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Differential Distribution of Free and Bound Glutathione and Cyst(e)ine in Human Blood

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ABSTRACT. The redox status of free and bound glutathione (GSH) and cyst(e)ine (Cys) is altered by oxidative stress, drugs, and disease. Most studies measure only their free forms and not the bound forms, which may have a crucial protective role. For this reason, we determined free and bound, reduced and oxidized GSH and Cys in whole blood, red cells, and plasma of human blood from healthy adults. Distinct compartments of GSH and Cys were found. In whole blood, >99% GSH was in red cells, of which 16% was bound. GSH values were the same for red cells in whole blood or in cells isolated from the same samples. Only 0.5% of GSH was in plasma, all of which was bound. In contrast, 97% of Cys was in plasma and only 3% in red cells. This was a remarkable separation of these closely related metabolites in the same tissue. In plasma, 60% of Cys was bound. Also, strong correlations were shown of bound vs free Cys and also vs free *plus* bound Cys. The bound Cys was more constant and suggested that it is a metabolic reserve. Our findings demonstrate the occurrence of significant bound forms of GSH and Cys and have implications for future studies in disease and toxicology. BIOCHEM PHARMACOL 52;3:401–406, 1996.

KEY WORDS. human blood; erythrocytes; plasma; glutathione; cyst(e)ine; free and bound

"The GSH† status of cells is defined by the total cellular concentration of GSH and the nature of the distribution of the possible forms in which GSH can occur in cells." [1]. Moreover, ". . . intracellular components which behave chemically like G-SH or G-S-S-G . . . e.g., cysteine, unusually reactive disulfides, etc., should be considered in any evaluation of the G-SH-G-S-S-G status of the cell." [2]. These comments reflect the objectives of the present investigation, which is a continuation of our studies of GSH status in normal human blood [3–5].

GSH and Cys bound to proteins have been of interest since the seminal work of Stein and Moore [6] and Eagle *et al.* [7]. However, the significance of these forms is unclear, and quantitative values are needed to elucidate their *in vivo* mechanisms.

Mixed protein disulfides may play a regulatory role with free thiols during oxidative stress to spare reducing capacity [8]. For example, in rat lung and liver exposed to oxidation, total GSH is unchanged, but the percentage of bound GSH increases in a compensatory manner [9–11]. Kosower and Kosower [12] suggested that the disulfides may serve as a reserve pool of GSH that is mobilized rapidly to replenish intracellular GSH.

Bound GSH and Cys have been investigated in a variety of animal tissues including liver, heart, muscle, spleen, gastric muscle, intestinal mucosa, and lens [13–17] and also of human tumor cells, lens, and platelets [18–21]. The relative amounts of free and bound forms in human plasma have been reported [22–34], but not in the red cell.

The objectives of this investigation were to quantify systematically the concentrations and distribution of free and bound GSH and Cys in normal whole blood, red cells, and plasma. Validated methods of sample collection, processing, and analysis were used to minimize experimental artifacts and to specifically account for metabolic changes.

MATERIALS AND METHODS

Reagents

GSH, GSSG, Cys, CSSC, potassium borohydride, and urea were obtained from the Sigma Chemical Co. (St. Louis, MO). Mono-chloroacetic acid, HPLC-grade methanol, MPA, and acetonitrile were from the Fisher Scientific Co. (Pittsburgh, PA, and Fair Lawn, NJ). Heptanesulfonic acid was from Alltech Associates (Deerfield, IL). All other chemicals were reagent grade, and deionized distilled water was used.

Sample Collection and Processing

This work combines data from two sets of experiments done 2 years apart. Study I included 8 subjects (3 females and 5

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† *Abbreviations:* GSH, reduced glutathione; GSSG, glutathione disulfide; Cys, Cysteine; CSSC, cystine; Hb, hemoglobin; MPA, metaphosphoric acid; RBC, red blood cells; and CV, coefficient of variation.

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TABLE 1. GSH and Cys recoveries from borohydride-treated blood

Sample	% Recovery*	
	GSH	Cys
Whole blood†	96.9 (100, 93.8)‡	112 ± 4.58 (3)§
Acid-insoluble pellet‖	92.9 ± 1.16 (3)	97.9 ± 5.62 (4)

GSH or Cys was added to either whole blood or acid-insoluble pellets. Samples were reduced with borohydride as described in Materials and Methods.

* $$\%\ Recovery = \frac{observed\ GSH\ or\ Cys}{(endogenous + added)} \times 100.$$

† Whole blood: Endogenous GSH was 1096 nmol/mL with 200 nmol added. Endogenous Cys was 146 nmol/mL with 50 nmol added.

‡ Mean (individual values).

§ Mean ± SEM (number of samples).

‖ Acid-insoluble pellets: Endogenous GSH ranged from 106–226 nmol/mL with 100–150 nmol added. Endogenous Cys ranged from 54–80 nmol/mL with 50–100 nmol added.

males) with ages ranging from 28–70 years. Study II had 23 subjects (22 females and 1 male) with ages ranging from 35–98 years. Blood samples were obtained from these healthy adults who signed Informed Consents approved by our Institutional Review Board. Blood was collected by venipuncture into heparinized vacutainers (Becton-Dickinson, Rutherford, NJ), placed quickly into ice, and processed within 60 min. The stability of GSH and GSSG in whole blood stored at 0° for up to 6 hr was demonstrated previously [5]. Cys and CSSC values were also stable with CV values of 2.5% for 3 hr of storage and 7.3% for 6 hr.

Portions were analyzed for red cell number and hematocrit with a Coulter ZBI counter (Coulter Electronics, Inc., Hialeah, FL). Hb was determined by the cyanmethemoglobin method [35]. Protein was precipitated by the addition of 800 μL of 5% (w/v) MPA to 200 μL of whole blood. After centrifugation, the resultant supernatants were analyzed for free GSH, GSSG, Cys, and CSSC. The acid-insoluble pellets were frozen until further treatment.

Red cells were separated after centrifugation of whole blood at 1000 *g* for 20 min in a refrigerated centrifuge. The plasma and buffy layer were removed, and plasma was deproteinized by addition of an equal volume of 5% MPA. In 10 randomly selected samples, the cells were suspended in an equal volume of cold 0.9% (w/v) NaCl and washed twice before final suspension in NaCl. The cells were counted, and Hb was determined as before. Contamination by white cells was <0.08%. These red cell suspensions were acid-precipitated and processed as described for whole blood.

Borohydride Reduction of Pellets

To remove residual GSH or Cys, acid-soluble pellets were washed twice by resuspension in 800 μL of 5% MPA and once in 800 μL of 1 mM EDTA. Each suspension was centrifuged, and the pellets were resuspended in 1 mL of 8 M urea in 1 mM EDTA and incubated in a water bath at 40° for 10 min. After addition of 25 μL of octanol, 0.5 mL of aqueous 1.3 M potassium borohydride was added slowly to prevent foaming. This mixture was incubated at 40° for 30 min, and 20% (w/v) MPA was gradually added to a pH < 3.0. The sample was centrifuged, and the resultant supernatant filtered through a syringe filter with 0.2 or 0.45 μm pore (Nalgene Co., Rochester, NY).

Analysis of GSH, GSSG, Cys, and CSSC

GSH, GSSG, Cys, and CSSC were measured directly and simultaneously by an HPLC method with dual electrochemical detection, which was described previously [3, 5]. In brief, whole blood and red cell acid supernatants were diluted with HPLC solvent, but the reduced pellet supernatants were undiluted. Aliquots (8–40 μL) were injected onto a reverse-phase column and eluted isocratically with a solvent of 96% (v/v) 0.1 M mono-chloroacetic acid, pH 3.0, 4% (v/v) methanol and an ion-pairing reagent of 2.0 mM heptane sulfonic acid with a flow rate of 1 mL/min. Alternatively, 2% (v/v) acetonitrile replaced methanol, and 98% of 0.1 M mono-chloroacetic acid was used. The detection limits were 10 pmol for Cys, CSSC, and GSSG, and 80 pmol for GSH. To enable statistical analyses, a value of 1 nmol/mL of blood was assigned to undetectable compounds. All concentration data are expressed in GSH or Cys equivalents (GSH + 2GSSG or Cys + 2CSSC, respectively) to include *both* reduced and oxidized forms.

Recovery Experiments

Previous results showed that borohydride had no effect on the recovery of GSH solutions alone. However, to evaluate

TABLE 2. Distribution of GSH and Cys in whole blood

	GSH*		Cys*	
	(nmol/mL)	(%)†	(nmol/mL)	(%)
Red cells	1192 ± 29.4‡	99.5 ± 0.10	5.79 ± 0.214	2.7 ± 0.30
Plasma	6.27 ± 1.18	0.51 ± 0.09	231 ± 15.2	97.2 ± 0.29

Red cells and plasma were separated and then analyzed as described in Materials and Methods. The contribution of each fraction was determined using the hematocrits and cell numbers of whole blood and red cell suspensions.

* GSH = total free and bound GSH equivalents. Cys = total free and bound Cys equivalents.

† Percentages of GSH or Cys in 1 mL whole blood.

‡ Mean ± SEM of 34 samples.

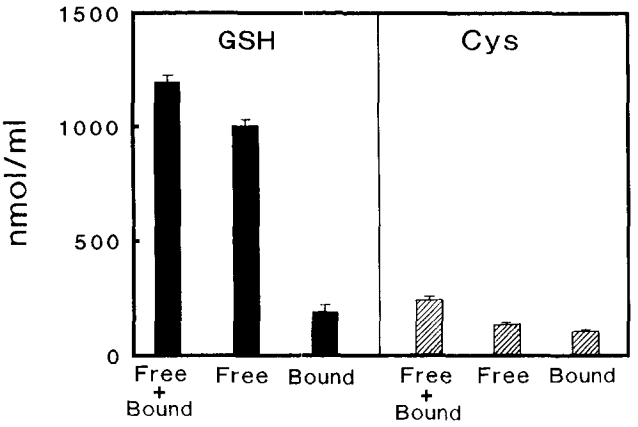

FIG. 1. GSH and Cys levels in whole blood. All values are expressed as GSH or Cys equivalents (GSH + 2GSSG or Cys + 2CSSC). Data are means ± SEM of 34 different samples.

borohydride treatment on both GSH and Cys concentrations in blood samples, recovery experiments were carried out with authentic GSH or Cys added to fresh whole blood or to acid-insoluble pellets. GSH (40 nmol) or Cys (10 nmol) was added to 200 μL of whole blood, which was precipitated with 800 μL of 5% MPA. The pellets were reduced with potassium borohydride as above. Also, GSH (20 and 30 nmol) or Cys (10 and 20 nmol) was added to washed pellets that were then reduced. These added amounts were measurable and did not mask the endogenous levels. Control blood samples and pellets were treated similarly, and GSH and Cys alone in 1 mM EDTA were analyzed.

Statistical Methods

Student's *t*-test was used for statistical analysis [36], and a P value of <0.05 was considered significant. Values are expressed as means ± SEM of 34 samples unless noted otherwise.

RESULTS

All subjects had normal hematological parameters. Mean values ± SEM were: red cell number = 4.49 ± 0.09 x 10^6/mm^3; hemoglobin = 13.5 ± 0.26 g/dL; and hematocrit = 39.1 ± 0.047%.

Borohydride treatment did not alter GSH or Cys concentrations as recoveries of 93–112% were obtained when known amounts were added to either whole blood *before* processing or to the acid-insoluble pellets *before* reduction with borohydride (Table 1).

The determination of percentages of GSH and Cys in red cells and plasma of *whole blood* utilized the hematocrits and cell counts from both whole blood and red cell suspensions. In whole blood, free *plus* bound GSH was 99.5% in red cells and less than 1% (6 nmol/mL) in plasma (Table 2; Fig. 1). The concentration of GSH per red cell was 2.70 ± 0.077 × 10^{-7} nmol of which 0.435 + 0.022 × 10^{-7} nmol (16.3%) was bound. The distribution and variability of free and bound GSH forms are shown in Table 3. As noted in previous work [5], GSSG levels were highly variable in contrast to GSH.

The concentrations of free plus bound GSH, bound GSH, and the percent bound were the same for red cells in *whole blood* and in red cells *isolated* from the same samples (Table 4). This was shown by the 98.8 ± 2.95% ratio of red cell to whole blood with a correlation coefficient (r) of 0.87 ($P < 0.02$) and a %CV of 9.3. The percent of bound GSH was 16.8 ± 2.03 in whole blood and 17.9 ± 1.30 in isolated cells. The percent of GSSG was 6.76 ± 0.89 in whole blood and 11.3 ± 1.16 in isolated cells ($P < 0.01$), the increase most likely due to oxidation artifacts that occurred during the several washings and centrifugations.

In contrast to GSH, only 3% of the Cys (or 6 nmol/mL) in whole blood was in *red cells*. In *isolated* cells the free plus bound Cys concentration per red cell was 3.41 ± 0.638 × 10^{-9} nmol (N = 10). Moreover, free CSSC was absent in the isolated cells. In *whole blood*, mean plasma Cys, 231 nmol/mL, accounted for 97% of all the Cys, of which 44.7 ± 1.32% was bound.

Free GSH was absent in all plasma samples and bound GSH was found in only half of them, and the concentration was only 10.3 ± 1.3 nmol/mL. In addition, GSSG was absent in all plasma samples.

The free plus bound Cys in plasma was 357 ± 14 nmol/mL. Bound Cys had a mean value of 213 ± 7 nmol/mL and comprised 60% of the total with a % CV of 11. The distribution and variability of the various forms of plasma Cys are shown in Table 5.

TABLE 3. Distribution and variability of GSH in whole blood

	Distribution		Concentration	
Fractions	**% of Total**	**% CV**	**μmol/10^{10} RBC**	**% CV**
Free + bound GSH	(100)*		2.70 ± 0.077†	16.7
Free GSH + GSSG	83.7 ± 0.800	5.57	2.27 ± 0.072	18.4
Free GSH	75.9 ± 1.18	9.09	2.05 ± 0.069	19.6
Free GSSG	7.81 ± 0.631	47.1	0.21 ± 0.018	49.9
Bound GSH	16.3 ± 0.795	28.4	0.44 ± 0.022	29.8

* One hundred percent equals (free GSH + GSSG) + bound GSH.
† Mean ± SEM of 34 samples.

TABLE 4. Comparison of free and bound GSH in whole blood and red cells

Fractions	Whole blood (μmol/10^{10} RBC)	Red cells (μmol/10^{10} RBC)	RBC/ Whole blood
Free GSH + GSSG	1.93 ± 0.12*	1.92 ± 0.13	0.99 ± 0.02
Free GSH	1.80 ± 0.10	1.69 ± 0.12	0.94 ± 0.03
Free GSSG	0.13 ± 0.02	0.21 ± 0.02†	1.79 ± 0.19
Bound GSH	0.38 ± 0.04	0.41 ± 0.04	1.15 ± 0.13
Free + bound GSH‡	2.31 ± 0.11	2.31 ± 0.14	0.99 ± 0.03

From each fresh sample, whole blood and red cells were processed, analyzed, and calculated separately.

* Mean ± SEM of 10 samples.

† Significantly different from whole blood, $P < 0.01$.

‡ Free (GSH + 2GSSG) + bound GSH, expressed in GSH equivalents.

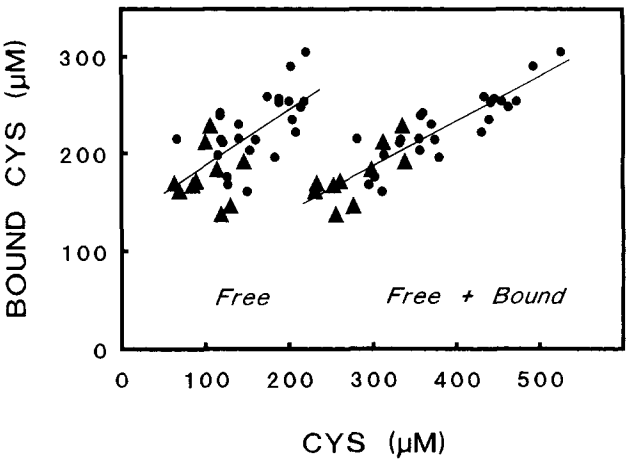

FIG. 2. Correlation of bound Cys with free and free + bound Cys. All values are expressed as Cys equivalents (Cys + 2CSSC). The data were obtained from 34 different samples. Key: (▲) samples from Study I; and (●) samples from Study II.

High correlations were found between bound vs free Cys ($r = 0.66$; $P = 0.04$), and also vs free plus bound Cys ($r = 0.89$; $P = 0.012$) (Fig. 2). Although both slopes were similar, it is apparent that there was greater variability in the free Cys vs the bound.

DISCUSSION

Distinctly different and discrete compartments were observed of GSH and Cys in whole blood, red cells, and plasma, and also of their free and bound forms. In whole blood, GSH occurred exclusively in red cells with <1% in plasma. Cys, in contrast, was almost all in plasma, with only 3% in red cells. In some samples, GSH was not detected in plasma nor Cys in red cells. It seems remarkable that these closely related metabolites are maintained in such separate compartments in the same tissue.

These results also demonstrated that GSH can be measured accurately in whole blood and obviates the separation and potential oxidation of red cell GSH. GSH levels of red cells in whole blood were the same as in cells isolated from the same samples. Also, bound GSH and free plus bound GSH concentrations were the same in both preparations.

TABLE 5. Plasma Cys—Distribution and variability

Fractions	Distribution % of Total	Distribution % CV	Concentration nmol/mL	Concentration % CV
Free + bound Cys	(100)*		357 ± 14.0†	22.2
Free Cys + CSSC	39.6 ± 1.10	16.2	143 ± 7.9	32.4
Free Cys	1.59 ± 0.21	76.0	5.1 ± 0.59	67.4
Free CSSC	38.0 ± 1.11	17.0	138 ± 8.3	34.2
Bound Cys	60.5 ± 1.11	10.7	213 ± 6.90	18.9

* One hundred percent equals (free Cys + CSSC) + bound Cys.

† Mean ± SEM of 34 samples.

We observed no loss in GSH equivalents with cell separation before analysis, contrary to others [37, 38]. Loss was most likely due to GSH oxidation to GSSG which we noted also in isolated cells, but without GSH loss or degradation. Thus, a decrease in the GSH/GSSG status with increase concentrations of free GSSG and percent GSSG probably indicates an experimental artifact.

Cys in red cells was usually absent or, when present, was 64% bound with trace concentrations from 16–90 nmol/10^{10} RBC. Early investigators measured only free Cys in red cells and overestimated it [39], but recently very low concentrations like ours have been reported [40, 41]. Other studies suggested that CSSC was lost during deproteinization [42] or via S-S binding to plasma proteins during hemolysis [43].

A small amount of GSH is associated with the cell membrane [44], but most of the bound GSH in red cells is

TABLE 6. Summary of free and bound Cys in plasma

Cys + CSSC Free	Bound (nmol/mL)	Total	Bound (%)	Reference
143 ± 8.0*	213 ± 6.9	357 ± 14	60 ± 1.11	This work
122	146†	268	54	22
152	80†	232	34	29
92	165	250	66	23
126	103†	229	45	30
114	161	275	58	31
129	115†	244	47	27
102	108†	210	51	32
90	151	241	63	33
98	105	215	49	34

* Values are means ± SEM, N = 34.

† Values were calculated by the difference of F-Cys and total Cys. Other values were direct measurements. The number of samples in these studies varied from 10–45.

probably a mixed disulfide with Hb. Early studies showed in normal human Hb that GSH complexes with the -SH of Cys-93 of the β chain [45–48].

Hb–GSH complexes may form due to oxidative stress, as a portion of intracellular GSH associated with oxyhemoglobin is released upon deoxygenation [12]. Our finding of 17% bound GSH in red cells was similar to the 20% difference in GSH concentration between oxyhemoglobin and deoxyhemoglobin.

When mixed disulfides of radiolabeled GSH and Hb were cleaved by GSSG-reductase and NADPH *in vitro*, 80–160 nmol GSH were bound/μmol of the Hb β chain [49]. In our study, if *all* the bound GSH were released from Hb, the calculated values would be 47 ± 2.7 nmol GSH/μmol of β chain.

The role of plasma as a major transport mechanism for GSH is unlikely because of its very low levels. We found plasma GSH levels were very low (10 nmol/mL) and protein-bound. Overall, these GSH levels were consistent with reports of others from 1986–1993 [22–28]. The rapid disappearance of endogenous and exogenous GSH *ex vivo* is well documented for both human and rodent plasmas, and although most becomes protein-bound, some is probably degraded [23, 24, 26, 33, 50–55]. As our blood samples were not reduced before processing, the finding of bound GSH was expected.

Most of the plasma Cys was found as mixed-protein disulfides, and our results are in agreement with others from 1981–1993 who measured both free and bound forms by various methods (Table 6). We found higher concentrations of free plus bound Cys, probably because our dual electrochemical detection method is very sensitive and measures directly the different metabolites.

Considering the diverse subject population, remarkably constant values were found for both the concentrations of bound Cys and the percent bound. This may be attributed to the relatively narrow range for normal albumin concentrations (3.6 to 4.8 g/dL). Free -SH in albumin accounts for about 500 nmol/mL of thiol [56] and provides sufficient binding sites for the amounts of bound Cys found.

In our study, strong correlations between the concentrations of bound Cys and free Cys, or free *plus* bound Cys support the concept that protein-bound Cys and free Cys may be in equilibrium [57] and act as a buffer system [58] that protects against oxidative challenges. This systematic investigation produced findings that have implications for medicine [59] and pharmacological studies involving thiols that may compete with Cys for binding sites [60].

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