both at 0.08 mmol/g albumin in the preparation used here), and heat treatment. It is possible that this introduces changes that affect BCG binding by the albumin, causing a shift in spectral band in the Vitros slide but not affecting the same process in the manual BCG assay used in this study. It should be noted that in the Vitros slide, the albumin concentration remains essentially unchanged in the 10-µL serum sample during color development, whereas in the manual BCG method, the serum sample is diluted 1:250 with color reagent.

If a change in the BCG-binding properties of serum albumin is the explanation, it raises the possibility that in vivo transport functions also are altered in the purified albumin preparations. To our knowledge, this important question has not been investigated to any extent, and certainly deserves further investigations.

References

Thiols as a Measure of Plasma Redox Status in Healthy Subjects and in Patients with Renal or Liver Failure, Anders Andersson,* Arne Lindgren,‡ Margret Arnadottir,¶ Hanne Prytz,* and Björn Hultberg* (Departments of *Clinical Chemistry, ‡Neurology, ¶Nephrology, and 4Internal Medicine, University Hospital, S-221 85 Lund, Sweden; *author for correspondence: fax 46-46189114, e-mail anders.andersson@klinikum.lu.se)

Plasma thiols have been the object of growing interest because numerous studies have indicated that even a mild degree of hyperhomocysteinemia is associated with an increased risk of developing occlusive vascular diseases (1–5). However, the mechanism behind the vascular injuries is still unknown. Studies of the possible pathogenetic mechanism of increased plasma homocysteine concentrations are difficult because little is known about the mechanism for the formation of different homocysteine species in vivo.

We recently published a method that measures reduced and total fractions of homocysteine, cysteine, glutathione, and cysteinylglycine (6). In a preliminary study, we found that patients suffering from stroke have hyperhomocysteinemia, whereas their reduced homocysteine was within the health-related reference interval (7). We hypothesized that the increased concentrations of the oxidized forms of homocysteine in plasma were attributable to a hyperoxidative state in the plasma. We also observed (8) that patients suffering from renal failure had concentrations of reduced homocysteine within the reference interval despite increased total homocysteine. The redox state of homocysteine in plasma may be influenced by other thiols (9), such as glutathione, which is involved in maintaining the intracellular thiols in reduced form.

In the present study, we therefore investigated the relationships between homocysteine, cysteine, and glutathione in 29 healthy subjects and 15 patients with renal or liver failure. We used a newly developed preparation procedure, especially designed to minimize several known pitfalls that frequently influence plasma glutathione determinations. Increased hemolysis during sample collection causes falsely increased plasma glutathione measurements because of the high glutathione contents in the blood cells. Plasma glutathione also decreases with time because of the activity of γ-glutamyltransferase in plasma. Furthermore, reduced glutathione disappears within minutes in cell-free plasma because of oxidation (7, 10–12). In the present study, we also determined thiols in whole blood and hemoglobin in plasma.

Specimens were collected from nine anuric hemodialysis patients (seven men and two women; mean age, 76 years; range, 55–79 years). The renal diagnoses were: polycystic kidney disease (n = 2), diabetic nephropathy (n = 2), glomerulonephritis (n = 2), nephrosclerosis (n = 1), bilateral nephrectomy attributable to cancer (n = 1), and light chain nephropathy (n = 1). No patient had clinical signs of heart failure or respiratory insufficiency, and there was no laboratory evidence of liver dysfunction in any of the cases. The subjects received daily supplementation with 5 mg of folic acid and 5 mg of pyridoxine.

Specimens were also collected from six male patients with liver disease (mean age, 56 years; range, 28–74 years). All of the patients had advanced liver disease with cirrhosis, one because of autoimmune hepatitis, and the others because of alcohol. None of the patients had pulmonary diseases or impaired renal function.

After the exclusion of two individuals because of increased sample collection hemolysis (353 and 2670 mg hemoglobin/L plasma), 29 apparently healthy individuals (14 men and 15 women; mean age, 64 years; range, 41–87 years) participated in the study. Their ages did not differ significantly from the age of the patients with renal or liver failure. Eight apparently healthy individuals participated in the comparison of the present method with the previously described method (6).

Blood was drawn (with a tourniquet applied) into an EDTA-Vacutainer Tube® that had been prechilled in ice water. Immediately (within 10 s) after sample collection, the tube was placed in ice water and chilled for 6 min. During the cooling period, 50 µL of blood was mixed with 450 µL of 33 g/L sulfosalicylic acid and placed at −70 °C for later analysis of reduced thiols in whole blood. After
the cooling period, another aliquot of blood was centrifuged for 3 min at 10,000 g, and 1.2 mL of the plasma was transferred to a prepared tube containing 0.3 mL of 150 g/L sulfosalicylic acid, 20 mmol/L t-serine, and 20 mmol/L sodium tetraborate. The acidified plasma was mixed and stored at −70 °C and analyzed within 4 days. The remaining plasma was stored at −70 °C for later determination of hemoglobin in plasma by derivative spectroscopy (13).

To determine total thiols in plasma, 500 μL of thoroughly mixed acidified plasma was neutralized with 1 mol/L NaOH to pH 7–8, mixed with 75 μL of 0.1 mol/L dithiothreitol, and incubated at room temperature. After 15 min, the solution was acidified with HCl (1 mol/L) and centrifuged for 3 min at 10,000 g. The supernatant was applied to HPLC as described previously (6). The remaining acidified plasma was centrifuged for 3 min at 10,000 g. The supernatant was used for the determination of reduced thiols as described previously (6). For the determination of reduced thiols in whole blood, the tube with acidified blood was thawed and centrifuged for 3 min at 10,000 g, and 20 μL of the supernatant was applied to the HPLC.

The Student independent t-test was used to test whether differences between the groups of patients and the healthy individuals were significant. Linear regression analysis was used to obtain correlation coefficients.

To study the influence of γ-glutamyltransferase on the measured glutathione concentration, we assayed plasma samples (n = 8), using a preparation containing serine/borate as described above and a preparation without serine/borate according to the previous method (6). Plasma samples were mixed with the preparations 2 h after the sample collections and assayed the same day by HPLC. Under these conditions, the mean total glutathione measured with the original method was 53% lower than the total glutathione measured with the modified method (P < 0.001), whereas the other thiols showed similar values when assayed with the different methods.

The concentrations of plasma hemoglobin (29–168 mg/L) in all 44 individuals did not show any significant correlation (r = 0.24; P = 0.15) to reduced plasma glutathione. When the samples were divided into one group with low (29–74 mg/L; n = 22) and one with high plasma hemoglobin (76–168 mg/L; n = 22), the plasma concentration of reduced glutathione did not differ (P = 0.11). Therefore, we consider that hemolysis corresponding to a plasma hemoglobin concentration <170 mg/L had no significant influence on the plasma concentration of glutathione.

Reported plasma concentrations in healthy subjects showed large variability, 0.34–31.7 μmol/L, when total glutathione was assayed by use of HPLC (6, 7, 12, 14–18) or colorimetric methods (19, 20) and when reduced glutathione, 1.9–6.9 μmol/L, was assayed by HPLC (7, 12, 14, 17, 21, 22). Our modified preparation procedure improves the reliability of the method, and the values obtained for reduced and total thiols are similar to those that we (6, 7) and others (17) have observed previously in healthy individuals.

Patients with renal failure showed decreased plasma concentrations of reduced cysteine and reduced homocysteine but increased concentrations of the total forms of these thiols compared with the healthy individuals (Table 1A). Both the decreased concentration of reduced plasma homocysteine and the increased concentration of total plasma homocysteine could be attributed to increased prooxidant activity in plasma from these patients, which might lead to a rapid oxidation of reduced homocysteine to disulfides. Homocysteine bound to plasma proteins through disulfide bonds is not as metabolically available as homocysteine in low-molecular weight disulfides or in its reduced form and therefore accumulates in the circulation (23). Another possible explanation for the decreased concentration of reduced plasma homocysteine is a lowered intracellular production of reduced homocysteine because of a more efficient metabolism of homocysteine caused by vitamin supplementation. All investigated thiols showed decreased reduced/total ratios in patients with renal failure compared with the healthy individuals (Table 1), which supports previous findings of

### Table 1A. Concentrations of reduced and total thiols, and the reduced/total ratio in plasma from various subjects.

<table>
<thead>
<tr>
<th>Reduced thiol</th>
<th>Total thiol</th>
<th>Reduced/total ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys GSH Cys-Gly Hcy</td>
<td>Cys GSH Cys-Gly Hcy</td>
<td>Cys GSH Cys-Gly Hcy</td>
</tr>
<tr>
<td>Controls</td>
<td>29</td>
<td>9.36 (1.58)</td>
</tr>
<tr>
<td>Renal patients</td>
<td>9</td>
<td>7.37 (0.43)</td>
</tr>
<tr>
<td>Liver patients</td>
<td>6</td>
<td>8.02 (0.60)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reduced thiol</th>
<th>Mean (SD), μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys GSH Cys-Gly Hcy</td>
<td>Cys GSH Cys-Gly Hcy</td>
</tr>
<tr>
<td>Controls</td>
<td>7.27 (2.12)</td>
</tr>
<tr>
<td>Renal patients</td>
<td>9.45 (2.12)</td>
</tr>
<tr>
<td>Liver patients</td>
<td>6.38 (1.50)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reduced thiol</th>
<th>Mean (SD), μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys GSH Cys-Gly Hcy</td>
<td>Cys GSH Cys-Gly Hcy</td>
</tr>
<tr>
<td>Controls</td>
<td>6.80 (0.96)</td>
</tr>
<tr>
<td>Renal patients</td>
<td>8.02 (0.60)</td>
</tr>
<tr>
<td>Liver patients</td>
<td>6.38 (1.50)</td>
</tr>
</tbody>
</table>

### Table 1B. Whole blood concentration of reduced thiols.a

| Reduced thiol, mean (SD), μmol/L |
|-----------------|------------------|
| Cys GSH Cys-Gly Hcy |
| Controls | 7.27 (2.12) | 917 (133) | 3.30 (1.9) |
| Renal patients | 9.45 (2.12) | 924 (275) | 4.24 (1.84) |
| Liver patients | 6.38 (1.50) | 738 (357) | 1.86 (0.79) |

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a GSH, glutathione; Hcy, homocysteine.

b,c Significantly different from controls: b P < 0.05; c P < 0.01.

d Reduced homocysteine was not detected in whole blood.
an impaired redox status in plasma from these patients (8, 22–27).

Patients with liver failure showed significantly lower concentrations of reduced glutathione in whole blood (Table 1B) and in plasma (Table 1A) compared with healthy individuals. The total plasma homocysteine concentration was increased, whereas the concentrations of total glutathione and total cysteine in plasma were not significantly changed. The reduced/total ratios of thiols in plasma were significantly decreased for glutathione and homocysteine (Table 1A). The decreased whole blood concentration of reduced glutathione in this study and in others (28, 29) might be attributable to lower hepatic production of glutathione in liver disease. The decreased reduced/total ratios and the increased total homocysteine concentration in plasma may indicate an increased oxidative activity, which previously has been reported to be present in plasma from patients with liver disease (30).

In the healthy individuals, reduced glutathione in whole blood showed a correlation with reduced cysteine in blood \( (r = 0.50; P < 0.01) \) and in plasma \( (r = 0.36; P = 0.05) \), but no correlation to any other analyte or ratio. The total plasma concentration of glutathione correlated \( (r = 0.84; P < 0.001) \) with reduced plasma glutathione, but neither total nor reduced plasma glutathione correlated with any form of homocysteine, cysteine, or their ratios. These findings show that glutathione does not influence the redox status of homocysteine or cysteine and suggest that glutathione is not an exclusive reductant in plasma. The concentration of reduced cysteine in whole blood showed a correlation with reduced plasma cysteine \( (r = 0.66; P < 0.001) \), reduced plasma homocysteine \( (r = 0.44; P < 0.05) \), and with the reduced/total ratios for plasma cysteine \( (r = 0.60; P < 0.001) \) and for plasma homocysteine \( (r = 0.47; P < 0.01) \). Furthermore, reduced plasma cysteine correlated to reduced homocysteine \( (r = 0.83; P < 0.001) \). The reduced/total ratio of plasma cysteine correlated with that of homocysteine \( (r = 0.90; P < 0.001) \), with the reduced form of homocysteine in plasma \( (r = 0.65; P < 0.001) \), and inversely with the total forms of cysteine \( (r = -0.65; P < 0.001) \) and homocysteine \( (r = -0.47; P < 0.01) \) in plasma. These findings suggest that reduced cysteine plays a role, at least partly, in the maintenance of the redox status in plasma.

References