CLIN. CHEM. 39/6, 980–985 (1993)

Redox Status and Protein Binding of Plasma Aminothiols during the Transient Hyperhomocysteinemia That Follows Homocysteine Administration

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We administered reduced L-homocysteine perorally (67 μmol/kg of body wt) to 12 healthy subjects and injected the same dose into one person, and determined the kinetics of the alterations in reduced, oxidized, and protein-bound concentrations of homocysteine, cysteine, and cysteinylglycine. After oral intake, reduced homocysteine increased rapidly ($t_{max} \leq 15$ min), reaching concentrations [3.97 (SD 2.99) μmol/L] 20-fold above fasting values, and then declined towards the normal concentration within 2 h. There was a similar increase in reduced cysteine and a moderate increase in reduced cysteinylglycine. During this response, we observed a positive correlation between the reduced/total ratio for homocysteine and cysteine. When homocysteine was injected, the increase in reduced homocysteine preceded the increase in reduced cysteine by about 3 min. After oral loading, oxidized homocysteine showed a transient increase (t_{max} = 30 min) that lagged behind the increase of reduced homocysteine. Oxidized cysteine and cysteinylglycine were stable or decreased slightly. Protein-bound homocysteine increased the least rapidly after homocysteine administration (t_{max} = 1–2 h), and returned to normal values slowly. Changes in protein-bound homocysteine essentially mirrored a concurrent decrease in protein-bound cysteine, suggesting displacement of bound cysteine. These data show that plasma homocysteine has a pronounced, direct effect on the redox status and protein binding of other plasma thiol components. Such effects should be recognized when studying the mechanisms behind the atherogenic effect of increased plasma homocysteine.

Indexing Terms: *cysteine · cysteinylglycine · premature cardiovascular disease · metabolism · heritable disorders*

Homocysteine is a sulfur amino acid and a product of *S*-adenosylmethionine transmethylation reactions (*1*). The plasma concentration of this compound is increased markedly in patients with the inborn error called homocystinuria, which is most commonly caused by deficiency of the homocysteine-catabolizing enzyme, cystathionine β-synthase (EC 4.2.1.22). Rare forms are caused by various defects in homocysteine remethylation (*2*).

Premature cardiovascular disease is a common cause of death in these individuals and may occur in early adolescence and even in childhood (*2*). The fact that homocystinuria caused by various genetic defects is associated with early vascular disease suggests that homocysteine is responsible for the vascular lesions (*3*). This hypothesis has motivated investigations to determine whether a moderate increase of plasma homocysteine (hyperhomocysteinemia) is a risk factor for cardiovascular disease. Studies performed up to now, including about 1800 patients and a comparable number of controls, have shown a significant relation between hyperhomocysteinemia and premature cardiovascular disease (*4, 5*).

Despite the growing evidence that plasma homocysteine is a cardiovascular risk factor, the mechanism behind the vascular injuries still remains conjectural. Mechanistic studies are hampered by the fact that little is known about which homocysteine species prevail(s) in vivo. In freshly prepared plasma from healthy subjects, the major fraction is protein bound and most free homocysteine exists as homocysteine–cysteine mixed disulfide (*6*).

We recently developed a procedure for determining reduced, oxidized, and protein-bound homocysteine and other thiol components in human plasma. The procedure is based on trapping thiols by collecting blood directly into evacuated tubes containing thiol-reactive agents (*7*). Using this method, we detected significant amounts of reduced homocysteine in plasma from healthy subjects (*7*). The reduced fraction was markedly increased after an oral intake of methionine (*8*). Perhaps more important, in eight patients with homocystinuria, reduced homocysteine was increased to 200 times the amount in healthy subjects (*9*). In both healthy subjects and patients, increased amounts of reduced homocysteine in plasma were associated with increased concentrations of reduced cysteine and cysteinylglycine and decreased protein-binding of these thiol components (*8, 9*).

The presence of large amounts of reduced homocysteine in homocystinuric patients and small amounts in healthy subjects complies with the idea that this species plays a role in atherogenesis. Moreover, the increased amount of reduced homocysteine may have marked effects on the redox status of other thiols, as demonstrated for cysteine and cysteinylglycine, and these remote chemical effects may disturb normal cellular function. Notably, there is recent evidence that reduced homocysteine and cysteine may play a role in atherogenesis by supporting cell-mediated generation of reactive oxygen species that modify low-density lipoproteins to a form that is taken up by macrophages via the scavenger receptor (*10, 11*).

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Received November 11, 1992; accepted January 11, 1993.

In the present work we investigated the effect of the increase in reduced, oxidized, and protein-bound homocysteine after homocysteine administration on the redox status of other thiol components in plasma. The magnitude and kinetics of this response together with a low plasma methionine concentration simplify the interpretation of the results. The data we obtained support the idea that homocysteine has an immediate and direct effect on the redox status and protein binding of other aminothiols in human plasma.

Materials and Methods

Materials and Subjects

Sources of most reagents were as given previously (*7*). L-Homocysteine thiolactone was purchased from Sigma Chemical Co., St. Louis, MO. It was converted into the reduced form by incubating with 5 mol/L NaOH for 5 min at room temperature (*12*). The solution was then placed on ice, neutralized by adding 5 mol/L HCl, and finally diluted to 200 mL, pH 4–5 (for oral use), or to 50 mL, 400 mmol/kg, pH 6–7 (for intravenous administration). The solution for intravenous use was sterile-filtered.

Six healthy men (ages 22–36 years, mean 29 years) and six healthy women (ages 27–34 years, mean 31 years) participated in this study. The participants had provided their written informed consent, and the protocol was approved by the regional ethical committee of western Norway.

Procedures

Administration of homocysteine, blood sampling, and processing. Homocysteine was given orally after an overnight fast at a dose of 67 μmol/kg body weight. Blood samples were collected immediately before and 15, 30, and 60 min and 2, 4, 6, 8, 12, 24, and 48 h after the homocysteine intake.

One subject was injected intravenously with homocysteine at 67 μmol/kg body weight. From this person, blood samples were drawn before and at various times after injection: 55 and 90 s; 3.75, 5.3, 10, 15, 30, 60, and 90 min; and 2, 3, 4, 6, 8, 12, 24, and 48 h.

Blood was routinely collected into three evacuated tubes containing the thiol-reactive reagents monobromobimane (mBrB) or *N*-ethylmaleimide (NEM), or no additive. The samples were then immediately centrifuged at 10 000 × *g* for 1 min to remove the blood cells.

Determination of reduced, oxidized, and protein-bound thiol components in plasma. Thiols in blood collected into a tube with mBrB react with this reagent and form fluorescent adducts. The blood cells are removed by centrifugation, and the plasma proteins by acid precipitation. Chromatographic analysis of the acid-soluble supernate yields the free, reduced forms of homocysteine, cysteine, and cysteinylglycine.

When blood is collected into a solution containing NEM, the reduced sulfhydryl groups are rapidly trapped as their NEM adducts. The plasma fraction is then deproteinized with acid, and the disulfides are reduced in the presence of $NaBH_4$ to their corresponding thiols, which are then derivatized with mBrB. With this procedure we determined the oxidized, free forms of homocysteine, cysteine, and cysteinylglycine.

Total amounts of homocysteine, cysteine, and cysteinylglycine in plasma were determined by reducing the disulfides in whole plasma with $NaBH_4$ and derivatizing the free thiols with mBrB.

The protein-bound fraction was calculated by subtracting the quantities of the reduced and the oxidized species from the total amount.

The thiol-mBrB adducts are separated by ion-paired liquid chromatography on an ODS-Hypersil column. Further details on these assays have been published previously (*7*).

Statistics. Variation in a particular analyte during the 48 h after the homocysteine loading was analyzed by the Friedman test (nonparametric analysis of variance) and by repeated-measures analysis of variance. The latter test was also used to evaluate the sex-related differences. In cases where significant ($P < 0.05$) changes were obtained, the values before loading and the maximal response after loading were compared by using the Wilcoxon matched-pair signed rank test. The relationship between the reduced/total ratio for various plasma thiols was assessed by using linear correlation. *P* values are given for a test for zero correlation; all *P* values are given as two-tailed, and values <0.025 are considered significant.

Results

Homocysteine loading. Six healthy men and six healthy women were included in this study. Their fasting plasma concentrations of different forms of homocysteine, cysteine, and cysteinylglycine are listed in Table 1. The values for total homocysteine, cysteinylglycine, and cysteine were within the normal ranges (*6, 13*). After the subjects were given a standard oral dose of freshly prepared reduced homocysteine, the plasma concentrations of reduced, oxidized, and protein-bound thiol components in men were not significantly different from those in women. In the rest of our presentation, the data from men and women are treated as a single population.

Reduced homocysteine and other thiol components. There was a rapid ($t_{max} < 15$ min), significant ($P < 0.005$) increase in reduced homocysteine in plasma from 0.04 (SD 0.026) to 3.97 (SD 2.99) μmol/L after the oral homocysteine loading. Then the concentration rapidly normalized, corresponding to a rate of $\sim 0.9\ h^{-1}$ (Figure 1, upper panel).

Reduced cysteine showed similar kinetics ($t_{max} = 15$ min), but the increase ($P < 0.005$) relative to the fasting concentration was less pronounced, from 4.68 (SD 1.93) to 8.64 (SD 3.93) μmol/L (Figure 1, middle panel). There was a concurrent moderate but significant ($P < 0.05$) increase [from 1.66 (SD 0.49) to 2.4 (SD 0.61) μmol/L] in reduced cysteinylglycine (Figure 1, lower panel).

We also investigated in the individual subjects the relation between the reduced/total ratios (the fraction of the total amount that exists in the reduced form) for the plasma thiol components 15 and 30 min after oral

Table 1. Fasting Concentrations of Homocysteine, Cysteine, and Cysteinylglycine in Plasma (μmol/L)

Homocysteine				Cysteine				Cysteinylglycine			
Total	Reduced	Oxidized	Protein-bound	Total	Reduced	Oxidized	Protein-bound	Total	Reduced	Oxidized	Protein-bound
Men											
7.9	0.07	1.7	6.1	221.5	7.7	101.7	112.0	26.4	1.8	8.4	15.6
7.6	0.05	1.0	6.6	232.0	3.9	81.8	146.3	31.0	1.6	7.0	22.4
6.8	0.08	1.0	5.7	265.3	6.6	108.6	150.1	26.9	1.8	7.3	17.8
10.1	0.03	1.5	8.6	272.5	3.5	99.9	169.0	34.2	1.5	8.2	24.5
10.1	0.01	0.8	9.4	263.0	2.2	66.7	194.0	25.8	0.9	5.6	19.2
8.4	—	0.2	8.1	253.8	2.6	56.7	194.6	32.0	1.2	7.2	23.6
Women											
11.3	0.03	1.7	9.6	257.4	3.5	91.4	162.4	24.6	1.2	6.2	17.3
6.0	0.05	2.0	4.0	236.1	4.3	94.4	137.4	28.2	1.7	9.7	16.8
7.5	0.05	0.8	6.7	230.6	6.3	76.9	147.4	24.7	2.1	6.9	15.7
12.1	—	1.2	10.8	295.1	4.1	95.6	195.4	25.3	1.2	6.9	17.2
5.8	0.03	0.8	5.0	209.8	3.7	50.7	155.5	34.5	2.4	9.4	22.8
8.8	0.05	0.4	8.3	293.2	7.9	81.5	203.8	29.3	2.0	6.5	20.8

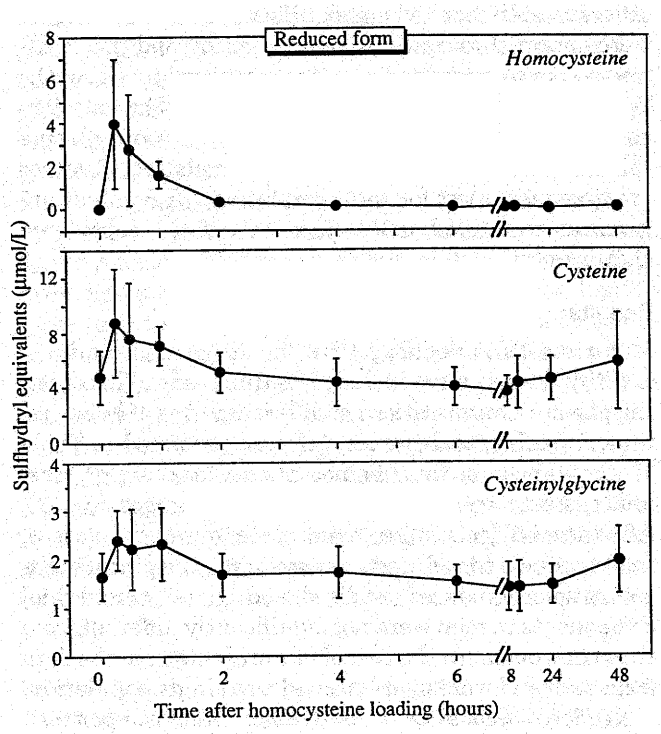

Fig. 1. Reduced homocysteine, cysteine, and cysteinylglycine in plasma after oral administration of homocysteine

Six men and six women were given a standard homocysteine dose of 67 μmol/kg of body weight. The data are given as mean ± SD from all 12 subjects

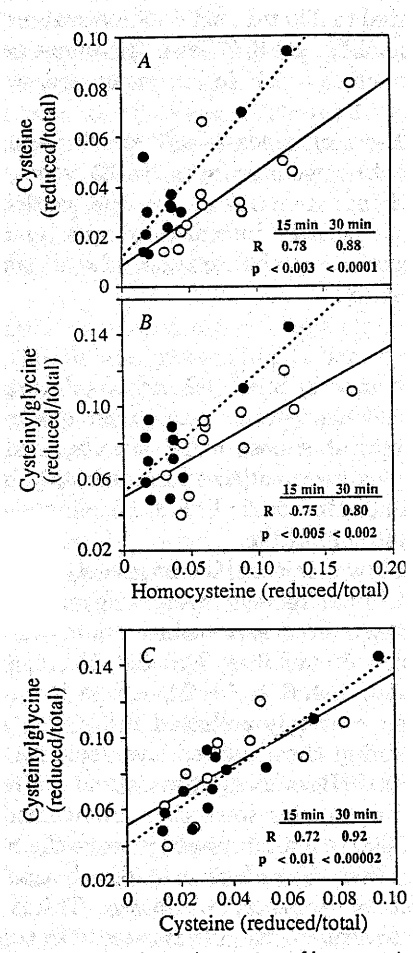

Fig. 2. Relation between the redox status of homocysteine, cysteine, and cysteinylglycine in plasma

The fraction of total homocysteine that exists in the reduced form (plotted as reduced/total ratio) was determined in plasma from 12 subjects, 15 (○) and 30 (●) min after oral homocysteine administration. Results from linear-regression analysis are shown (*A*) between homocysteine and cysteine, (*B*) between homocysteine and cysteinylglycine, and (*C*) between cysteine and cysteinylglycine

homocysteine intake (Figure 2). There was a significant correlation by linear-regression analysis between the reduced/total ratio for homocysteine and cysteine; the relation vs the ratio for cysteinylglycine was weaker. However, the reduced/total ratios for cysteine and cysteinylglycine were closely related, especially after 30 min (Figure 2).

The initial kinetics of the increase of the reduced species could be distinguished in the plasma from the subject who was injected with homocysteine. Reduced homocysteine peaked after <1 min and preceded the increase in reduced cysteine and cysteinylglycine, which was maximal after about 4 min. Then there was a parallel decline in the plasma concentrations of all three components (Figure 3).

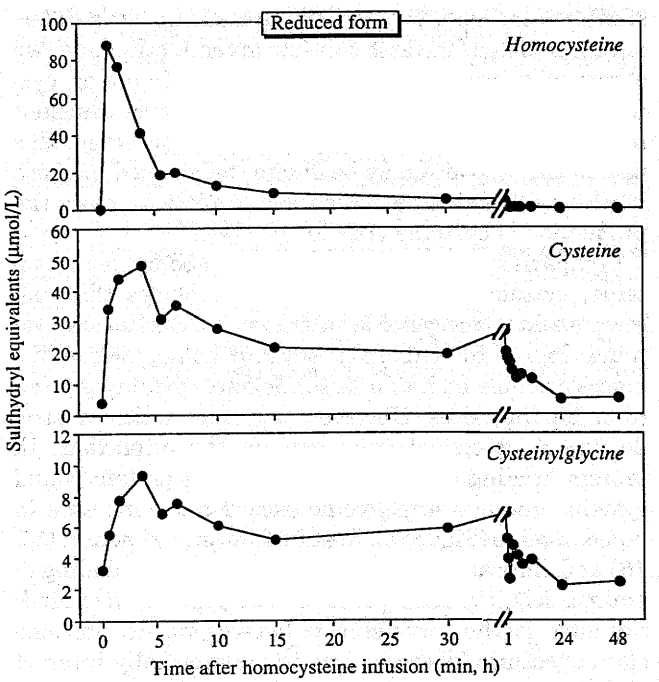

Fig. 3. Reduced homocysteine, cysteine, and cysteinylglycine in plasma after intravenous administration of homocysteine to one subject

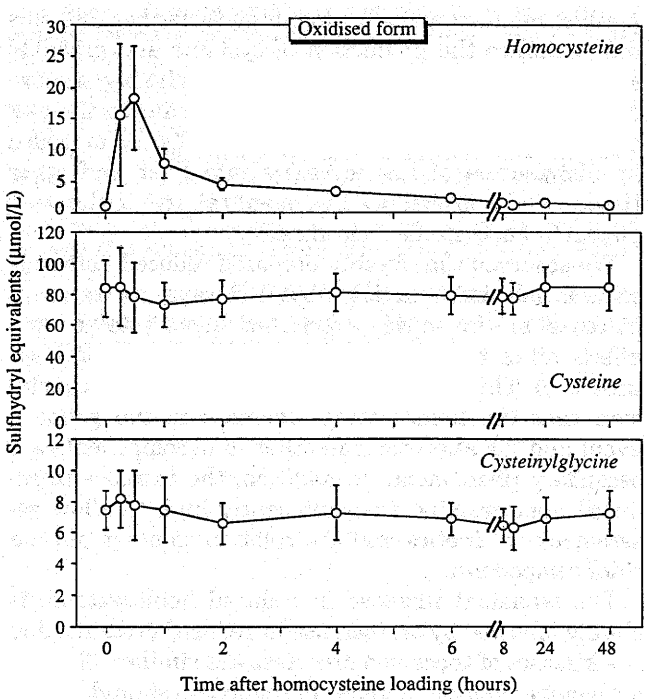

Fig. 4. Oxidized homocysteine, cysteine, and cysteinylglycine in plasma after oral administration of homocysteine
Subjects, dose, and results expressed as in Fig. 1

Free oxidized species. Oxidized homocysteine increased significantly (P <0.005 at 30 min) for a brief time after the oral homocysteine loading but the response (t_{max} = 30 min) lagged behind the increase in the reduced form, and the decline towards the preload values proceeded more slowly. The amounts of oxidized cysteine and cysteinylglycine were not significantly altered (Figure 4).

Protein binding. Protein-bound homocysteine showed the slowest response after loading (t_{max} = 1–2 h, P <0.005) and gradually (at a rate of about 0.15 h^{-1}) approached the preload value. Protein-bound cysteine decreased (t_{min} = 1 h, P <0.005), and the decrease mirrored the increase in protein-bound homocysteine. A moderate decrease was also observed for protein-bound cysteinylglycine. Notably, the decrease in protein-bound cysteine plus cysteinylglycine was somewhat greater than the corresponding increase of protein-bound homocysteine (Figure 5).

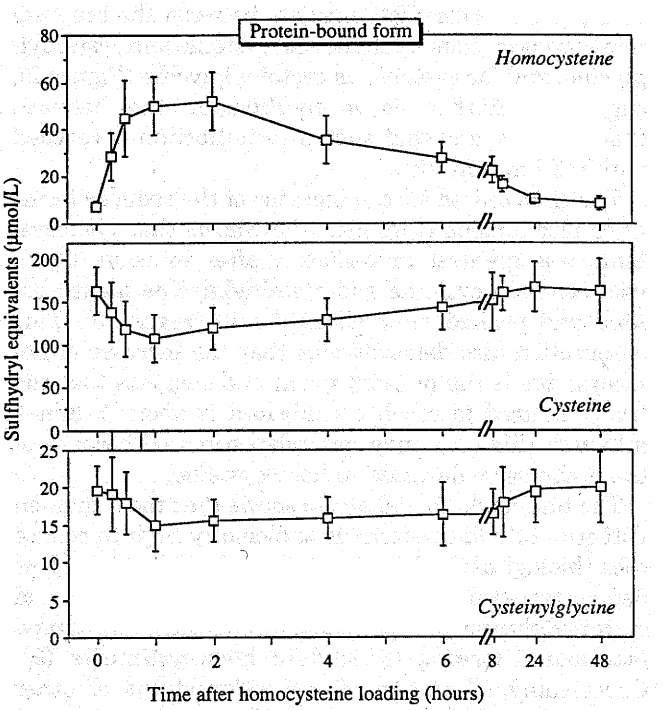

Fig. 5. Protein-bound homocysteine, cysteine, and cysteinylglycine in plasma after oral administration of homocysteine
Subjects, dose, and results expressed as in Fig. 1

Discussion

We have previously demonstrated that small amounts of reduced homocysteine exist in plasma from healthy subjects (*7, 8*), and this thiol increases significantly during the transient hyperhomocysteinemia induced by methionine loading. There is a concurrent complex effect on the protein binding of other thiol components such as cysteine and cysteinylglycine in plasma (*8*).

The homocysteine response after methionine loading is preceded by the enzymic formation of *S*-adenosylmethionine, followed by the formation of *S*-adenosylhomocysteine, which is finally hydrolyzed to homocysteine (*1*). Accordingly, the homocysteine response is a slow process, reaching a maximum in 2–6 h (*8*), which therefore does not allow evaluation of the kinetics of interaction between homocysteine and other thiol components in vivo. Furthermore, the amount of reduced homocysteine is only slightly increased (*8*), which makes estimates on the relation between thiols in plasma difficult. Finally, high plasma concentrations of

methionine may add a confounding factor because this may enhance the production of cysteine and probably cysteinylglycine in the liver (*14*). Conceivably, administered homocysteine may also serve as a cysteine precursor, but this metabolic conversion must be preceded by distribution of homocysteine into liver and other tissues, and is probably a late event relative to the peak plasma homocysteine concentration.

We observed that freshly prepared reduced homocysteine in solution is rapidly absorbed, causes a transient increase in the various forms of homocysteine, and affects other thiol components in human plasma (Figures 1–5). This experimental design allows the conclusion that the homocysteine increase is the primary event and the alterations in other thiol components are secondary phenomena. In addition, the increase in reduced homocysteine was sufficiently high to allow assessment of kinetics and the relation to other plasma thiol components.

The transient increase in reduced homocysteine is closely followed by an increase in reduced cysteine, and the kinetics of these two processes are similar. There is a concurrent small increase in reduced cysteinylglycine (Figure 1). This suggests that reduced homocysteine undergoes a disulfide interchange reaction with cysteine-mixed and symmetric disulfides. The observation that a positive correlation exists between the reduced/total ratios for homocysteine and cysteine and cysteinylglycine, and for cysteine vs cysteinylglycine (Figure 2), suggests that there is an equilibrium state between these species, and that such an equilibrium is reached within 15 min in vivo.

The time course for the increase of the reduced forms after intravenous administration shows that homocysteine was greatest immediately after infusion (55 s), whereas both cysteine and cysteinylglycine lagged behind and peaked after about 4 min (Figure 3). This observation also demonstrates that the increase of homocysteine is the primary event and suggests that the time required to reach equilibrium is about 3 min—although this time may be underestimated because of the progressive decrease in homocysteine.

The finding discussed above shows that the reduction potential of homocysteine is sufficiently high to reduce other biological thiol components at physiological pH, as demonstrated in the present work for cysteine and cysteinylglycine, and previously in subjects receiving a methionine loading (*8*) and in homocystinurics (*9*). Conceivably, alteration of the redox status of other biological thiol components in addition to cysteine and cysteinylglycine during hyperhomocysteinemia may occur, and should be considered potentially important for the tissue damage caused by a high concentration of homocysteine.

The oral homocysteine intake caused a marked increase in protein-bound homocysteine that outlasted the transient increase in reduced homocysteine (Figures 1 and 5). There was a concurrent decline in protein-bound cysteine and to some extent cysteinylglycine (Figure 5). Protein-bound cysteine and cysteinylglycine showed a similar response after methionine loading, but a difference was noted. In most subjects given methionine, we observed a temporary increase in protein-bound cysteine after the initial decline (*8*). Because no transient increase after homocysteine intake was observed, this part of the response may be due to the marked increase in plasma methionine, which may rapidly increase the production of cysteine (*14*).

Comparison of the curves for protein-bound homocysteine, cysteine, and cysteinylglycine shows that the increase in homocysteine mirrored the decline in cysteine (Figure 5). This suggests that homocysteine displaces cysteine and, to a lesser degree, cysteinylglycine from binding sites. However, a simple stoichiometric displacement cannot fully explain the alterations in protein binding because the decrease in protein-bound cysteine and cysteinylglycine exceeded the increase in homocysteine (Figure 5). Results from experimental (*15, 16*) and clinical studies (*17*) suggest complex binding of aminothiols to plasma proteins. The population of binding sites for homocysteine is heterogeneous, and one class of saturable sites seems to preferentially interact with homocysteine (*15–17*).

In conclusion, the increase of plasma homocysteine alters the redox status and protein binding of other thiol components in plasma, and probably also affects thiol–disulfide interchange reactions involving structural proteins and enzymes essential for normal cellular function. This may play a role in the tissue damage (including atherogenesis) induced by high homocysteine concentrations. Conceivably, efficient trapping of high concentrations of homocysteine in plasma through interaction with plasma protein(s) may represent a protective process that counteracts such damage.

We thank S. E. Volset and Jan Aarseth for performing the statistical analysis, and E. Blomdal for technical assistance. This work was supported by grants from the Norwegian Cancer Society and the Norwegian Council on Cardiovascular Diseases. M.A.M. is a research fellow of the latter organization.

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