A was initially held at 100% for 5 min. Then, from 5 to 6 min, the mobile phase was changed to 20% A + 80% B and held there for 7 min. Then, from 7 to 8 min, the eluent was brought back to 100% A and held there until 19 min for reequilibration. This long reequilibration was required to reduce the baseline noise. The Turbo ion spray source was maintained at 500°C with 5,500 V ion spray voltage. Nitrogen gas at a pressure of 70 psi was used to nebulize the sample into the source. The heater gas (N₂) was maintained at 90 psi. The curtain gas (N₂) pressure was 35 psi, and the collision-activated transitions of the analytes were monitored for the presence of product ions.
dissociation gas pressure was held at medium flow. Declustering potential was 65 V, entrance potential was 10 V, and collision cell exit potential was 12 V. Analyst software (version 1.4.2; Applied Biosystems) was used for data registration. Mass spectra were acquired under three modes. First, we recorded the enhanced resolution mass spectra of the intact molecules. Second, multiple reaction monitoring (MRM) ion pairs were monitored to acquire data used to calculate isotopic enrichments from precursor→product pairs. The ion pairs monitored were 1) for carboxymethyl-GSH: 366.1→237.1, 367.2→237.1, and 367.1→238.1; 2) for cyanomethyl-GSH derived from GSSR: 347.2→238.1, 348.2→218.2, and 348.2→219.1; 3) for carboxyethyl-homoglutathione: 380.1→233.1; 4) for cyanomethyl-homoglutathione: 361.1→232.1; and 5) for ophthalmate: 290.3→161.1, 291.3→161.1, and 291.3→162.1. Third, product ion spectra (MS-MS) were used to calculate the enrichments of fragments of the carboxymethyl-GSH derivative; from the precursor ions at mass-to-charge ratio (m/z) 366.1 and 367.1, we monitor m/z 134 [cysteine (CYS) at collision energy 33], 237 [glycine (GLY)-CYS at collision energy 19], and 291 [glutamyl (GLU)-CYS at collision energy 21], with a mass width of 5 amu, from m/z 100 to 400.

The 2H enrichment of plasma water was assayed by exchange with unlabeled acetone in alkaline medium, followed by extraction and GC-MS of deuterated acetone (35).

Calculations. We calculated the M1 2H enrichment of GSH derivatives from MRM data on the basis of the fragmentation patterns of the M and M1 parent ions of each derivative. The calculations for the carboxymethyl-GSH derivatives are based on the transitions of the M parent ion (366.1→236.1) and M1 parent ion (367.1→237.1 and 238.1). The M fraction is calculated from the average peak intensity of the 366.1→237.1 MRM pair. The M1 fraction is calculated as the sum of the average peak intensities of the two MRM pairs 367.1→237.1 and 367.1→238.1. The mole percent enrichment of the GSH derivative is calculated as M1/(M + M1). The contribution of M2 and higher mass isotopomers to the calculation is inconsequential.

In experiments where M3 GSH was used, we measured the abundances of the M to M3 species. For the M2 isotopomer, we calculated the sum of the average peak intensities of three MRM pairs (368.1→237.1, 238.1, and 239.1). For the M3 isotopomer, we calculated the sum of the average peak intensities of four MRM pairs (369.1→237.1, 238.1, 239.1, and 240.1). The M3 mole percent enrichment of the GSH derivative was calculated as M3/(M + M1 + M2 + M3).

The above calculations use the average intensities of the peaks at retention time ± 0.1 min. Data were processed using a Visual Basic script (1), which simplifies peak integration by opening the files sequentially, and then transferring the average intensity of the selected peaks to an Excel spreadsheet.

We calculated the M1 enrichment of the constitutive amino acids of GSH from the product ion spectra of the derivatives. Calculations used data from the most abundant product ions: m/z 134 (CYS), 237 (GLY-CYS), and 291 (GLU-CYS), monitored with a mass width of 5 amu. The enrichment of GLY was calculated from the difference in enrichment of GLY-CYS and CYS. The enrichment of GLU was calculated from the difference in enrichment of GLU-CYS and CYS.

We fit the time course labeling data to a model for a single compartment using nonlinear regression implemented with the Origin statistical package (OriginLab, Northampton, MA). We used the Box-Lucas 1 model, which fits the data to the equation E(t) = Einf(1 - e^-kt), where t is time and E(t) is the enrichment of the isotopomer at time t and Einf is the plateau enrichment. This equation describes the labeling of the pool with the tracer. The parameters to be estimated are the plateau enrichment and k, which is equal to flux/ (pool size). We used the calculated 95% confidence intervals for the parameters as the criteria for determining difference when comparing plots.

**RESULTS**

Method development. The first goal of our study was to develop a sensitive technique to measure low concentrations and low 2H labeling of both GSH and GSSR in plasma. This imposed the following constraints. First, since the GSH concentration in plasma is much lower than that in red cells, we had to treat blood samples carefully to avoid any hemolysis that would be revealed by the color of plasma (see METHODS). Second, we needed to protect the easily oxidizable thiol group of GSH. Third, we wanted to measure the concentration and labeling of all DTT-reducible “bound” forms of GSH, i.e., GSSR, which includes GSSG, and express these concentrations in GSH equivalents. This required reducing GSSG and other oxidized forms of glutathione, including disulfide addition to proteins, and possibly the sulfenic acid, as well as being able to differentiate the liberated GSH from extant GSH. Thus, we had to use different protecting agents for extant GSH and for GSH released from GSSR. Fourth, we wanted to use low-molecular-weight protective agents to minimize the natural isotopic enrichment of the two protected forms of GSH. Fifth, we chose to conduct the assays by LC-MS to avoid adding derivatizing groups that would increase the natural enrichment of GSH assayed by GC-MS (6, 13).

Although N-ethylmaleimide is frequently used to protect GSH (5, 14), it adds 7% natural M1 isotopic enrichment to the GSH molecule: (6 × 1.1% for the 6 carbon atoms) + (1 × 0.4% for the nitrogen atom). Similarly, 1-methyl-4-vinylpyridine (31) would add 8.1% M1 enrichment. Therefore, iodoacetate (21), which adds only two carbons to the GSH molecule, in the formation of the carboxymethyl derivative, was preferable. This minimizes the natural M1 enrichment of the derivative group (2.2%). To protect GSH released from GSSR, we used iodoacetonitrile, resulting in a natural enrichment of the protective group of 2.6% in the cysteaminyl derivative. Protection with iodoacetamide was rejected because of the low sensitivity of the assay of the derivative. Also, we found that the carboxamidomethyl derivative of GSH can partially hydrolyze to the carboxymethyl derivative.

To release GSH from GSSR and to neutralize excess iodoacetate, we used 50 mM DTT (10-fold excess over iodoacetate). On that basis, we worked out the sample processing sequence shown in Fig. 1. Using homoglutathione as internal standard, concentration calibration curves for GSH, GSH formed from the reduction of GSSG, and ophthalmate were linear over a wide range of substrate concentrations (Supplementary Fig. S1; Supplemental data for this article can be found at the AJP-Endocrinology and Metabolism web site).

To measure the labeling of GSH and its constituent amino acids from 2H enriched body water, we used the product ion spectra shown in Supplementary Fig. S2. Supplementary Fig. S2A shows the precursor GS-carboxymethyl with [M + H]+ molecular ion peak at m/z 366.1 and the product ions at 134.3 (CYS carboxymethyl), 237.1 (GLY-CYS carboxymethyl), and 291.0 (GLU-CYS carboxymethyl). Supplementary Fig. S2C shows the precursor GS-cyanomethyl with [M + H]+ molecular precursor ion at m/z 347.2 and product ions at 115.2 (CYS cyanomethyl), 218.3 (GLY-CYS cyanomethyl), and 272.3 (GLU-CYS cyanomethyl). Supplementary Fig. S2, B and D, show the corresponding spectra for M3 GSH.
Figure 2 compares the mass spectra of the molecular ion clusters of GSH and GSSR in the plasma of two rats, a control rat (continuous line) and a rat that had its body water 2.5% 2H enriched for 31 days (Fig. 2, dotted line, offset to the right for clarity). To avoid confusion with the nomenclature of mass isotomers, note that the unlabeled [M + H]+ species is designated as the unlabeled mass isotopomer M. The M1 and/or 15N. The increases in the M1 signals in the GSH and GSSR of the rat whose body fluid was 2.6% 2H enriched (Fig. 2, dotted line) reflects the 2H enrichment resulting from 2H incorporation over 31 days. Since, on average, 6 H atoms from water are incorporated in GSH (5), the increase in the M1 signal is substantial.

Animal experiments. Table 1 shows the concentrations of GSH and GSSR in the plasma and liver of the rats used in the two tracer experiments described below. The GSH and GSSR concentrations in erythrocytes were 0.221 ± 0.12 and 0.013 ± 0.009 μmol/ml (SE; n = 20), respectively. On the basis of a blood content of 18% of rat liver in vivo, and a hematocrit of 40%, one calculates that the fraction of liver GSH/GSSR, which is located in erythrocytes, is only ~3.5%. This small fraction was not taken into account in kinetic calculations described below.

Supplementary Fig. S3 shows the time course of labeling of body water as a result of bolus injection of 2H2O (to achieve an initial enrichment of 2%) and keeping the rats on 3.25% 2H-enriched drinking water. One rat was euthanized at each time point. The stabilization of the 2H-enriched body water at 200 h indicates much slower turnover of the GSH and GSSR pools in erythrocytes than in liver. The 95% confidence intervals for the k (48 h) in erythrocytes are lower and do not overlap with the confidence intervals in plasma and liver. In contrast, GSH and GSSR in erythrocytes are well equilibrated as in liver and plasma. The slow labeling of the erythrocyte pool is due to the low rate of synthesis or of import of plasma GSH into erythrocytes relative to the GSH + GSSR pool size in erythrocytes. The kinetic analysis cannot distinguish between erythrocyte synthesis and plasma transport.

The time course of labeling over the longer time course of 31 days (Fig. 3B) indicates that GSH and GSSR in plasma and liver continue to accumulate label after the plateau for biosynthesis has been reached. We will show below that this is caused by the slow labeling of the glutamate and cysteine moieties of GSH. The plasma and liver GSH and GSSR data for the 31-day labeling do not fit the single-pool model. This statement is based on the fact that the data points are not randomly distributed relative to the line for the single exponential (Fig. 3B). For plasma and liver, all data points fall below the line at times 40 to 200 h and then above the line at later times. The 31-day labeling profile does not fit a single-pool model apparently.

Table 1. Data from 2 rat experiments tracing the kinetics of GSH with M3 GSH or 2H2O

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Rat Weight, g</th>
<th>Liver Weight, g</th>
<th>Liver [GSH], μmol/g</th>
<th>Liver [GSSR], μmol/g</th>
<th>Plasma [GSH], μmol/ml</th>
<th>Plasma [GSSR], μmol/ml</th>
<th>Total pool GSH + GSSR in ECF + liver, μmol/kg body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3 GSH (n = 9)</td>
<td>271 ± 7</td>
<td>10.9 ± 0.18</td>
<td>4.21 ± 0.14</td>
<td>0.14 ± 0.009</td>
<td>0.012 ± 0.006</td>
<td>0.0025 ± 0.0002</td>
<td>177 ± 6.4</td>
</tr>
<tr>
<td>2H2O (n = 20)</td>
<td>211 ± 7</td>
<td>9.75 ± 0.23</td>
<td>4.4 ± 0.15</td>
<td>0.14 ± 0.004</td>
<td>0.0095 ± 0.0005</td>
<td>0.0005 ± 0.0003</td>
<td>215 ± 9.7</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. M3 GSH, [13C5,15N-glycine]glutathione; GSSR, DTT-reducible forms of GSH. The concentrations of oxidized “bound” forms of GSH (GSSR, which includes GSSG and protein mixed disulfides) are expressed in μmol GSH equivalents per gram or per milliliter. The liver concentrations of GSH and GSSR have been corrected for the amounts of these compounds located in the erythrocytes of the liver vascular space (3.5% of total; see RESULTS). The sizes of the pools of GSH and GSSR in the extracellular fluid (ECF) were calculated on the basis of 200 ml ECF/kg and assuming that the concentrations of GSH and GSSR are the same in plasma and interstitial fluid (the 2 components of ECF).
respectively, after body fluids were loaded with 2H (Fig. 6). The concentrations of ophthalmate were 17.9 ± 3.3 nmol/g liver and 2.2 ± 0.2 nmol/ml plasma. These concentrations are in the same range as those reported by Soga et al. (see Fig. 9 in Ref. 32).

We measured the rate of appearance (Ra) of plasma GSH in nine anesthetized rats (271 ± 4 g, SE) infused with M3 GSH. Figure 7 shows the rapid stabilization of the M3 labeling of plasma GSH. The Ra of plasma GSH, calculated from the plateau of M3 enrichment, was 2.06 ± 0.08 μmol·min⁻¹·kg⁻¹ (SE; n = 9). The M3 labeling of plasma GSSR was ~90% of the labeling of GSH during the infusion of M3 GSH. The decay of M3 GSH enrichment after interruption of tracer infusion at 24 min yielded half-lives of GSH (6.4 ± 0.1 min) and GSSR (8.2 ± 0.2 min). The corresponding fractional turnover rates were 0.11 ± 0.002 and 0.09 ± 0.002 min⁻¹ for GSH and GSSR, respectively. Supplementary Fig. S4 shows the plasma concentrations of GSH and GSSR (unlabeled, M3 labeled, and total) in the same experiments. Although the enrichment of GSH was higher than in a pure tracer experiment, the increase in total GSH concentration did not influence the concentration of unlabeled GSH. Thus, the calculated Ra of plasma GSH is valid.

To get some insight on the fates of the GSH and GSSR that left plasma after the end of the infusion of M3 GSH (Fig. 7), we conducted two in vitro experiments where we added a bolus of M3 GSH to 5 ml of normal human plasma or to 5 ml of 4% dialyzed bovine serum albumin dissolved in saline. Supplementary Figs. S5 (albumin) and S6 (plasma) show the concentrations of the unlabeled and labeled forms of GSH and GSSR as well as the total concentration of the GSH + GSSR forms. Supplementary Fig. S7 shows the M3 enrichments of GSH and GSSR in the human plasma and albumin experiments. In the albumin solution, the total concentration of the GSH and GSSR species remained constant (Supplementary Fig. S5, top curve), whereas the concentrations of the GSH and GSSR species decreased and increased, respectively. In the same experiment, the M3 enrichments of GSH and GSSR progressively equili-

Table 2. Kinetic parameters of the labeling of GSH/GSSR and OA from 2H-enriched body water in rats

<table>
<thead>
<tr>
<th></th>
<th>Eυat ± SE, %</th>
<th>k ± SE, h</th>
<th>Adjusted R²</th>
<th>τo of labeling, h</th>
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<tbody>
<tr>
<td></td>
<td>31 Days</td>
<td></td>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>GSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>14.1 ± 0.84</td>
<td>0.051 ± 0.011</td>
<td>0.85</td>
<td>14</td>
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<tr>
<td>Plasma</td>
<td>12.6 ± 0.68</td>
<td>0.065 ± 0.015</td>
<td>0.85</td>
<td>11</td>
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<tr>
<td>RBC</td>
<td>12.7 ± 0.44</td>
<td>0.0094 ± 0.00082</td>
<td>0.98</td>
<td>74</td>
</tr>
<tr>
<td>GSSR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>12.2 ± 0.61</td>
<td>0.072 ± 0.014</td>
<td>0.84</td>
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<tr>
<td>Plasma</td>
<td>12.3 ± 0.63</td>
<td>0.068 ± 0.016</td>
<td>0.85</td>
<td>10</td>
</tr>
<tr>
<td>RBC</td>
<td>12.6 ± 0.65</td>
<td>0.0087 ± 0.0011</td>
<td>0.95</td>
<td>80</td>
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<tr>
<td>OA</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>13.3 ± 0.39</td>
<td>0.53 ± 0.13</td>
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<tr>
<td>Plasma</td>
<td>12.4 ± 0.32</td>
<td>0.36 ± 0.066</td>
<td>0.85</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. RBC, red blood cells; OA, ophthalmate. The enrichment data of Fig. 3 were fitted to a single exponential saturation curve E = Eυat (1 – e⁻ᵏᵗ) under two conditions: 1) data from 0 to 31 days and 2) data from 0 to 2 days.

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In plasma (Supplementary Fig. S6), the total concentration of the GSH + GSSR species decreased from 20 to 12 μM over 60 min. The decrease in the concentration of the GSH species was much greater than the increase in the concentrations of the GSSR species. In the same experiment, the M3 enrichments of GSH and GSSR equilibrated at a lower level than in the experiment in albumin. This results from the presence of substantial amounts of unlabeled GSSR in plasma at zero time (Supplementary Fig. S6).

Human investigation. In normal human subjects whose body water was 0.5% enriched by ingestion of 2H2O over 1 h, we measured the time profile of 2H labeling of plasma GSH, GSSR, and ophthalmate over 72 h (Fig. 8). We were able to follow accurately the low labeling of plasma GSH, GSSR, and ophthalmate because, in the MRM data acquisition mode, the signal-to-noise ratio is much higher than in the enhanced mass spectrum mode. Therefore, accurate measurement of plasma peptide enrichment can be obtained with low 2H enrichment of body water. The labeling of GSH was faster than that of GSSR. Because of the low 2H enrichment of body water, we could not measure the enrichment of the amino acid moieties of the glutathione molecule. The rate of labeling of ophthalmate was similar to that of GSH. None of the three compounds reached steady-state labeling after 72 h. The data were fitted to a monoeXponential saturation curve, yielding parameters shown in Table 4. The eight plasma concentrations of GSH, GSSR, and ophthalmate (nmol/ml) of each subject were averaged, and the means ± SE of these averages were 2.4 ± 0.1, 1.9 ± 0.04, and 2.8 ± 0.1, respectively (n = 6).

**DISCUSSION**

Although the use of hydrogen isotopes for measuring the synthesis of biomolecules has been known since the 1930s (30), its application to the synthesis of macromolecules [proteins (4, 28, 33, 34), peptides (5, 33), and nucleic acids (25)] has expanded only recently. One main advantage of using 2H-enriched body water for measuring the synthesis of proteins and peptides is the rapid equilibration of 2H enrichment in all body cells (29). Also, some amino acids such as alanine (9) become rapidly labeled from 2H-enriched body water by a combination of transamination, keto-enol tautomerism, and interactions with various pathways. For example, glutamate and glutamine become labeled by equilibration with α-keto-glutarate, which itself becomes labeled via reactions of the citric acid cycle. Once incorporated into proteins and peptides, the 2H enrichment of constitutive amino acids is stable even during acid hydrolysis of the proteins or during partial digestion with enzymes such as trypsin (28).

Cabral et al. (5) recently reported the LC-MS measurement of the 2H enrichment of GSH labeled from 2H-enriched body water. From the time course of GSH labeling, they determined that, on average, six H atoms from water are incorporated during the synthesis of GSH. Our study expands on study by Cabral et al (5) by using LC-MS-MS technology and by measuring the labeling of bound GSH, which includes GSSG. The simultaneous measurement of the mass isotopomer distribution of extant GSH and bound GSH in the same LC-MS-MS run allows analysis of the 2H labeling of the two forms of GSH under identical analytical conditions. The concentrations of GSH, GSSR, and ophthalmate can be measured under the same protocol using homo-GSH as internal standard (Supplementary Figs. S1, A, B, and C).

Our data reveal the very rapid equilibration of the labeling of the free and bound GSH pools in liver in vivo (Fig. 3). This is
despite the fact that glutathione disulfides are a heterogeneous group of substances. Although our protocol could not differentiate the labeling of the cytosolic and mitochondrial GSH pool, these could be differentiated in hepatocytes incubated in 2H-enriched medium with amino acids (9) before rapid fractionation of mitochondria with digitonin (37). However, such a protocol requires the assessment that the amino acids present in the incubation or culture medium have equilibrated their 2H labeling with that of medium water (9).

The rapid labeling of liver GSH and GSSR in vivo (Fig. 3) reflects the rapid turnover of its large GSH/GSSR pools. It also shows that the pools of bound GSH rapidly equilibrate with the pool of free GSH. In contrast, the turnover of erythrocyte GSH/GSSR is much slower than that of liver, as shown previously by bolus injection of [14C]glycine (8, 24). The labeling profile of plasma GSH is close to that of liver GSH. Since most of the plasma GSH is formed in the liver (19), we view the labeling profile of plasma GSH, from 2H-enriched body water, as a proxy of the labeling profile of liver GSH. This opens the way to clinical investigations on the rate of liver GSH synthesis in health and disease.

Our long-term experiment in rats under steady-state 2H-enriched body water (31 days) revealed very different rates of labeling of the amino acids making up the GSH molecule (Figs. 4 and 5). Glycine equilibrated rapidly with body water. The equilibration of glutamate with body water was biphasic, with a very rapid component followed by a slower component. Long-term labeling of glutamate was limited to ~2.5 protons from water on average per molecule (since the plateau of glutamate labeling (Fig. 4) was 2.5 times that of body water (2.5%; Supplementary Fig. S3)). These hydrogen atoms are presumably distributed on the five carbon-bound hydrogens of glutamate. Labeling of H on C2 results from reversible cycling between glutamate and α-ketoglutarate. Labeling of the two H on C3 results presumably from some keto-enol tautomerism of α-ketoglutarate. Labeling of the two H on C4 comes from the methyl of acetyl-CoA, which is labeled via reactions of fatty acid oxidation, glycolysis, and alanine transamination. The slow labeling of the glutamate moiety of GSH (10 days to plateau; Fig. 4) probably reflects the time needed to label the large pool of muscle glutamine that is not in isotopic equilibrium with plasma glutamine and glutamate during short-term experiments (7). The latter is constantly released into the plasma and is converted to glutamate in the periporal cells of the liver. The very slow labeling of cysteine (13 days to plateau; Fig. 4) results presumably from the high $K_m$ for cysteine (22 mM) of cysteine-α-ketoglutarate aminotransferase (2). It is likely that each H in the GSH molecule that is derived from water on average per molecule [since the plateau of glutamate labeling (Fig. 4) results presumably from the high $K_m$ for cysteine (22 mM) of cysteine-α-ketoglutarate aminotransferase (2)].

Table 3. Kinetic parameters of the $^2$H labeling of the amino acid fragments of rat plasma GSH

<table>
<thead>
<tr>
<th></th>
<th>Einf ± SE, %</th>
<th>$k$ ± SE, h</th>
<th>Adjusted $R^2$</th>
<th>$%$ of labeling, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>1.7±0.056</td>
<td>0.25±0.063</td>
<td>0.91</td>
<td>2.8</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4.9±0.29</td>
<td>0.027±0.0048</td>
<td>0.84</td>
<td>26</td>
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<tr>
<td>Glutamate</td>
<td>6.2±0.34</td>
<td>0.052±0.011</td>
<td>0.85</td>
<td>13</td>
</tr>
<tr>
<td>GSHcalc</td>
<td>11.9±0.51</td>
<td>0.077±0.014</td>
<td>0.87</td>
<td>9.0</td>
</tr>
<tr>
<td>GSSRcalc</td>
<td>11.7±0.79</td>
<td>0.060±0.015</td>
<td>0.72</td>
<td>12</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. GSHcalc and GSSRcalc, calculated GSH and GSSR. The enrichment data of Fig. 4 (glycine, cysteine, glutamate, and total labeling of the GSH fragments) were fitted to a single exponential saturation curve $E = E_{inf} (1 - e^{-kt})$. The fittings were calculated under two conditions: 1) data from 0 to 31 days and 2) data from 0 to 2 days (48 h).

Fig. 6. $^2$H labeling of plasma and liver ophthalmate in the rat experiment described in Fig. 4. Inset shows the early labeling period.

Fig. 7. M3 labeling of plasma GSH and GSSR in rats infused with $[13C_2,15N]$glycine-glutathione. The infusion of labeled GSH was stopped at 24 min to allow measurement of the decay in GSH and GSSR labeling ($n = 9$).
from body water has its own kinetics of incorporation. Because of the slow labeling of glutamate and cysteine, their plateaus of labeling do not reflect the levels that would be achieved in a closed system at the actual $^2\text{H}$ enrichment of body water. These plateaus reflect the balance between slow enzymatic labeling and the constant dietary supply of unlabeled cysteine, glutamate, and of amino acids that can be converted to glutamate.

The complexity of the time profile of GSH labeling from $^2\text{H}$-enriched body water (Fig. 5, A and B) results in a large relative error in the calculation of the fractional turnover of plasma GSH (Table 2). Therefore, the kinetics of $^2\text{H}$ labeling of plasma GSH cannot be used to calculate a precise turnover rate of plasma GSH. On the other hand, the slower turnover rate of erythrocyte GSH compared with plasma GSH (apparent half-labeling time of $\approx 70$ vs. 10 h; Table 2) results in a much lower relative error in the calculation of its fractional turnover and in a higher adjusted $R^2$ (Table 2).

The rapid $^2\text{H}$ labeling to final plateau of the glycine moiety of plasma GSH ($k = 0.27$ h; Table 3 and Figs. 4 and 5B) suggests that its kinetics could be used to calculate the $R_a$ of the GSH $+$ GSSR system in the extracellular fluid $+$ liver compartment. This is justified since the labeling of GSH and GSSR is very similar in plasma and liver. Using the data of Table 1, the $k$ of the glycine moiety of plasma GSH ($0.27$ h; Table 3), and the formula $R_a = k$ (pool), the apparent $R_a$ of the GSH/GSSR system in liver $+$ plasma amounts to 1.00 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ (95% confidence limits: 0.69 to 1.32 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$). This rate falls in the same range as the $R_a$ of plasma GSH calculated with an infusion of M3 GSH, i.e., 2.06 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$. Note that the $k$ of the glycine moiety of GSH (0.27 h) is somewhat underestimated because the labeling of the glycine moiety of GSH is preceded by the labeling of free glycine at the site of GSH synthesis in the liver. This probably explains why the $R_a$ of the glycine moiety of GSH labeled from $^2\text{H}$ water is about one-half of the $R_a$ of GSH measured with an infusion of M3 GSH. The latter rate is also a minimal estimate of the $R_a$ of GSH $+$ GSSR in the liver since most, if not all, plasma GSH is released by the liver (19). The estimate is minimal because it does not include the amount of GSH excreted in bile. Then, a comparison of the $R_a$ of plasma GSH ($2.06 \pm 0.08 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) with the pool of extracellular fluid GSH at $t = 0$, i.e., $[[0.012 \mu\text{mol/mL}] \times (200 \text{ ml/kg}) = (2.4 \mu\text{mol/kg})]$, reveals that the whole pool of extracellular GSH was replaced in about 1.15 min. This calculation assumes that the concentrations of GSH in plasma and interstitial fluid are similar.

Since most if not all plasma GSH/GSSR is formed in the liver (19), one can calculate an apparent turnover of the GSH/GSSR present in the liver $+$ extracellular fluid, i.e., 177 $\mu\text{mol/kg body wt}$ (Table 1, far right column). In comparing this pool with the $R_a$ of plasma GSH ($2.06 \pm 0.08 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$), one calculates that the $R_a$ of plasma GSH accounts for at least 1.1%/min of the liver GSH pool. Since, in addition to releasing GSH in plasma, the liver puts out GSH conjugates in bile, the turnover of hepatocyte GSH is very rapid.

In the same rats whose body water was 2.5% enriched (Supplementary Fig. S3), the enrichment of plasma ophthalmate plateaued at about 13% (Fig. 6). Thus, the synthesis of ophthalmate incorporated five $\text{H}$ from body water. Assuming that the labeling of the glycine and glutamate moieties of GSH and ophthalmate are the same, one calculates that the 2-amino-butyrate moiety of ophthalmate had incorporated two $\text{H}$ from body water.

Anderson and Meister (3) have studied the fate of unlabeled or $[^{14}\text{C}]$-glutamate/GSH added to fresh plasma and concluded that a component of the disappearance of plasma GSH results from intravascular metabolism mostly to GSSG. To get more insight on this process, we added M3 GSH either to fresh human plasma or to a control 4% bovine serum albumin solution kept at 37°C. In the bovine serum albumin solution, the total concentration of GSH $+$ GSSR (labeled $+$ unlabeled) remained constant (Supplementary Fig. S5, top curve). However, in plasma, the total concentration of GSH $+$ GSSR decreased, but much more slowly than in the in vivo experiment (Supplementary Fig. S6). This confirms the contribution of intravascular metabolism of GSH to its turnover. In vivo, the bulk of the disappearance of plasma GSH involves the uptake of GSSG by the kidney (23). Also, the isotopic equilibration of the GSH and GSSR is more rapid in plasma than in a bovine serum albumin solution. The more rapid events in plasma compared with a solution of bovine serum albumin are likely contributed by several factors. For example, the decline in GSH in favor of GSSR may be driven by reaction of GSH with cysteine to form GSS-cysteine. Cystine is present at much higher concentrations in plasma than GSSG and cysteine (16), contributing to the oxidizing environment of plasma. The plasma also contains the glutathione metabolic

![Graph](image-url)

**Fig. 8.** Labeling of GSH, GSSR, and ophthalmate from $^2\text{H}$-enriched body water in normal humans. The subjects ingested $^2\text{H}_2\text{O}$ over the 0- to 1-h period. The amount of $^2\text{H}_2\text{O}$ ingested resulted in the 0.5% $^2\text{H}$ labeling of plasma water. The dotted curves show the fitting of the data to a monoexponential saturation function.

### Table 4. Kinetic parameters of $^2\text{H}$ labeling of plasma GSH, GSSR, and OA in healthy humans

<table>
<thead>
<tr>
<th>$E_{\text{inf}} \pm \text{SE}, %$</th>
<th>$k \pm \text{SE}, \text{h}$</th>
<th>Adjusted $R^2$</th>
<th>$r_0$ of labeling, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH 2.09 $\pm$ 0.19</td>
<td>0.035 $\pm$ 0.0078</td>
<td>0.85</td>
<td>20</td>
</tr>
<tr>
<td>GSSR 2.01 $\pm$ 0.25</td>
<td>0.024 $\pm$ 0.0057</td>
<td>0.89</td>
<td>29</td>
</tr>
<tr>
<td>OA 2.02 $\pm$ 0.08</td>
<td>0.062 $\pm$ 0.0074</td>
<td>0.91</td>
<td>11</td>
</tr>
</tbody>
</table>

Data are presented as means $\pm \text{SE}$. The 0- to 72-h values of $^2\text{H}$ enrichment of GSH, GSSR, and OA of the 6 subjects were fitted to 3 monoexponential saturation curves.
The incorporation of labeled glycine into glutathione in vivo was measured with C14-glycine. 


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