Blood Glutathione Disulfide: In Vivo Factor or in Vitro Artifact?

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Background: The reported mean concentration of glutathione disulfide (GSSG) in human blood/erythrocytes varies widely (1 to >500 μmol/L), as does that of reduced glutathione (GSH) to a lesser extent. We have identified and investigated possible pitfalls in measurement of both GSH and GSSG.

Methods: We measured GSH and GSSG using a spectrophotometer with a modification of the GSH recycling method; the same samples were also measured by reversed-phase HPLC after derivatization of thiols (dithiothreitol was used to reduce disulfides) with monobromobimane. The thiol-bimane adduct was measured by a fluorescence detector.

Results: Measured GSH/GSSG concentrations were affected by the following: (a) oxidation of thiols in acidified samples; (b) oxidation after restoring neutral-alkaline pH; (c) oxidation during acid deproteinization; (d) shift in the GSH/GSSG equilibrium because of irreversible blocking of free thiols; and (e) reaction of electrophiles with amino groups. In particular, oxidation during sample deproteinization with acid influenced and produced artifacts (30–150 μmol/L GSSG was produced by this procedure); this phenomenon was directly correlated with the presence of oxygenated hemoglobin, being minimized by both oxygen deprivation and incubation in an atmosphere of 5% carbon monoxide.

Conclusions: GSSG is present in healthy human blood at low concentrations (2–6 μmol/L), and most published data on GSSG may be affected by artifacts. © 2002 American Association for Clinical Chemistry

Given the role of glutathione in the protection against oxidative stress and detoxification of xenobiotics, its availability in the reduced form (GSH) may be a key factor in the maintenance of health. It has been established in several different animal models, as well as in humans, that a decrease in GSH concentrations may be associated with aging and the pathogenesis of many diseases, including rheumatoid arthritis, muscular dystrophy, amyotrophic lateral sclerosis, AIDS, Alzheimer disease, alcoholic liver disease, cataractogenesis, respiratory distress syndrome, progeria, and Werner syndrome (1–9).

Because blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both GSH and glutathione disulfide (GSSG) in blood has been considered essential as an index of whole-body glutathione status and a useful indicator of disease risk in humans.

The oxidation-reduction status of blood glutathione is central to many investigations involving oxidative stress and free radical pathologies (2, 3, 10–39). Low GSH, high GSSG, and a lower GSH/GSSG ratio have been found in blood from patients with various pathologies (2, 3, 10–39). Table 1 summarizes some of the results obtained by different investigators for the concentrations of GSH and GSSG in blood samples: the extremely high variability of GSH and, moreover, of GSSG is evident. GSSG and GSH concentrations range from 1 to 500 μmol/L and from 150 to 1500 μmol/L, respectively, in controls, as well as in patients with different pathophysiologic conditions (2, 3, 10–39). The main problem is not represented by the wide distribution of GSH and GSSG values in different individuals of the same group, but by the fact that mean values for GSH and particularly GSSG obtained by different investigators span up to two orders of magnitude. This justifies new efforts to verify the methods used for

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3 Nonstandard abbreviations: GSH, reduced glutathione; GSSG, glutathione disulfide; CDNB, 1-chloro-2,4-dinitrobenzene; GR, glutathione reductase; NEM, N-ethylmaleimide; FDNB, 2,4-dinitrofluorobenzene; MBB, monobromobimane; RBC, red blood cell; IAA, iodoacetic acid; TCA, trichloroacetic acid; SH, sulfhydryl; and Hb, hemoglobin.
Data were normalized to a 45% hematocrit blood containing 5 × 10^6/mm^3 RBCs and 150 g/L hemoglobin. In some reports, GSH and GSSG concentrations were determined in the RBCs rather than in whole blood. In this case, the contribution of glutathione from white cells was considered negligible. Where only GSH/GSSG ratio was reported, values were normalized to a 1 mmol/L total GSH-containing blood. Some of these values were approximately deduced from graph data.

a Responders to chemotherapy.

b Nonresponders to chemotherapy.

c AIDS.

d (14) Treatment with aminoglutethimide (AGT) and p-phenyl-N-(2chloroethyl)-1-nitrosourea was from Bristol-Myers Squibb; all chemicals of analytical grade were from Sigma.

Materials and Methods

Chemicals

HPLC-grade reagents were obtained from BDH; 1,3-bis(2-chloroethyl)-1-nitrosourea was from Bristol-Myers Squibb; and all chemicals of analytical grade were from Sigma.

Animals

Male Wistar rats were purchased from Charles River. Human blood was collected by venipuncture from healthy volunteers who gave informed consent. Arterial and venous rat blood was drawn from the abdominal
aorta and portal vein, respectively, after light anesthesia with diethyl ether. The procedures followed were in accordance with institutional guidelines. All blood samples were collected in disodium EDTA as the anticoagulant.

Red blood cells (RBCs) were obtained from whole blood washed with phosphate-buffered saline plus glucose (final concentration, 10 mmol/L).

GSH AND GSSG DETERMINATIONS
The GSH recycling method, CDNB endpoint method, and two HPLC methods [the methods of Reed et al. (44) and Newton et al. (45)] with spectrophotometric or fluorometric detection were used for GSH and/or GSSG determination.

The GSH recycling method was performed as described previously (40). Briefly, for total GSH measurement, sample aliquots were added to a buffered solution (0.1 mol/L phosphate buffer, pH 7.4) containing NADPH (final concentration, 0.1 mmol/L) and GR (final concentration, 0.5 kU/L). The reaction was started by the addition of 5,5'-dithio-diphenylcarbazide (final concentration, 0.2 mmol/L). GSSG was titrated by the same method, but samples were pretreated with NEM (final concentration, 10 mmol/L), and the excess of NEM was extracted with dichloromethane (0.5 mL of sample plus 5 mL of dichloromethane). The reaction was monitored at 412 nm, and the GSH or GSSG concentration was calculated on the basis of a calibration curve obtained with authentic GSH or GSSG. For the CDNB endpoint method, only acidified clear supernatants were used: sample aliquots (0.1 mL) were neutralized and buffered at pH 7.4 with 1 mL of 0.2 mol/L phosphate buffer, pH 7.8; the reaction was then started by the addition of CDNB and glutathione S-transferase (final concentrations, 0.2 mmol/L and 0.5 kU/L, respectively) and monitored at 340 nm (ε = 9600) until no variation in absorbance was evident (3–4 min).

For the first HPLC method (44), samples were incubated at pH 8.0 with iodoacetic acid [([IAA]; final concentration, 50 mmol/L) for 45 min, and then 0.2 mL of each sample was diluted with 0.2 mL of FDNB (15 mL/L in ethanol) for 3 h. Samples were then applied to a Bio-Rad Biosil NH2 column (250 × 4.6 mm) and eluted with a gradient of 1.5 mol/L acetate in water and methanol (44): 0–10 min, 70% solvent A (800 mL/L methanol, 200 mL/L water) and 30% solvent B (1.5 mol/L acetate buffer, pH 4.6, in solvent A); 10–35 min, linear gradient, 30% to 95% solvent B. For the second HPLC method (45), samples were incubated in 0.2 mol/L phosphate buffer (pH 7.4); acidified samples were adjusted to pH 7.3–7.5, both with phosphate buffer and by the addition of small amounts of 1 mol/L NaOH) with MBB (final concentration, 1 mmol/L), then loaded onto a C18 reversed-phase column and eluted with a methanol gradient as described previously (45) with slight modifications: 0–5 min, isocratic elution with 75% solvent A (2.5 mL/L acetic acid adjusted at pH 3.09 with 1 mol/L NaOH) and 25% solvent B (methanol); 5–25 min, linear gradient of 25% to 100% solvent B. After its conversion to GSH by treatment with DTT (final concentration, 1 mmol/L) for 15 min, GSSG was titrated by HPLC after derivatization with MBB, as described above.

HPLC determinations were carried out with a Hewlett Packard Series 1100 HPLC instrument equipped with both a diode array and a fluorescence detector.

ENZYME ASSAYS
Enzymatic determinations of GR were carried out at 37 °C on purified enzyme from water-hemolyzed RBCs (1 mL of RBCs + 9 mL of water). Enzyme activity was monitored at 340 nm by NADPH consumption as reported previously (46).

All spectrophotometric determinations were carried out with a JASCO V550 UV-Vis apparatus.

Results
The first step in glutathione analysis is usually protein precipitation by acidification [trichloroacetic acid (TCA), perchloric acid, metaphosphoric acid, or sulfosalicylic acid], yielding a clear, protein-free supernatant after centrifugation. Glutathione and other thiols and disulfides are then measured by spectrophotometry or by HPLC. HPLC determination allows the simultaneous detection of GSH and other low-molecular weight thiols (e.g., cysteine) and disulfides, but requires derivatization of samples. In any case, neutral-alkaline pH must be restored before measurement and/or derivatization.

Oxidation of thiols during sample manipulation represents the major problem in such determinations, frequently leading to an overestimation of disulfides. The blocking of thiol groups with various agents (see Introduction) is often used to prevent this phenomenon. Trapping of sulfhydryl (SH) is, in most cases, achieved after proteins have been discarded and neutral-alkaline pH restored; however, in a few early reports [see Ref. (10)], investigators treated samples with SH quenchers before deproteinization by acids.

The overall pathways followed in the titration of thiols and disulfides in blood or RBC samples are shown in Fig. 1; circled letters identify pitfalls that can take place during sample manipulation: (a) oxidation of thiols in acidified samples; (b) oxidation after restoration of neutral-alkaline pH; (c) oxidation during acid deproteinization; (d) shift of the GSH/GSSG equilibrium attributable to irreversible blocking of free thiols; and (e) reaction of electrophiles with amino groups.

OXIDATION OF THIOLS IN ACIDIFIED SAMPLE
Acidification is required for most procedures for thiol and/or disulfide titration. Only a few methods (e.g., nuclear magnetic resonance) are suitable for obtaining measurements in intact cells or nondeproteinized samples (47). Because samples are usually processed after minutes to hours from acidification, oxidation of thiols in acidified
extracts may occur. To assess this phenomenon, blood samples were treated with various acids. Different final acid concentrations (50 – 200 g/L) were used, and the rate of oxidation was followed at 0 °C or at room temperature. We found that a concentration of at least 80 – 100 g/L was required to prevent time-dependent GSH oxidation (not shown). Moreover, TCA was the best precipitant in that GSH loss with time was minimal (Fig. 2). At 0 °C, GSH oxidation was ~3–4% 20 h after the addition of TCA. Metal chelators (deferoxamine and tripotassium EDTA) were able to minimally decrease the oxidation process (not shown). Some reports [see Ref. (48)] also suggest the use of metaphosphoric acid; however, sample dilution is required before acidification, and, in any case, oxidation was higher than in TCA-treated samples (not shown).

**Oxidation after restoration of neutral-alkaline pH**

Neutralization of samples is necessary for all procedures for glutathione titration, both spectrophotometric and HPLC; generally, neutralization-slight alkalization is achieved by the addition of strongly buffered solutions or solid sodium bicarbonate.

After neutralization, rapid oxidation of thiols occurred at a pH-dependent rate (Fig. 3A); at pH 8.0–8.5 in particular, which is necessary for derivatization with IAA or MBB for HPLC determinations, thiol content decreased by 50% within a few minutes. Under all conditions, the GSH that disappeared was entirely recovered as GSSG (not shown).

This phenomenon is negligible if GSH is quenched within a few seconds (e.g., by NEM) after restoration of neutral-alkaline pH or if titration procedures are very quick and carried out at pH 7.0–7.4 (e.g., the spectrophotometric, CDNB endpoint method).

The chemical species involved in thiol oxidation are unknown. However, metal chelators (deferoxamine and tripotassium EDTA) and anaerobic conditions (not shown) were able to decrease the reaction rate by up to 50–60%.

Analysis of the plot of $t_{1/2}$ vs thiol concentration indicated that the thiol oxidation was regulated by second-order kinetics (Fig. 3B).
Oxidation during acid deproteinization

The acid treatment of tissues containing high concentrations of heme groups, e.g., blood, may lead to the formation of oxidizing species (radical species) and, as a consequence, to the oxidation of thiol groups. To prevent these effects, the blockade of free thiols could be carried out before the deproteinization of biologically active samples.

In Table 2, we report the effect of NEM pretreatment on GSSG concentrations after TCA acidification of blood or RBCs. In NEM-pretreated samples, GSSG was \(3-10 \mu\text{mol/L}\), whereas GSSG was \(70-150 \mu\text{mol/L}\) when NEM pretreatment was omitted. The use of metal chelators (final concentration, 1 mmol/L deferoxamine and tripotassium EDTA) did not significantly change these results (not shown). It appears evident that alkylation by NEM under nondenaturing conditions prevents GSH oxidation, thus justifying the low GSSG concentrations. However, we cannot exclude the possibility that the GSSG concentration was low because of the action of other artifacts (e.g., shift of the GSH/GSSG equilibrium and/or reaction of electrophiles with amino groups) in NEM-pretreated samples.

**Shift of GSH/GSSG equilibrium because of irreversible blocking of free thiols**

As hypothesized above, the addition of NEM could have influenced the GSH/GSSG equilibrium in biologically active samples. Cellular concentrations of GSH and GSSG are regulated by the flavoenzyme GR, which catalyzes the following reaction:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + \text{GSH}
\]

If we blocked GSH with NEM (or other thiol blocking agents), the GR/NADPH couple could, in theory, drive the redox equilibrium (not in acid-denatured samples) from GSSG toward GSH, yielding artificially low values for GSSG.

To verify this hypothesis, we added GSSG to freshly prepared RBC hemolysate (1 mL of RBCs + 9 mL of water), and the recovery of GSSG was evaluated (Fig. 4). The recovery of added GSSG was 85-100% in samples acidified 30 min after the contemporaneous addition of NEM plus GSSG, whereas it was complete in samples treated with NEM and GSSG and acidified after 0.5 min. In parallel experiments, the use of other thiol blocking reagents (i.e., IAA and MBB) was studied. IAA is slower than NEM in alkylating SH groups and requires up to 15-45 min for complete reaction; hemolysates supple-

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**Table 2. Differences in blood or RBC GSSG concentrations with or without NEM pretreatment.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>NEM pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood (human)</td>
<td>Yes 3.33 ± 1.07, No 84.1 ± 35.3</td>
</tr>
<tr>
<td>Whole blood (rat)</td>
<td>Yes 5.4 ± 1.44, No 78.3 ± 30.0</td>
</tr>
<tr>
<td>RBCs (human)</td>
<td>Yes 3.27 ± 1.51, No 75.2 ± 38.1</td>
</tr>
<tr>
<td>RBCs (rat)</td>
<td>Yes 5.01 ± 1.73, No 87.8 ± 30.5</td>
</tr>
</tbody>
</table>

* Blood (or 45% hematocrit RBCs; 1 mL) samples were rapidly mixed with 1 mL of a solution containing 100 mmol/L phosphate buffer, pH 7.4, and 50 mmol/L NEM (in nonpretreated samples, NEM was replaced with 25 mmol/L NaCl in phosphate buffer). After 10 s, samples were acidified with TCA (final concentration, 100 g/L). After proteins were discarded by centrifugation, NEM was extracted with dichloromethane, and GSSG was measured with the Tietze method (40). Values are expressed as \(\mu\text{mol/L}\) in blood or a 45% hematocrit RBC solution. Number of replicates, 6 (single measure on different samples).

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Fig. 4. Shift of GSH/GSSG equilibrium in RBC hemolysate.

(A), GSSG was added to the hemolystate (1 mL of RBCs + 9 mL of water), and the recovery of GSSG was evaluated under different conditions. \(\square\), GSSG and NEM (final concentration, 10 mmol/L) were added to the hemolysates, then acidified (final concentration, 100 g/L TCA) after 30 min; \(\square\), GSSG and NEM (final concentration, 10 mmol/L) were added to the hemolysates, then acidified with TCA after 0.5 min. \(\square\), GSSG and IAA (final concentration, 100 mmol/L), then acidified (final concentration, 100 g/L TCA) after 30 min. The percentage indicates the recovery of added GSSG. Number of replicates, 4. TCA-extracted supernatants were then processed for GSSG measurements; NEM was extracted with dichloromethane. The HPLC method of Reed et al. (44) or the Tietze spectrophotometric recycling method (40) was used; both methods gave very similar results. Data shown are from measurements carried out with the HPLC method (44). (B), same conditions as in A, but RBCs were pretreated with 1,3-bis(2-chloroethyl)-1-nitrosourea (final concentration, 20 \(\mu\text{mol/L}\)) for 2 h before hemolysis.
mented with GSSG and IAA showed a poor GSSG recovery after 45 min. MBB was more efficient than IAA, but less efficient than NEM in preventing GSSG loss in the same experiment (data not shown). Pretreatment of RBCs with the GR inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (49) partially prevented the GSSG loss in NEM-treated hemolysates; conversely, the GSSG loss was greatly reduced in IAA-treated samples (Fig. 4B), thus indicating that, in this case, the enzyme was responsible for the GSSG reduction in these samples.

GR is known to be inhibited by thiol reagents (42), in particular by NEM. To verify this process, human RBC reductase was purified, and the time required for its inhibition was evaluated under various conditions (Table 3). IAA was unable to efficiently inhibit the enzyme under all conditions, whereas NEM inhibition was strongly substrate dependent, being protected by GSSG and increased in the presence of NADPH (Table 3).

As indicated by the data in Table 3 and Fig. 4, it is reasonable to infer that the inhibition of GR by IAA is probably slow and, conversely, that the inhibition of GR by NEM is efficient.

**Table 3. Inhibition of glutathione reductase.**

<table>
<thead>
<tr>
<th>Substrate, μmol/L</th>
<th>NADPH</th>
<th>GSSG</th>
<th>t_{50}, min</th>
<th>t_{90}, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM (2 mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;120</td>
<td>&gt;120</td>
</tr>
<tr>
<td>10</td>
<td>500</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>&lt;0.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>&lt;0.2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td>0.2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>IAA (50 mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;120</td>
<td>&gt;120</td>
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<tr>
<td>50</td>
<td>10</td>
<td>&gt;30</td>
<td>&gt;120</td>
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<tr>
<td>200</td>
<td>100</td>
<td>&gt;60</td>
<td>&gt;120</td>
<td></td>
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<tr>
<td>MBB (2 mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;120</td>
<td>&gt;120</td>
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<tr>
<td>200</td>
<td>10</td>
<td>2</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>10</td>
<td>&gt;30</td>
<td></td>
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</tbody>
</table>

The thiol group is a far stronger nucleophile than the amino groups. Under some circumstances, i.e., high pH, long incubation time, and high concentrations of the SH blocker, electrophiles can react with amines of glutathione (and other thiols). Some procedures (17, 44) use incubation of samples for minutes to hours at an alkaline pH. We therefore evaluated the possibility that NEM and IAA modify GSSG by attacking amino groups, leading to its underestimation. Samples of GSSG at pH 8.0 were incubated for various times with 50 mmol/L NEM or 100 mmol/L IAA, then derivatized or directly loaded onto an HPLC instrument. Elution profiles showed a progressive reduction of the GSSG peak area in NEM-treated samples (Fig. 5); the reduction was proportional to the incubation time. The peak area of GSSG was only slightly influenced by IAA pretreatment (not shown). The reaction of NEM with the amino group of GSSG was also confirmed by the formation of a new peak with a lower retention time.

Treatment of a GSSG calibrator for 45 min with 50 nmol/L NEM produced a 60% reduction of peak area, with formation of new peaks with lower retention times.

**Influence of Hb-ligand state**

We next evaluated the influence of hemoglobin (Hb) status on the glutathione redox state. The concentrations of GSH and GSSG (and total GSH) on arterial or venous rat blood with or without NEM pretreatment are shown in Table 4.

Concentrations of arterial or venous GSSG were very different in non-NEM-pretreated samples. Conversely, similar concentrations in both arterial or venous blood were found in NEM-pretreated samples. Arterial blood, kept under carbon monoxide (5% in air), showed GSSG concentrations similar to those of venous blood. Also in this case, metal chelators (deferoxamine and tripotassium EDTA) did not significantly influence the results (not shown). It was therefore evident that thiol oxidation...
Table 4. Influence of oxy-Hb on GSH oxidation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pretreatment</th>
<th>GSH, μmol/L</th>
<th>GSSG, μmol/L</th>
<th>Total GSH, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial</td>
<td>None</td>
<td>756 ± 85</td>
<td>75 ± 27.9</td>
<td>914 ± 84</td>
</tr>
<tr>
<td>Venous</td>
<td>None</td>
<td>945 ± 95</td>
<td>31.2 ± 21.5</td>
<td>996 ± 101</td>
</tr>
<tr>
<td>Arterial</td>
<td>NEM (50 mmol/L)</td>
<td>ND</td>
<td>5.6 ± 1.33</td>
<td>ND</td>
</tr>
<tr>
<td>Venous</td>
<td>NEM (50 mmol/L)</td>
<td>ND</td>
<td>6.6 ± 1.55</td>
<td>ND</td>
</tr>
<tr>
<td>Arterial (with 5% CO)</td>
<td>None</td>
<td>996 ± 85</td>
<td>15 ± 12.1</td>
<td>1041 ± 140</td>
</tr>
</tbody>
</table>

*Venous (from vena cava) and arterial (from abdominal aorta) blood (1 mL) was drawn simultaneously and acidified with 1 mL of 100 g/L TCA solution. NEM pretreatment before acidification (where applied) was carried out as specified in Table 2. For CO treatment, 2 mL of arterial blood was introduced in a tonometer (150 mL volume), and then 5% of air volume was replaced with carbon monoxide. The sample was shaken for 20 min, and then aliquots were withdrawn and acid denatured with TCA. GSH and GSSG were titrated with the CDNB endpoint and Tietze (40) method, respectively. Number of replicates, 4.

**Discussion**

Oxidative stress is a facet of many diseases, such as chronic heart failure, rheumatoid arthritis, amyotrophic lateral sclerosis, and diabetes; it also has recently been implicated in AIDS (50). Oxidative stress has a profound effect on the thiol balance and can lead to a decreased SH/SS ratio in many body organs. Various studies (2, 3, 10–39) have pointed out the importance of measuring blood glutathione for both pathologic and physiologic purposes. Moreover, the hypothesis that a decrease in blood GSH and an increase in GSSG may reflect similar variations in less accessible tissues, serving as an indicator of overall GSH/GSSG status, has reinforced the importance of GSH/GSSG measurement in human blood (10–39).

Because of this hypothesis, many studies have analyzed whole blood or RBC GSH and GSSG values, comparing concentrations obtained in healthy individuals with those affected by various diseases. In addition, glutathione under particular physiologic conditions (e.g., physical exercise, advanced age, and in newborns) was analyzed (15, 38, 51). Decreased concentrations of GSH and increased concentrations of GSSG were found to a significant extent under these physiologic conditions. In particular, physical exercise (12, 15), advanced age (3), cold exposure (19), diabetes (52, 53), cardiovascular diseases (37), cancer, and chronic diseases (14, 31) are only some examples in which a correlation between the disease (or the physiologic condition) and significantly different concentrations of GSSG and GSH and/or GSH/GSSG ratios was found.

However, the results of these studies, as reported in Table 1, suggest that because large differences in GSH and particularly in GSSG have been found in different investigations (also for control values in healthy people), an accurate revision of the methods used is needed. The fact that GSSG values in human blood can span up to two orders of magnitude, even in controls, based on the...

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Fig. 6. Hb-O₂ influence on GSH titration.

Rat RBC samples were degassed and kept under N₂, and then O₂ (partial pressure in MPa shown on x axis) was added. After every addition, samples were shaken for 20 min, and aliquots were withdrawn and treated with TCA (final concentration, 100 g/L). In the clear supernatant, GSH was titrated with the CDNB endpoint method, and GSSG was titrated with the Tietze method (40). GSH; □, GSSG; △, total GSH [GSH + (2 × GSSG)]. Number of replicates, 4. Some error bars were omitted for clarity.
method used justifies new efforts to identify and eliminate all possible pitfalls. The variations cast doubt on many previous speculations on this subject, and it is essential to revise the methods before attributing any interpretation on the role of an altered GSH or GSSG concentration in blood.

The present study was designed to examine the most frequent pitfalls in sample collection, manipulation, and GSH/GSSG titration in human blood (or RBC) samples.

Oxidation of thiols in acidified samples was found to be rather marginal (Fig. 2) and can be limited, at minimum, by the use of an ice-cold 100 g/L TCA solution; under these conditions, only 2–3% of GSH was transformed into GSSG after 24 h. Conversely, the oxidation of TCA (or other precipitating acid)-treated samples after restoration of neutral–alkaline pH leads to a rapid decrease of thiol concentration: in 5–10 min, >50% of GSH was transformed into GSSG (Fig. 3). We have no information on the oxidant species involved in this phenomenon; metal chelators (EDTA, EGTA, deferoxamine, diethylene-triamine pentaacetic acid) and/or O2 deprivation can slow the process by up to 30–40%, but are not able to prevent it (not shown). This fact may explain why poor GSH recovery (20–80%) and extremely high GSSG concentrations were found in blood by the investigators (44).

Acidification may produce artifacts; in particular, it leads to oxidation/conjugation of the SH group of GSH. Data in Table 2 clearly suggest the presence of a wide discrepancy in the concentrations of GSSG in the same blood sample if treated with NEM before acidification. GSSG values decreased from 60–70 μmol/L to 3–5 μmol/L in NEM-pretreated samples. It appears from these data that there are two possibilities: (a) oxidation of GSH takes place during acid denaturation of proteins; or (b) NEM is able to artifically lower the concentrations of GSSG.

The activity of GR in human blood and RBC is ~0.4 and ~0.8 kU/L, respectively (54); thus it is theoretically able to reduce 0.1 mmol/L GSSG within a few seconds. If the addition of a thiol-blocking agent (i.e., NEM, IAA, MBB), with consequent depletion of free GSH, is carried out on a non-acid-denatured sample, the presence of an active reducing system (GR plus NADPH) could in theory shift the equilibrium and reduce GSSG to GSH. GSH in turn could be trapped by the conjugation with the alkylation or arylating agent. Titration of GSSG in GSSG-added hemolysates (Fig. 4) indicates that GSSG can be reduced by cellular reductases and also after treatment with SH alkylating or arylating agents.

NEM appeared to be sufficiently rapid in inactivating GR; however, the recovery of added GSSG (Fig. 4) after NEM treatment was complete only when samples were acid denatured within a few minutes after the addition of NEM. This lower recovery could also be attributable to the reaction of NEM with amino groups of GSSG. Other frequently used thiol reactants (e.g., IAA) led to poor recovery of GSSG; this also occurred when high concentrations (50–100 mmol/L) were used.

These results are consistent with the rate of inhibition of GR, the activity of which is largely dependent on the presence of its substrates; in fact, GR activity is protected by GSSG and increased by NADPH. NEM is a rapid inhibitor in the presence of NADPH and in the absence of GSSG. Conversely, IAA required hours to completely inhibit the enzyme. Both substances had no effect on the purified enzyme in the absence of NADPH (Table 3).

These experiments demonstrate that oxidation of GSH takes place during acid denaturation of blood samples and that NEM is scarcely able to reduce the concentration of GSSG. High doses of NEM coupled with short incubation times before acid precipitation of proteins are the ideal conditions for the prevention of both artifactual reduction of GSSG in non-acid-denatured samples and oxidation during acidification.

Reaction of electrophiles with amino groups involves the possibility of various electrophiles, used to block free SH groups of GSH, reacting with amino groups of the molecule. Amino groups are weaker nucleophiles than thiol groups; alkaline pH values, however, can increase the reaction rate of amino groups, and under some conditions (i.e., pH >7.5 and high concentrations), this reaction becomes relevant. The fact that NEM is able to react with GSSG, leading to depletion of its peak area, is shown in Fig. 5. This is important, in particular, when HPLC methods with FDNB derivatization are applied because FDNB reacts with amino groups with a reaction time of some hours at alkaline pH. Thus, extraction of NEM from acidified samples is a necessary step before sample processing.

Taken together, our data therefore suggest the following: (a) oxidation of GSH is very fast when TCA-precipitated blood is restored to neutral pH; (b) oxidation of GSH takes place during acid denaturation of the blood samples; (c) only NEM treatment of blood before acidification can prevent GSH oxidation with minimal shift of equilibrium; and (d) some attention must be given to the eventual reaction of NEM with amino groups of GSSG.

After ascertaining that unacceptable variability is generated in these procedures, the next step is determining the correct guidelines for GSH and GSSG measurements in blood or RBCs. It is clear that because GSH is much more concentrated than GSSG, a minimal percentage GSH oxidation can lead to a 10- to 100-fold overestimation of GSSG. It is therefore important that every procedure for GSSG determination must include the blocking of free SH groups of GSH before acidification. The agent used for this purpose must penetrate cell membranes, react rap-
idly, and preferably, inactivate cellular GR rapidly. We recommend that the best substance in this case is represented by NEM: it reacts within seconds, the conjugate is stable in an acid environment, and it rapidly inactivates GR (Table 3). The use of NEM before acidification to prevent SH-group oxidation and disulfide titration has also been applied on other systems, e.g., plasma samples, as described by Mansoor and coworkers (55, 56).

However, some caution must be taken because NEM reacts with amino groups at pH >7.5. Thus, if titration procedures require long incubations at alkaline pH, NEM must be extracted before alkalization of the medium (i.e., with dichloromethane). The concentrations of GSSG in human blood treated with NEM for 10 s and then acidified with TCA are shown Table 5. The range was 2–5 μmol/L (the values are similar for rat blood, 4–8 μmol/L; data not shown). The same values were obtained in the presence of metal chelators (deferoxamine and tripotassium EDTA). For GSSG measurements, we used the three most popular methods: (a) the GSH recycling method (40); (b) the HPLC method of Reed et al. [GSSG derivatization with FDNB; see Ref. (44)]; and (c) the fluorescence detection method of Newton et al. [reduction of GSSG and derivatization with MBB; see Ref. (45)]. We obtained the same values with the first two methods and a slightly higher value with the third. The overestimation with the fluorescence detection method may be attributable to the fact that GSSG is titrated after its reduction to GSH with dithiothreitol; thus, GSH can in part come from mixed disulfides (e.g., with cysteine) or as a consequence of a slight instability of the NEM-GSH adduct.

After assessment of the appropriate method for measuring GSSG, the next task is determination of the most practical procedure for GSH detection. Oxidation of GSH during acid denaturation is able to reduce the GSH concentration, producing GSSG and, probably, GSH-protein mixed disulfides. This event implies an underestimation of GSH up to 20–30%; even if this artifact is less marked than that produced for GSSG, protection of GSH before acidification is recommended. NEM-GSH adducts cannot be detected by spectrophotometry; moreover, HPLC-based determination is not easily performed because of the tendency of NEM-GSH adducts to slowly decompose into subproducts (17). Nevertheless, the high GSH concentration allows other procedures: (a) direct titration of GSH in hemolysates with the GSH recycling method (40); (b) blocking of SH with IAA (the IAA-GSH can be detected by HPLC) and then TCA acidification; and (c) derivatization (thereby blocking) of SH with the fluorescent-labeling agent MBB, then TCA acidification and measurement by HPLC with fluorescence detection. The use of IAA or MBB can lead to a shift in equilibrium. This phenomenon is attributable to the irreversible blocking of free thiols because GR is not completely inhibited, and the reaction of IAA and MBB with thiols requires minutes to hours (Table 3). However, this occurrence can be rather marginal given the minimal concentration of GSSG present in samples. In Table 5, the data for GSH titration obtained with the three methods are reported; the values are similar. In this case, as GSH was titrated in hemolysates, the eventual action of transpeptidases (which can degrade glutathione) was assessed by the use of transpeptidase inhibitors (50 mg/L acivicin) and by HPLC analyses of glutathione degradation products (i.e., cysteine and cysteinyl-glycine); under our conditions, the action of transpeptidases was negligible (not shown).

Artifacts other than those described in the present study may also occur; a typical example is plasma GSH oxidation. GSH concentrations are ~2–3 and ~15–25 μmol/L in human and rat plasma, respectively. Usually, GSH oxidation occurs within minutes of blood collection (57), thus leading to an overestimation of GSSG in whole blood if it is not processed quickly. We verified that rat GSSG increased from 5 to 20 μmol/L when blood was maintained at room temperature for 1 h; this phenomenon was not present in washed rat blood cells (not shown). In human blood, this GSSG overestimation was lower, given the minimal concentration of GSH in plasma, but it could be amplified by the presence of a slight hemolysis (cellular GSH is 2–3 mmol/L).

Many studies have analyzed blood or RBC GSH and GSSG values and have encountered titration problems. Asensi et al. (17), for example, suggested that the addition of a NEM-containing solution of perchloric acid was able to prevent GSH oxidation, and they measured a GSSG concentration of 19 ± 5 μmol/L in rat blood. It is thereby possible that a NEM-containing perchloric acid solution is not fully able to prevent oxidation during acid deproteinization. Moreover, because NEM is not extracted, its reaction with amines of GSSG may decrease GSSG concentrations.

The problem of GSH/GSSG titration has been also

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<th>Table 5. Methods for GSH/GSSG measurement.</th>
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* Measurement of GSH and GSSG in human blood. For GSH titration, samples were hemolyzed by 10-fold dilution with water. The hemolyzate was then used directly for GSH measurement (method A, Tetze method) or derivatized with FDNB (method B) or MBB (method C). After derivatization (methods B and C), proteins were removed by TCA (final concentration, 100 g/L) acidification. For GSSG titration, samples (1 mL) were rapidly mixed with 1 mL of 100 mmol/L phosphate buffer, pH 7.4, containing 50 mmol/L NEM and acidiﬁed with TCA (final concentration, 100 g/L) after 10 s. In the clear supernatant, NEM was extracted with dichloromethane, and GSSG was measured directly (method A) or after derivatization (methods B and C).

* Conditions for the methods were as follows (number of replicates, 8): method A, hemolysate aliquots (for GSH titration) or acidified supernatants (for GSSG) were added to a buffered solution and measured with the GSH recycling method (see methods); method B, samples were buffered and incubated with FDNB and processed by HPLC as described in Materials and Methods; method C, samples were buffered and incubated with monobromobimane, and then charged onto an HPLC instrument as specified in Materials and Methods (MBB-fluorescence method [45]). GSSG was titrated after its conversion to GSH by treatment with dithiothreitol.
evaluated by many other investigators (10, 36–39), and various analytical procedures have been developed. Some studies (16, 42, 44) placed particular emphasis on the validation of the methods; in particular the following were carefully analyzed: reproducibility, accuracy, recovery, and sensitivity, as well as the processing method. Although numerous reports (16, 17, 58) suggest a thorough revision of the methods used, only a few studies (16, 58, 59) have considered the problems of possible artifacts during sample collection and manipulation and titration procedures. Interestingly, Mills et al. (59) found some results convergent with ours, but the interpretation was different. They suggested that the low concentrations of GSSG after the addition of NEM could be attributable to artifacts. Conversely, our results demonstrate that the real concentrations of GSSG are low (3–4 μmol/L) and that the addition of NEM can prevent artifacts from being generated by oxygenated Hb and acid protein precipitation. Paradoxically, earlier investigations (10) appear to have measured GSSG with greater accuracy than more recent studies. In our previous investigations (27, 60, 61), concentrations of GSSG in human blood were always higher than those seen in the present study. We believe that these artifacts were generated during the sample denaturation (acidification). Moreover, nonsignificant differences were usually found in blood and RBC GSH and/or GSSG concentrations of apparently healthy individuals or patients with various pathologies (diabetes, macular degeneration, and lateral amyotrophic sclerosis; unpublished results).

In conclusion, titration of GSH and GSSG is influenced by several factors; among these, the most important is oxidation during the acidification process. In this phenomenon (Fig. 6 and Table 4), the oxygenated form of Hb is most likely implicated, GSH oxidation being minimized by the subtraction of O2 (N2 atmosphere) or the addition of 5% CO (a 5% CO atmosphere can displace >99% of O2 from Hb). Moreover GSH, GSSG, and total glutathione concentrations [GSH + (2 × GSSG)] follow exactly the O2 dissociation curve of Hb (Fig. 6), confirming the role of oxy-Hb in this process. In addition, one must take into consideration that total glutathione is depleted when blood is acidified in the presence of oxy-Hb; this suggests that glutathione-protein mixed disulfides may be artificially formed during acidification. Thus, basal values of glutathione-protein mixed disulfides measured previously (27) may (at least in part) also derive from artifacts.

Overall, the results of our study demonstrate that many factors can influence concentrations of GSH and, particularly, GSSG in blood (RBCs), suggesting that almost all differences found in other investigations may be attributable to these artifacts more than to pathophysiologic in vivo factors. We have also demonstrated that very low values of GSSG occur in human blood and that higher concentrations are likely attributable to various artifacts. Because GSSG concentrations in human blood/RBCs are in the range of 2–6 μmol/L, it is difficult to evaluate with great precision its biological role, given that many factors after blood withdrawal can influence GSSG concentrations. Consequently, the measurement of GSSG in human blood as an index of whole-body oxidative stress is quite meaningless, and the notion that some pathophysiologic conditions can alter the GSH/GSSG homeostasis of blood still needs to be confirmed.

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References


