An elevated concentration of plasma total homocysteine is an independent risk factor for cardiovascular disease. Greater than 80% of circulating homocysteine is covalently bound to plasma protein by disulfide bonds. It is known that albumin combines with cysteine in circulation to form albumin-Cys\(^{34}\)-S-S-Cys. Studies are now presented to show that the formation of albumin-bound homocysteine proceeds through the generation of an albumin thiolate anion. Incubation of human plasma with L-\(^{35}\)S-homocysteine results in the association of >90% of the protein-bound \(^{35}\)S-homocysteine with albumin as shown by nonreduced SDS-polyacrylamide gel electrophoresis. Treatment of the complex with \(\beta\)-mercaptoethanol results in near quantitative release of the bound \(^{35}\)S-homocysteine, demonstrating that the binding of homocysteine to albumin is through a disulfide bond. Furthermore, using an \textit{in vitro} model system to study the mechanisms of this disulfide bond formation, we show that homocysteine binds to albumin in two steps. In the first step, the albumin thiolate anion rapidly displaces cysteine from albumin-Cys\(^{34}\)-S-S-Cys, forming albumin-Cys\(^{34}\) thiolate anion and homocysteine-cysteine mixed disulfide. In the second step, an albumin thiolate anion attacks homocysteine-cysteine mixed disulfide to yield primarily albumin-Cys\(^{34}\)-S-Hcy and to a much lesser extent albumin-Cys\(^{34}\)-S-S-Cys. The results clearly suggest that when reduced homocysteine enters circulation, it attacks albumin-Cys\(^{34}\)-S-S-Cys to form albumin-Cys\(^{34}\) thiolate anion, which in turn, reacts with homocysteine-cysteine mixed disulfide or homocysteine to form albumin-bound homocysteine.

Homocysteine is a sulfur-containing amino acid formed during methionine metabolism (1). It is catabolized to cysteine through the transulfuration pathway, or it may be remethylated back to methionine (2). An elevated level of plasma total homocysteine (tHcy)\(^{1}\) is a strong independent risk factor for cardiovascular disease (3, 4) and an emerging risk factor for Alzheimer's disease (5, 6). tHcy is the sum of free homocysteine and protein-bound homocysteine. Free homocysteine is made up of reduced homocysteine (\(\text{SH}(-)\) (<1% of tHcy), and low molecular weight oxidized disulfide (\(-\text{S-S}\-) forms including homocysteine (5–10% of tHcy) and homocysteine-cysteine mixed disulfide (5–10% of tHcy). Greater than 80% of tHcy in circulation is bound to protein by disulfide bonds (7–9). A small amount of homocysteine may also be bound to plasma proteins via amide linkage as a result of homocysteine thiolactone reacting with the e-amino group of protein lysine residues (10).

The upper limit of normal tHcy is \(\leq 0.012 \text{ mM} (11, 12)\). However, in patients with homocystinuria, tHcy levels approach 0.5 mM (13). The overall \textit{in vitro} binding capacity of human plasma proteins for homocysteine is >0.4 mM (14). Almost all pathophysiology studies utilize free reduced homocysteine (reviewed in Ref. 15), whereas little or no attention has been paid to protein-bound homocysteine, despite the fact that it is the most abundant form of circulating homocysteine both in normal and hyperhomocysteinemic subjects.

Albumin is the most abundant protein in plasma. Typical plasma concentrations range from 0.6 to 0.75 mM, and albumin makes up more than 50% of the total plasma protein (16). It is a nonglycosylated, single-chain polypeptide tightly folded into three domains that are structurally defined by 17 intrachain disulfide bonds formed between 34 cysteine residues. Albumin contains one additional cysteine residue at Cys\(^{34}\) that does not participate in intrachain disulfide bonding. Albumin Cys\(^{34}\) accounts for the bulk of free thiol (\(\text{SH}\)) in plasma (17). The crystal structure of human serum albumin shows that Cys\(^{34}\) is situated in a partially protected site in a seven residue turn between helices h2 and h3 of subdomain 1A (18) and sits in a crevice 9.5–10 Å deep (17).

The p\(K_{a}\) of the thiol group of Cys\(^{34}\) is abnormally low (\(-5\) (19). This is in contrast to the p\(K_{a}\) of most of the low molecular weight aminothiols present in plasma. Thus, at physiological pH, albumin-Cys\(^{34}\) exists primarily as thiol anion and is highly reactive with metals, thiols, and disulfides (20). In fact, about one-third of the albumin molecules in the plasma carry disulfide-bonded thiolis at this Cys\(^{34}\) residue (20). These ligands probably become disulfide bonded in the plasma, because the albumin that is formed and secreted from the liver is in the free thiol form (17). Certain drugs containing thiol groups also bind to Cys\(^{34}\) of albumin (19, 21, 22). Thus, Cys\(^{34}\) of albumin seems

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\(^{1}\) The abbreviations used are: tHcy, plasma total homocysteine; TES, 2-(\(\beta\)-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminoethanesulfonic acid; DTPA, diethylenetriaminepentaaetic acid; HPLC, high performance liquid chromatography; FD, fluorescence detection.
to be the most probable binding site for low molecular weight thiols including homocysteine. In an earlier study where plasma proteins were resolved by gel filtration chromatography, it appeared that homocysteine was associated with albumin; however, the mechanism of homocysteinylilation was not addressed (23). In this study we show that albumin is homocysteinylated when its thiolate anion attacks homocysteine-cysteine mixed disulfide or homocysteine.

**EXPERIMENTAL PROCEDURES**

Reagents—L-Homocysteine, t-homocysteine thiolactone, TES, Trizma® base, sodium borohydride, diethylenetriaminepentacetic acid (DTPA), 5,5'-dithiobis(2-nitrobenzoic acid), and human serum albumin were purchased from Sigma. Monobromobimane was obtained from Molecular Probes (Eugene, OR). Perchloric acid, HPLC grade acetonitrile, and HPLC grade methanol were from Fisher. All other chemicals used in this study were of reagent grade.

**Human Serum Albumin—**Crystalline human serum albumin (Sigma; item number A-1653 and lot number 88H7610) was used in these studies. We determined that this albumin preparation contained 0.23 mol $^{-2}$SH/mg protein, 0.33 mol S-$\text{-S}$-homocysteine/mg protein and 0.015 mol S-$\text{-S}$-homocysteine/cysteine protein. This human serum albumin had 1.5 mol fatty acids/mol albumin. The metal content of this albumin was also determined using inductively coupled plasma mass spectrometry (24). The samples were digested with nitric acid in polytetrafluoroethylene test tubes with $^{22}$Ga as an internal standard. The albumin was found to contain 3.62 ppm of copper, 191.5 ppm of calcium, 12.96 ppm of iron, 0.015 ppm of cobalt, and 0.35 ppm of nickel. Albumin is also known to carry other thiols (e.g. glutathione and cysteinylglycine) along with other metabolites (e.g. nitric oxide) on Cys86; however, the concentrations of these compounds were not determined in this study.

**Preparation of L-$^{35}$S-Homocysteine—**L-$^{35}$S-Homocysteine was prepared from L-$^{35}$S-methionine as described by Mudd et al. (25) with slight modifications. Briefly, 0.02 mmol of l-methionine was mixed with 1 mmol of l-$^{35}$S-methionine (1 mCi) and refluxed with 5 ml of hydrochloric acid for 24 h. The solution was then dialyzed against argon atmosphere. The reaction mixture was then adjusted to pH 7 with 10 min on ice, and centrifuged for 10 min at 12,000 rpm. The protein pellet was washed with 0.10 ml of 0.1 M Tris buffer (pH 8.5) and the concentrations of albumin-bound thiols were estimated by HPLC, as described below. The perchloric acid solubile fraction was immediately stored at $-20 \degree$C. The amount of total free thiol in this fraction was determined using the method of Ellman (27).

**Quantification of Homocysteine and Homocysteine-Cysteine Mixed Disulfide—**To specifically determine the amount of homocysteine and homocysteine-cysteine mixed disulfide formed during the reaction of human serum albumin with homocysteine, 0.75 mM human serum albumin was incubated with 0.5 mM l-$^{35}$S-homocysteine. After 3 h of the reaction, aliquots were withdrawn from the reaction mixture, and albumin was precipitated by adding 1.5 $\text{m}$ perchloric acid. The supernatant was subjected to descending paper chromatography using the same conditions as mentioned above. The standards used were homocysteine and homocysteine-cysteine mixed disulfide. The areas corresponding to the individual disulfides were cut from the paper and eluted with water, and their radioactivity was determined by counting in a liquid scintillation counter.

**HPLC Determination of Thiols—**Albumin-bound homocysteine and albumin-bound cysteine were determined by HPLC with fluorescence detection as described by Jacobsen et al. (31). Briefly, 0.1 ml of the solubilized albumin pellet (obtained after precipitating the reaction mixture with perchloric acid as mentioned above) was treated with 0.35 mM sodium borohydride in 0.10 M sodium hydroxide followed immediately by the addition of 0.035 ml of 1.0 $\text{m}$ HCl. After addition of 0.05 ml of 7 mM monobromobimane in 4 mM sodium EDTA (pH 7.0), the solution was incubated at 42 $\degree$C for 12 min. Albumin was precipitated by the addition of 0.05 ml of 1.5 $\text{m}$ perchloric acid. After centrifugation (12,000 rpm, 10 min), the supernatant was adjusted to pH 4 by the addition of 0.025 ml of 2.0 $\text{m}$ Tris-HCl base. The samples (0.10 ml) were then transferred to injector vials for automated HPLC analysis. Standard curves were generated with known amounts of cysteine and homocysteine to calculate the concentrations of the two thiols in the reaction mixture. Albumin concentration was determined by the bicinchoninic acid method (32).

**RESULTS**

**Identification of Albumin as a Binding Protein for Homocysteine in Human Plasma—**We recently determined the equilibrium binding capacity of plasma proteins for homocysteine but did not identify the specific proteins responsible for the binding (14). To identify specific homocysteine-binding proteins, human plasma was incubated with $^{35}$S-l-homocysteine. Albumin
was found to be the predominant (>90%) homocysteine-binding protein in plasma (Fig. 1). Treatment with β-mercaptoethanol resulted in the near quantitative removal of the bound homocysteine, indicating that the binding of homocysteine to albumin was through a disulfide linkage.

Binding of Homocysteine to Human Serum Albumin—Binding of homocysteine to human serum albumin was studied as a function of both time and concentration. Time course studies (Fig. 2A) revealed that the binding of homocysteine to albumin increases with time. With concentrations of homocysteine greater than 0.1 mM, an equilibrium of homocysteine binding is reached in about 10 h. The formation of albumin-bound homocysteine also increases with increasing homocysteine concentrations (0.025–1 mM), and saturation of binding is achieved between 0.5 and 1 mM homocysteine. Concomitantly, the concentration of free reduced thiol in the medium decreased, and no free reduced thiol could be detected after 3 h of reaction (Fig. 2B), when 1 mM homocysteine was used.

As mentioned under "Experimental Procedures", the albumin used in these studies contained 0.33 mol cysteine/mol albumin. Interestingly, upon the addition of homocysteine, this cysteine was rapidly displaced from albumin. The amount of cysteine displaced from albumin-Cys-S-Cys began to increase slowly until it reached in about 1 h of reaction and then decreased (Fig. 4B) as the concentration of albumin-Cys34-S-S-Hcy per mol albumin. To account for this difference, we hypothesized that albumin thiolate anion was being formed during the reaction. To test this, we measured the formation of albumin thiolate anion in the reaction mixture using the method of Ellman (Fig. 4B). We found that the formation of albumin thiolate anion followed a bell-shaped curve. It steadily increased from an initial concentration of 0.23 mol/mol albumin to 0.43 mol/mol albumin during the first hour of reaction and then decreased (Fig. 4B) as the concentration of albumin-Cys34-S-S-Hcy increased.

The homocysteine in the reaction and the cysteine released from albumin undergo oxidation to homocystine, cystine, and homocystine-cysteine mixed disulfide and after 3 h of reaction, free reduced thiol could not be detected in the system (Fig. 2B). To study the fate of homocysteine in the reaction mixture, we reacted 0.75 mM albumin with 0.5 mM L35S-homocysteine and found that after 3 h of reaction, 0.14 mm homocystine and 0.095 mm homocystine-cysteine mixed disulfide were formed in addition to the formation of 0.21 mol albumin-Cys34-S-S-Hcy per mol albumin (Fig. 4A). Because the formation of albumin-
bound thiol continues to increase even in the absence of detectable free reduced thiol (Figs. 2 and 3), we hypothesized that the oxidized forms of homocysteine and cysteine were participating in the overall reaction. Therefore, we decided to test the reaction of albumin with homocysteine. We incubated 0.75 mM albumin with 0.25 mM l-homocysteine (equivalent to 0.5 mM homocysteine) and found that albumin did indeed react with homocysteine (Fig. 5A), but the reaction rate was much slower compared to that with homocysteine. Moreover, homocysteine did not displace cysteine from albumin-Cys$^{34}$-S-Cys (data not shown). Thus, the reaction of homocysteine with albumin alone could not explain the data shown above. Another possibility was that the albumin thiolate anion formed during the initial stage of the reaction could react with the oxidized forms of the thiol of the thiol that were concurrently being produced during the course of the reaction.

To test this hypothesis, albumin thiolate anion was prepared by treating albumin with dithiothreitol (5 mol/mol albumin). Albumin thiolate anion was then reacted either with homocysteine or homocysteine. With 0.5 mM homocysteine, only 0.02 mol albumin-Cys$^{34}$-S-S-Hcy was formed/mol albumin after 1 h of reaction. However, when 0.25 mM homocysteine was used, 0.12 mol of albumin-Cys$^{34}$-S-S-Hcy was formed/mol albumin (Fig. 5B). The rate constant for the reaction with homocysteine was 0.175 s$^{-1}$. This suggests that initially homocysteine reacts with albumin forming albumin thiolate anion, which then reacts with the oxidized form of the thiol to yield the final products.

To investigate whether trace metals associated with albumin might play a role in homocysteinilation, we treated albumin with the metal chelator DTPA. DTPA treatment and dialysis removed >97% of the metals from the albumin preparation as determined by inductively coupled plasma mass spectrometry. In the absence of trace metals, formation of homocysteine or cysteine by autooxidation was inhibited. As shown in Fig. 6, the initial rate of the reaction was almost identical when native and DTPA-treated albumin was used. However, with DTPA-treated albumin, the formation of albumin-Cys$^{34}$-S-S-Hcy slowed considerably after the initial 30 min of reaction. This was in contrast to the reaction with native albumin, where the levels of albumin-Cys$^{34}$-S-S-Hcy continuously increased up to 10 h. This suggests that, in the absence of homocysteine, which is normally formed as a result of trace metal-catalyzed autooxidation of homocysteine, albumin thiolate anion lacks a suitable target for nucleophilic attack.

**Reaction of Albumin Thiolate Anion with Cystine, Homocysteine, and Homocysteine-Cysteine Mixed Disulfide**—When albumin enters circulation it is likely that most, if not all of the Cys$^{34}$ is in the thiolate anion form (17). Once entering the circulation, albumin thiolate anion can react with either cysteine, homocysteine, or homocysteine-cysteine mixed disulfide. To mimic this situation in vitro, 0.25 mM albumin thiolate anion was reacted with 0.125 mM cysteine or 0.125 mM homocysteine. During the reaction of albumin thiolate anion with cysteine, 0.69 mol of albumin-Cys$^{34}$-S-S-Cys/mol of albumin was formed after 24 h (Fig. 7A). In the same time period 0.43 mol of albumin-Cys$^{34}$-S-S-Hcy/mol albumin was obtained when homocysteine was used (Fig. 7B). However, when both homocysteine and cysteine were present in equal concentrations (0.0625 mM) in the same mixture, albumin thiolate anion (0.25 mM) preferentially attacked homocysteine to form albumin-bound homocysteine (Fig. 7C). In fact, after 24 h of reaction, formation of albumin-bound homocysteine (0.5 mol/mol albumin) exceeded the formation of albumin-bound cysteine (0.19 mol/mol albumin) by about 2.6-fold (Fig. 7C). In homocysteinurics, the concentration of oxidized homocysteine is reported to be slightly greater than oxidized cysteine (13). Thus, albumin thiolate anion reacts efficiently with cysteine alone. However, when both homocysteine and cysteine are present in the reaction mixture, formation of albumin-bound homocysteine predominates. Similarly, when albumin thiolate anion was incubated with homocysteine-cysteine mixed disulfide, 0.518 mol of albumin-Cys$^{34}$-S-S-Hcy/mol albumin was formed after 24 h of reaction, whereas during the same time only 0.175 mol of albumin-Cys$^{34}$-S-S-Cys/mol of albumin was obtained (Fig. 7D). It is obvious that albumin thiolate anion preferentially attacks the sulfur atom of homocysteine in homocysteine-cysteine mixed disulfide.

**DISCUSSION**

Our studies using $^{35}$S-l-homocysteine clearly indicate that homocysteine binds to albumin through a disulfide bond in human plasma. The binding is saturable, and maximal binding is found at 0.5 mM homocysteine. The formation of albumin-
Cys\(^{34}\)-S-S-Hcy appears to be biphasic. Within the first 3 min there is a rapid formation of albumin-Cys\(^{34}\)-S-S-Hcy, followed by a short plateau (Fig. 4A, inset) that is then followed by the slow second phase of albumin-Cys\(^{34}\)-S-S-Hcy formation. After 10 h, the reaction reaches equilibrium (Fig. 4A). Jakubowski (10) also found that albumin was homocysteinylated by homocysteine. In his study 35S-L-homocysteine thiolactone was used, but a portion of the thiolactone undergoes hydrolysis to 35S-L-homocysteine, which in turn can form albumin-bound homocysteine.

Mechanistically, homocysteine first displaces cysteine from albumin-Cys\(^{34}\)-S-S-Cys, but this displacement can occur by two different pathways (Reactions 1 and 2). The homocysteine sulfhydryl group can react with the sulfur of albumin-bound cysteine, forming homocysteine-cysteine mixed disulfide and free albumin thiolate anion (Reaction 1), and/or it can react with the sulfur of albumin Cys\(^{34}\), forming albumin-bound homocysteine and free cysteine thiolate anion (Reaction 2).

Based on our experimental results, we propose that Reaction 1 predominates under the experimental conditions. The amount of homocysteine-cysteine mixed disulfide formed is also consistent with this observation.

**Fig. 4.** Homocysteinylation of albumin. 0.5 mM l-homocysteine was incubated with 0.75 mM albumin for the indicated time. Aliquots were withdrawn, perchloric acid was added, and the precipitated albumin was solubilized in Tris-HCl (0.5 M, pH 8.5). A, albumin-bound homocysteine was measured by HPLC-FD of the solubilized precipitate. B, albumin thiolate anion was determined by treating the solubilized precipitate with Ellman's reagent. The data represent the means ± S.D. (n = 3).

**Fig. 5.** Reaction of albumin with homocysteine and homocysteine. Either 0.5 mM l-homocysteine or 0.25 mM l-homocysteine were incubated with 0.75 mM albumin (A) or 0.75 mM albumin thiolate anion (B) in TES buffer (0.05 M, pH 7.4) at 37°C. At the indicated time points (1 h for B) aliquots were withdrawn, albumin was precipitated by adding perchloric acid, and the concentration of albumin-bound homocysteine was determined by HPLC-FD. The data represent the means ± S.D. (n = 3).
thiolate anion will be much more thermodynamically stable than cysteine thiolate anion, favoring Reaction 1. Moreover, as proposed by Christadoulou et al. (34, 35), albumin thiolate anion is further stabilized by forming a salt bridge with His39.

These results are in agreement with the in vivo observations of Mansoor et al. (36), who found that after intravenous administration of homocysteine into healthy individuals, there was a decrease in protein-bound cysteine. They also observed that the decrease in protein-bound cysteine exceeded the increase in protein-bound homocysteine. Our proposed mechanism explains these observations. In the initial phase of the reaction, homocysteine preferentially attacks the cysteine sulfur of albumin-Cys34-S-S-Cys, generating albumin thiolate anion and homocysteine-cysteine mixed disulfide (Reaction 1).

In the second phase of the reaction, albumin thiolate anion attacks homocysteine-cysteine mixed disulfide preferentially on the homocysteine sulfur (Reaction 3) to form albumin-bound homocysteine and cysteine thiolate anion.

\[
\text{Alb-Cys}_{34}^\text{S} + \text{Hcy-S-S-Cys} \rightarrow \text{Alb-Cys}_{34}^\text{S-S-Hcy} + \text{Cys}^\text{S}\]

Reaction 3

Albumin thiolate anion can also attack the cysteine sulfur of homocysteine-cysteine mixed disulfide to form albumin-bound cysteine and homocysteine thiolate anion (Reaction 4).

\[
\text{Alb-Cys}_{34}^\text{S} + \text{Hcy-S-S-Cys} \rightarrow \text{Alb-Cys}_{34}^\text{S-S-Cys} + \text{Hcy}^\text{S}\]

Reaction 4

However, because the pK_a of free homocysteine (8.7) is higher than that of free cysteine (8.15) (33), cysteine thiolate anion

Benesh and Benesh report thiol pK_a values of 10.0 and 8.53 for homocysteine and cysteine, respectively (38).

**FIG. 6.** Effect of DTPA on homocysteinylation of albumin. 0.5 mM l-homocysteine was incubated with 0.75 mM albumin thiolate anion with or without 5 mM DTPA in TES buffer (0.05 mM, pH 7.4) at 37 °C. At the indicated time points, aliquots were withdrawn, albumin was precipitated by adding perchloric acid, and the concentration of albumin-bound homocysteine was determined by HPLC-FD. The data represent the means ± S.D. (n = 3).

**FIG. 7.** Reaction of albumin thiolate anion with cystine, homocystine, and homocysteine-cysteine mixed disulfide. 0.25 mM albumin thiolate anion was reacted at 37 °C with 0.125 mM cystine (A), 0.125 mM homocystine (B), a mixture of 0.0625 mM cystine and 0.0625 mM homocystine (C), and 0.25 mM homocysteine-cysteine mixed disulfide (D). At the indicated time points, aliquots were withdrawn, albumin was precipitated with perchloric acid, and the concentration of albumin-bound thiol was determined by HPLC-FD. The data represent the means ± S.D. (n = 3).

**FIG. 8.** Buried and exposed conformations of albumin-Cys34. Cys34 exists in buried and exposed conformations as proposed by Christodoulou et al. (35). When albumin-Cys34 thiolate anion (exposed) attacks the mixed disulfide, the homocysteinylated product is stabilized in the exposed conformation. This figure is modified from Christadoulou et al. (35).
Mechanism of Albumin-S–S-Homocysteine Formation

(formed in Reaction 3) will be thermodynamically more stable at pH 7.4 than homocysteine thiolate anion (formed in Reaction 4). Thus, as shown in Fig. 7D, the formation of albumin-bound homocysteine (Reaction 3) is preferred over formation of albumin-bound cysteine (Reaction 4). This was also confirmed by the fact that the amount of albumin-bound cysteine increased by only 0.05 mol/mol albumin after 24 h of reaction (Fig. 3A). Moreover, it has been reported that when a thiolate anion attacks an unsymmetrical disulfide, the thiol that leaves will be the one having the lowest $pK_a$ (37). Thus, we conclude that when treated with mixed disulfide, albumin thiolate anion preferentially attacks the sulfur of homocysteine in the mixed disulfide.

In addition to reacting with homocysteine-cysteine mixed disulfide, albumin thiolate anion also reacts with homocystine and cysteine. Reaction with homocystine results in the formation of albumin-bound homocysteine (Reaction 5), while reaction with cysteine will lead to the formation of albumin-bound cysteine (Reaction 6).

\[
\text{Alb} - \text{Cys}_34\text{S}^- + \text{Hcy} \rightarrow \text{Alb}-\text{Cys}_34\text{S}^-\text{Hcy} + \text{Hcy}^- \\
\text{REACTION 5}
\]

\[
\text{Alb} - \text{Cys}_34\text{S}^- + \text{Cys}^- \rightarrow \text{Alb}-\text{Cys}_34\text{S}^-\text{Cys} + \text{Cys}^- \\
\text{REACTION 6}
\]

Cys$^{34}$ is situated in a pocket consisting of 4 amino acids and is protected from solvent in a crevice. Our results fit the model proposed by Christodoulou et al. (34, 35), where the Cys$^{34}$ exists in exposed and buried forms (Fig. 8). The thiolate anion form of Cys$^{34}$ is primarily in the buried form and is in close proximity to His$_{39}$ with which it can form a salt bridge for stabilization. Disulfide bond formation of Cys$^{34}$ with homocystine results in the formation of Cys$^{34}$ thiolate anion also reacts with homocystine-cysteine mixed disulfide.

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