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978-0-521-65319-0 - Homocysteine in Health and Disease
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Historical Overview and Recent Perspectives

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This book provides a comprehensive account of current knowledge about the place of the sulfur-containing amino acid, homocysteine, in health and disease. Markedly elevated levels of homocysteine in human plasma and urine were first described in certain rare inborn errors of metabolism. More recently, mild elevations have been found to be associated with coronary, cerebral, and peripheral vascular disease, renal disease, dementia, neural tube defects, and other disorders. As an introduction, it may be appropriate to review briefly how knowledge about this amino acid has evolved.

Homocysteine Metabolism

Homocysteine occupies a pivotal position in the metabolism of the essential amino acid, methionine. It is at the junction point of the transsulfuration pathway and the formation of cysteine and excretion of sulfur on the one hand and the remethylation of homocysteine to methionine with conservation of the carbon skeleton on the other (22). These findings followed the identification in 1932 by Butz and du Vigneaud of homocysteine as a biologically important amino acid (5). With a clearer picture of methionine metabolism and the various cofactors involved came the realization that an elevation of homocysteine could reflect deficiencies of the various vitamins of functional relevance in the pathway — vitamin B_6, cobalamin, and folic acid. It later became evident that renal function was an important contributor to homocysteine homeostasis. The discovery of the inborn error of metabolism, homocystinuria, initiated the upsurge of interest in homocysteine because of the precocious vascular disease, which was one of the major clinical features associated with the disorder.

Discovery of the Homocystinurias and Development of the Homocysteine Theory of Vascular Disease

The inborn errors leading to homocystinuria involve one of two pathways, transsulfuration or remethylation. In the transsulfuration pathway, there is a deficiency of cystathionine β-synthase, the enzyme mediating the transformation of homocysteine to cystathionine. Defects in remethylation result from inborn errors of metabolism or transport of cobalamin or folic acid, which affect the remethylation of homocysteine to methionine (Metabolic Diagram, Reaction 4). These are rare disorders, but the least rare is cystathionine β-synthase deficiency, cases of which were first reported in 1962. Two of the earliest described patients are shown in Figure 1.1 (7). Additional cases were quickly identified, and by 1965 the principal clinical features had been described (24). The first studies identified high concentrations of homocystine, the oxidized form of homocysteine, in the urine of some children with mental retardation. These children also had high circulating concentrations of homocysteine, measured as the oxidized compound, homocystine, and the mixed disulfide, cysteine-homocysteine. Methionine was also increased. In 1964 Mudd and colleagues identified the enzyme defect and established that this form of homocystinuria was due to a deficiency of cystathionine β-synthase (22). This provided the explanation for the elevations of both homocysteine and methionine found in this disorder.

Four major clinical hallmarks were frequently present in the disorder: dislocation of the ocular lens, a marfanoid habitus and other skeletal abnormalities, a degree of mental retardation, and most important, thromboembolic disease, which was the usual cause of premature death (22). Severe cases may have all of these features, but mild cases may have few or none. In 1969, Mudd and colleagues described a case of a different kind in which homocystinuria was combined with methylmalonic aciduria, but plasma methionine was low (23). This first case of a remethylating disorder was in an infant who died at 7 weeks of age. This case prompted the postulation by McCully that the vascular complications were a consequence of the ele-

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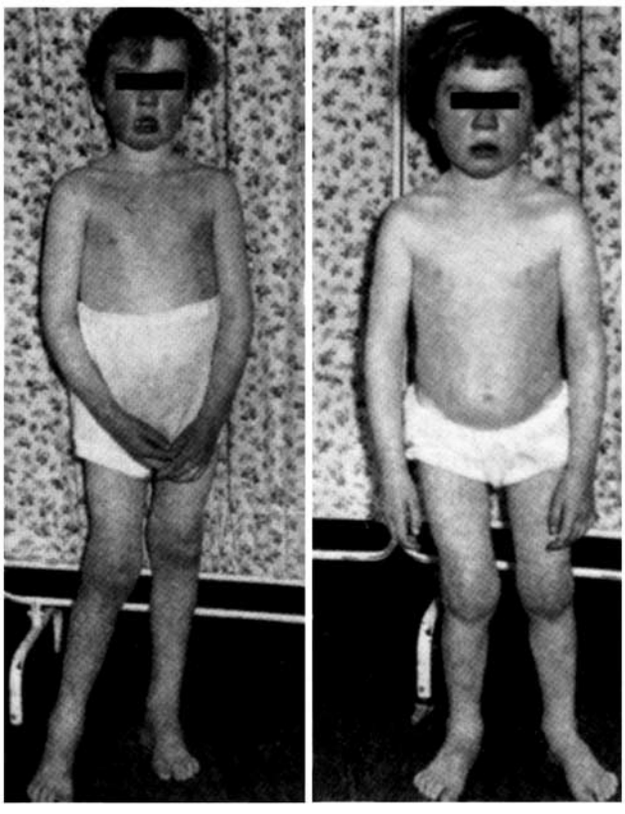

Fig. 1.1. The two homocystinuric sisters identified by Carson et al. (7) in the early 1960s (6-year-old sister on the left and 4-year-old on the right). The abnormalities included mental retardation, seizures, and dislocation of ocular lenses. Visible features include mottled skin and "knock knees." (Reproduced from Carson et al. [7], with permission.)

vated homocysteine rather than the result of any of the other complex metabolic changes occurring in cystathionine β-synthase deficiency (19).

At autopsy on the infant, McCully documented the presence of widespread vascular lesions. It was known at this time that cystathionine β-synthase-deficient patients also had precocious vascular disease but that their markedly elevated circulating levels of homocysteine were associated with increased circulating methionine as would be predicted with diminished cystathionine β-synthase activity. McCully noted that in two discrete disorders of metabolism associated with vascular disease, elevated homocysteine was common to both. He suggested that the elevated homocysteine was responsible for the vascular changes (19).

To establish that the increased homocysteine was responsible for the vascular changes, it was necessary to show that lowering the elevated levels would reduce the associated cardiovascular risk. It was soon established that in a proportion of patients with cystathionine β-synthase deficiency, the elevated homocysteine levels could be markedly reduced with large doses of pyridoxine (vitamin B_6) (24). In 1985, a landmark review by Mudd and colleagues of more than 600 patients showed that about half the patients were responsive to treatment with pharmacological doses of pyridoxine and were subsequently referred to as pyridoxine-responsive cystathionine β-synthase-deficient patients. The remaining patients had little or no response to pyridoxine (25). Although there is some overlap between these two groups, in general this distinction remains true and reflects, in part, the presence or absence of some residual cystathionine β-synthase activity (24). In this major review, Mudd and colleagues showed that after the age of about 10 years, the risk of a vascular event progressively increased. By the age of 30 years, approximately 50% of untreated cystathionine β-synthase-deficient patients had had either a cerebral thrombosis, a venous thrombosis, or a myocardial infarction. Mudd and colleagues were able to establish that this risk was reduced in treated pyridoxine-responsive patients (25).

Pyridoxine-responsive patients also require folic acid (37) and cobalamin (43) to maintain maximal lowering of homocysteine levels. A low methionine diet plus methionine-free amino acid supplementation is effective treatment for pyridoxine nonresponsive patients, but is usually tolerated only if introduced in infancy (24). However, homocysteine levels in patients diagnosed later can be lowered by the addition of betaine to the other therapies (42). Betaine enhances the remethylation of homocysteine to methionine by an alternative pathway (Metabolic Diagram, Reaction 5). By using this combined therapy, we were able to establish that treatment in patients not responsive to pyridoxine also reduced cardiovascular risk strikingly, even though this regimen frequently increased circulating methionine to quite high levels (43). The most recent analysis of the effect of long-term treatment in altering the natural history of vascular events in 84 cystathionine β-synthase-deficient patients attending centers in Australia ($n = 32$), the Netherlands ($n = 28$), and Ireland ($n = 24$) involved a total of 1,314 patient years of treatment. This shows quite unequivocally that treatment to lower the high homocysteine levels in these patients dramatically reduced cardiovascular risk (46). Nevertheless, in all these studies, total homocysteine levels achieved with treatment remained much higher than those seen in what is now known as "mild hyperhomocysteinemia."

Mild Homocysteine Elevation (Hyperhomocysteinemia) and Cardiovascular Risk

The accepted risk factors — elevated lipids, smoking, hypertension, diabetes, gender, and age — do not explain the occurrence and severity of a sizable propor-

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tion of cases of vascular disease, particularly coronary disease, in the general population. The recognition that untreated young homocystinuric patients develop aggressive vascular disease, resulting in a high mortality rate, led us to consider the possibility that even a very modest elevation of homocysteine could itself be a risk factor; or that it could amplify the effects of other risk factors to produce early onset vascular disease.

To explore this possibility we studied a group of patients with early onset (< 50 years) coronary disease established by coronary angiography and measured cysteine-homocysteine as a reflection of circulating homocysteine before and after the challenge of a methionine load (100 mg/kg) (44). The findings were compared with those in a normal age-matched control group known to be free of coronary artery disease. The approach of a methionine challenge had already been used by Sardharwalla and colleagues for the detection of heterozygotes for homocystinuria resulting from cystathionine β-synthase deficiency (29). We found significant increases in cysteine-homocysteine 4 hours after the load in coronary patients compared with the control group, results consistent with a reduced ability to metabolize homocysteine in some patients with premature coronary artery disease when this pathway is stressed. In another study, total free homocysteine measurements showed that 2 of 20 patients with premature coronary artery disease (identical twins) had elevated fasting and postmethionine load homocysteine levels and that these were normalized by oral folic acid (41).

These early findings have been confirmed in numerous subsequent studies, and it is now firmly established that 20% to 30% of patients with coronary, cerebrovascular, and peripheral vascular disease have mild homocysteine elevation, either in the fasting state or after a methionine load (28). Most heterozygotes for cystathionine β-synthase deficiency had been shown to exhibit abnormalities of methionine metabolism (29, 41). It had been recognized that heterozygotes could occur with a frequency of up to 1% in the general population (24); therefore, it had been postulated that patients with vascular disease who had mild hyperhomocysteinemia might include considerable numbers of cystathionine β-synthase heterozygotes. However, this was investigated further by Mudd and colleagues, who reviewed a large number of obligate heterozygotes and could not find definite evidence for increased risk. They concluded that if there was any increased risk, it would have to be small (24). Recent studies have not found any evidence of mutations in the cystathionine β-synthase gene in patients with vascular disease and mild hyperhomocysteinemia (13).

In 1995, an important meta-analysis of 27 studies in more than 4,000 subjects concluded that homocysteine elevation was an independent risk factor for vascular disease in the coronary, cerebral, and peripheral circulation (3). This finding has been confirmed in most of the prospective studies conducted so far (28).

Notwithstanding the clear-cut association between the modest homocysteine elevation and vascular disease, whether these small increases do of themselves confer vascular risk or are secondary phenomena is still to be determined. Mildly increased levels of plasma homocysteine are strongly associated with some of the standard risk factors, and there is the need to control for these to determine any independent homocysteine contribution. The large population-based Hordaland study showed that elevated plasma homocysteine was also associated positively with male sex, age, smoking, blood pressure, elevated total cholesterol, and lack of exercise (27). This relationship has been further investigated by a multicenter case-control study that showed that elevations of homocysteine interacted with conventional risk factors, but conferred a similar but independent effect on vascular disease (15). Another study by Nygård and colleagues showed that the risk appeared dose-related, even within the normal range, although the numbers were small in some of the outcome groups (26). Among the large number of recently reported studies, an interesting new one showed a significant and positive relationship between homocysteine levels and increased carotid artery wall thickness in a large randomly selected sample from the general population (20).

A finding that could cast doubt on the direct cause-and-effect aspect relates to a mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, a thermolabile variant (14). About 11% to 12% of the white population is homozygous for this variant, which is associated with a modest homocysteine elevation when folate levels are below the population median. A meta-analysis of the results of studies of the distribution of this variant revealed that homozygosity does not confer enhanced cardiovascular risk (4) even though the levels of homocysteine were mildly increased in the 13 studies in which it had been measured. There is a possibility that these surprising results could be explained if the MTHFR mutation conferred protection by another mechanism related to thymidine and DNA synthesis (see Metabolic Diagram). Also arguing somewhat against a direct cause-and-effect relationship of homocysteine in the pathogenesis of vascular disease is the apparently small vascular risk in cystathionine β-synthase-deficient patients during treatment, even when their homocysteine levels, although lowered, are still very high. However, in these studies (43, 46) only 19 of 84 patients were 40 years old or older.

The mechanism for an effect of homocysteine has remained elusive, but recent interesting data showed

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that small increases in circulating homocysteine are associated with reduced endothelial-dependent vasodilatation (32), and that this may also occur transiently when homocysteine levels are increased after a methionine load (8). These effects may result from homocysteine-induced oxidative stress as supported by the finding by Kanani et al., that oral ascorbic acid prevented the endothelial dysfunction associated with a twofold to threefold rise in homocysteine after a standard methionine load (18). Usui et al. suggested that homocysteine-induced oxidative changes affected endothelial nitric oxide-dependent vasodilatation and that there was an antioxidant effect of folic acid (33). They showed that a single large dose of folic acid prevented impairment of nitric oxide-mediated vasodilatation following a methionine load without altering the associated acute increase in homocysteine levels. An antioxidant effect of folate could also explain the increase in endothelial-dependent brachial artery dilatation found in 18 healthy adults after 6 weeks of oral folate, which lowered the plasma homocysteine levels significantly (1). It is also well known that B-vitamin deficiencies occur frequently in the older age group among whom mild homocysteine elevation and vascular disease are prevalent (6, 30).

The effect of lowering homocysteine levels on cardiovascular risk should be resolved within the next few years by the results of the seven major folate (± cobalamin) trials currently under way in patients with vascular disease (9). These trials will show whether folate medication, which lowers homocysteine levels in most people (28), is protective; but they will not address the issue of whether there is a direct effect on vascular disease of mildly elevated homocysteine levels.

Renal Disease

Also relevant to the homocysteine hypothesis is the possible contribution of elevated plasma homocysteine to the greatly enhanced cardiovascular risk seen in patients with chronic renal failure. In chronic renal failure, vascular risk is increased 20-fold (36) for reasons that are by no means clear. The notion that homocysteine elevation may contribute to renal failure came from the knowledge that the kidneys metabolize homocysteine and are responsible for about 70% of plasma homocysteine clearance under physiological conditions. We reasoned that the plasma homocysteine concentration would be increased when clearance is reduced. To explore this possibility, in the late 1970s we measured homocysteine levels in patients with chronic renal failure and transplant recipients with mildly impaired renal function; homocysteine levels were significantly elevated (39, 40). These increased levels were markedly reduced by oral folate, and their decline was positively and linearly related to the extent of renal function impairment (38). These findings have been confirmed in many subsequent studies. The recent demonstration that in end-stage renal failure it is remethylation, rather than transsulfuration, that is impaired is in agreement with the homocysteine-lowering effect of folate but not pyridoxine in this situation (34). It remains a strong possibility that elevated homocysteine levels, which are generally of much greater magnitude than the elevation usually found in patients with vascular disease, contribute to vascular disease in patients with renal insufficiency. Data from prospective studies are consistent with this notion (2, 21).

Neural Tube Defects and Other Adverse Pregnancy Outcomes

The pioneering work of Smithells and colleagues has clearly established that preconceptional folate supplementation prevents more than 50% of all neural tube defects (31). This suggested that modest homocysteine elevation might be a marker for an increased risk of neural tube defect. The possibility that the 11% to 12% of women homozygous for the MTHFR mutation are at increased risk of having children with neural tube defects when their folate intake is below the population median has been explored by van der Put and colleagues (35). Their data show that homozygosity for this common 667C→T mutation confers about a twofold increase in neural tube defect risk in the Dutch population.

Preliminary studies have suggested that other adverse pregnancy events and fetal malformations could be associated with elevated homocysteine levels. These include recurrent early pregnancy loss, abruptio placenta, early-onset preeclampsia (11), and nonsyndromic orofacial clefts (45). The mechanisms in clefting and neural tube defect may well be more related to DNA synthesis, whereas abruptio placenta and other pregnancy problems could be a manifestation of placental vasculopathy.

Alzheimer's Disease, Vascular Dementia, and Hyperhomocysteinemia

Evidence has recently been accumulating that patients with histologically confirmed Alzheimer's disease have significantly higher circulating levels of homocysteine and lower levels of folate and cobalamin than control subjects. The stability of the findings over time makes it unlikely that the elevated homocysteine levels are a consequence of the disease (10). This was a well-designed study by the Oxford group, whose study had large numbers and involved long follow-up evalua-

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tion. The findings have been confirmed by another rather different approach (17). In addition, hyperhomocysteinemia has been associated with subcortical vascular encephalopathy secondary to small vessel disease (12). If treatment to lower the elevated homocysteine levels prevented or delayed onset of Alzheimer's disease or some forms of vascular dementia, this would be a most significant discovery that would have considerable public health significance.

Conclusion

Lessons learned from the uncommon inborn errors of metabolism resulting in markedly elevated circulating homocysteine have led to the acquisition of new knowledge in many areas. These new insights have set in motion a reappraisal of the etiology and diverse manifestations of cardiovascular risk, in particular the relevance of mild homocysteine elevation to thrombogenesis and endothelial dysfunction. Studies of patients with homocystinuria have provided further insights into methionine metabolism and the relevance of the B vitamins to modest homocysteine elevation, particularly in the elderly. There has also been greater understanding of the role of the kidney in homocysteine homeostasis, as well as its possible contribution to the pathogenesis of the vascular disease frequently seen in renal insufficiency. The common MTHFR gene variant affecting folate metabolism and thus the remethylation of homocysteine to methionine, has been identified. As a result, we have become aware of the increased folate requirements of the 11% of Caucasians who are homozygous for this polymorphism, as well as the concomitant increased risk of offspring with neural tube defect, which is largely preventable by folate supplementation. A number of other disorders that may be associated with mild hyperhomocysteinemia, including dementia, were discussed at the recent conference in Nijmegen (16). The mechanisms are not yet established with certainty. Different mechanisms may be involved for the different associations, and the key issue of cause and effect is yet to be resolved. The role of folate supplementation in the prevention of the increased risks of vascular disease is being actively explored. This book deals with these and other issues that define the role of homocysteine homeostasis in health and disease.

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978-0-521-65319-0 - Homocysteine in Health and Disease
Edited by Ralph Carmel and Donald W. Jacobsen
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SECTION ONE. CHEMISTRY

2

Practical Chemistry of Homocysteine and Other Thiols

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The sulfhydryl-containing amino acids, or aminothiols, maintain intracellular and extracellular redox homeostasis and are part of the armamentarium of antioxidant defense systems. They are also precursors and intermediates in numerous metabolic pathways and facilitate the removal of noxious compounds. The "good thiols," glutathione and cysteine, are well known and abundant. The intracellular concentration of glutathione is usually in the 1 to 10 mmol/L range, and plasma total cysteine ranges from 200 to 300 μmol/L. Homocysteine is perhaps less well known because it is normally found at relatively low concentrations within the cell (≤ 1 μmol/L) and in the circulation (5 to 15 μmol/L). However, elevated plasma total homocysteine (hyperhomocysteinemia) has acquired the reputation as the "not-so good thiol" because of its association with cardiovascular disease (6, 54), end-stage renal disease (4, 13), hypothyroidism (25, 46), neural tube defects (16, 63), and cognitive dysfunction including Alzheimer's disease (8, 34).

This chapter focuses on the structure and physiological chemistry of homocysteine and closely related thiols, with special emphasis on the circulating forms of homocysteine. How homocysteine reacts with intracellular constituents is largely unknown, but the forms of homocysteine found in plasma and serum are now well characterized and offer clues to possible reaction mechanisms that could occur within the cell. Other chapters in this book describe specialized chemically related topics such as homocysteine thiolactone (see Chapter 3), homocysteine and lipid oxidation (see Chapter 4), and nitrosothiols (see Chapter 5).

Discovery of Homocysteine

Homocysteine was discovered 70 years ago by Butz and du Vigneaud at the University of Illinois (5). By heating methionine in sulfuric acid, a compound was isolated and crystalized that had chemical properties similar to those of cysteine and cystine. Using elemental analysis data and other chemical properties, the investigators concluded that they had synthesized "*bis*-(γ-amino-γ-carboxypropyl) disulfide" and suggested that it be called homocystine since it had the structure of the "next higher symmetrical homolog of cystine." They also suggested that homocystine might support growth on cysteine-deficient diets in advance of the discovery of the transsulfuration pathway.

Homocysteine thiolactone was first prepared from methionine by Baernstein (2) in 1934 and further characterized by the du Vigneaud group (14, 56). L-homocysteine thiolactone, a very stable form of homocysteine, can be converted to L-homocysteine by alkaline hydrolysis (2, 43). L-homocysteine is thus available for cell culture and other studies. Although racemic D,L-homocysteine is commercially available and is widely used for in vivo, ex vivo, in situ, and in vitro studies, the presence of the unnatural D-isomer of homocysteine may complicate the interpretation of experimental results.

Structure of Homocysteine and Related Thiols

Homocysteine is a key branch-point intermediate in the ubiquitous four-step methionine cycle (see Metabolic Diagram), the function of which is to generate one-carbon methyl groups for transmethylation reactions essential to all life forms. In mammals homocysteine can be diverted from the methionine cycle into the two-step transsulfuration pathway to generate the nonessential aminothiol cysteine. The essential amino acid methionine, therefore, is the precursor of homocysteine and cysteine, and all three compounds have structural similarities (Figure 2.1).

Methionine contains a sulfide sulfur, the general structure of which can be designated R-S-R′. Homocysteine and cysteine are sulfhydryl compounds (R-SH), and the words *homocysteine* and *cysteine* have precise chemical meanings, referring only to the R-SH forms shown in Figure 2.1. However, in recent years,

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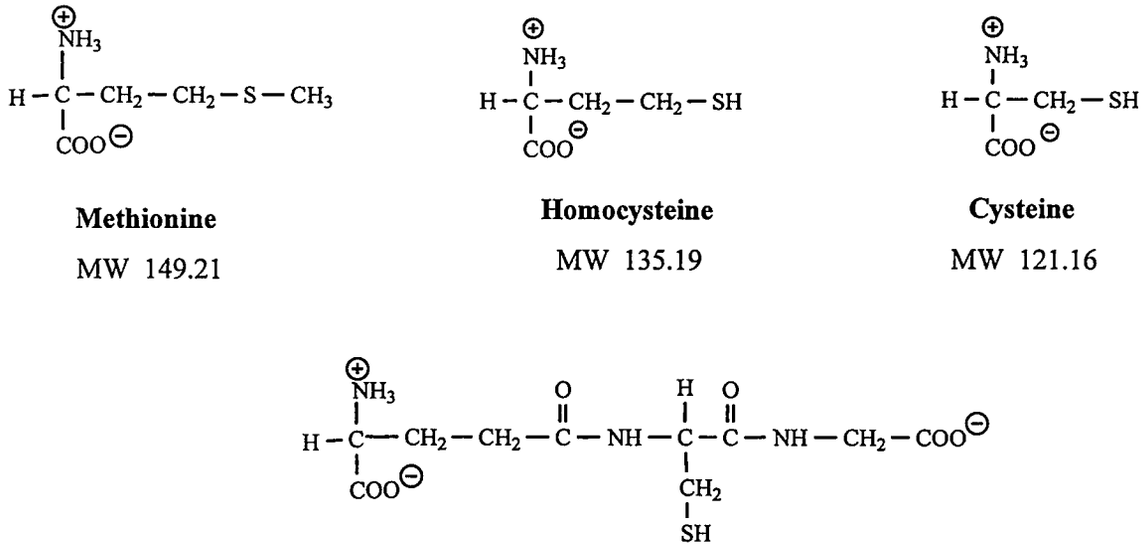

Fig. 2.1. The structures of methionine, homocysteine, cysteine, and glutathione.

these words have taken on generic meaning as well; and it is common, particularly in the clinical literature, to use the word *homocysteine* when referring to all circulating forms of homocysteine. In this book the term *plasma total homocysteine* refers to all circulating forms of homocysteine (*vide infra*).

Compounds that contain a free sulfhydryl group are known as "thiols." Other low-molecular-weight biological thiols include glutathione (Figure 2.1), coenzyme A, and the dithiol, dihydrolipoic acid. Glutathione, a tripeptide-containing glutamic acid, cysteine, and glycine, is the most abundant intracellular thiol and is utilized in a number of host defense systems including protection against reactive oxygen species and xenobiotics.

A general chemical property of thiols is their ability to oxidize in the presence of an electron acceptor such as molecular oxygen to form disulfides (-S-S-) (*vide infra*). Thus, homocysteine will autooxidize to form homocystine and cysteine will autooxidize to form cystine (Figure 2.2). Glutathione will also undergo autooxidation to form oxidized glutathione (Figure 2.2). The latter is formed intracellularly as a result of glutathione peroxidase activity during breakdown of reactive oxygen species. But in the reducing environment of the cell and the presence of glutathione reductase, oxidized glutathione is converted back to glutathione.

Homocysteine can oxidize with other thiols such as cysteine and glutathione to form mixed disulfides, and these compounds are referred to as "homocysteine-cysteine mixed disulfide" and "homocysteine-glutathione mixed disulfide" (Figure 2.3).

The other major class of thiols and disulfides consists of those found in peptides and proteins that contain cysteine. The intramolecular disulfide bonds formed by the oxidation of cysteine residues in peptides and proteins are primary structural elements (covalent bonds) that contribute to the final three-dimensional conformations. Peptides and proteins may also contain "free cysteine residues" that can autooxidize (e.g., serum albumin, to form disulfide dimers,) or can react with low molecular thiols to form stable disulfide bond complexes. Thus, > 70% of the homocysteine and approximately 50% of the cysteine in the circulation are bound to plasma protein cysteine residues through disulfide bonds (Figure 2.4).

Homocysteine thiolactone, the product of aminoacyl tRNA synthetase editing reactions (see Chapter 3), is the five-membered condensed ring form of homocysteine. Although this compound may be exported to the circulation, there is no reliable evidence for its existence in human plasma, perhaps because of nonspecific esterase activity found in plasma and on the surface of the vascular endothelium (15). Also, it was recently reported that human serum paraoxonase hydrolyzes homocysteine thiolactone as well (29a).

Nomenclature

Homocysteine and homocystine refer to the reduced and oxidized forms of homocysteine, respectively, and likewise for cysteine and cystine. Other oxidized forms of homocysteine include the mixed disulfides with free

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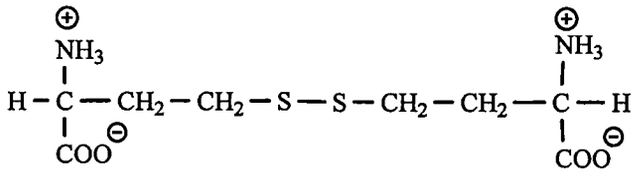
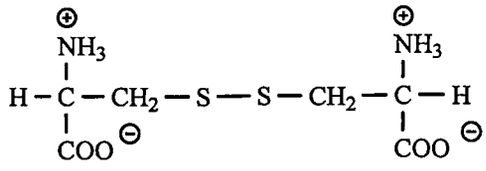


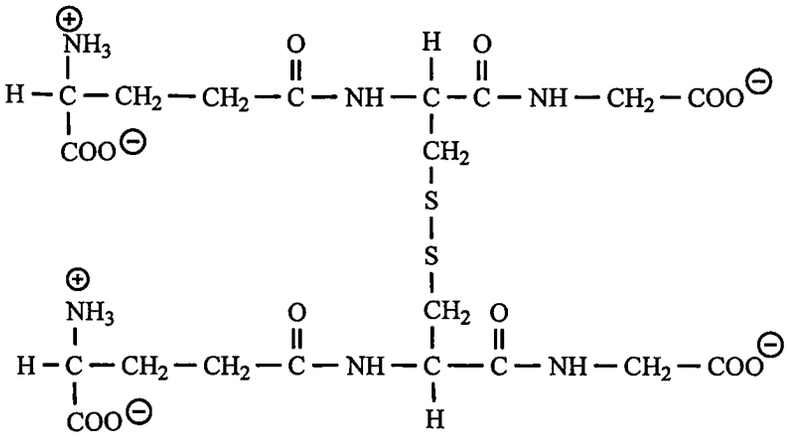
Homocystine

MW 268.36



Cystine

MW 240.30

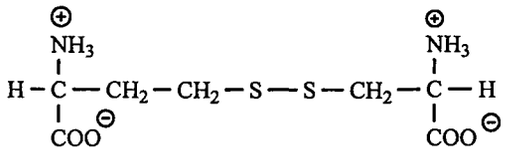


Oxidized glutathione

MW 610.62

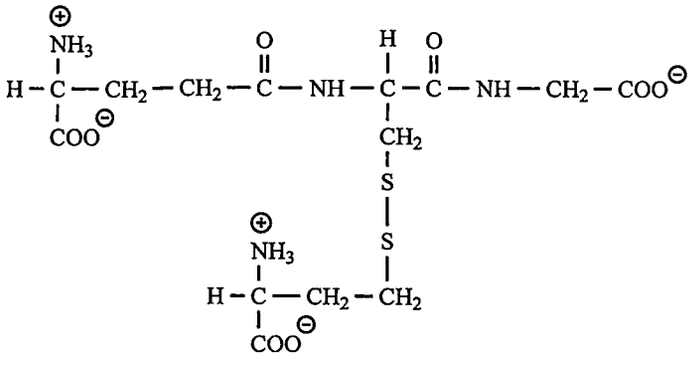
Fig. 2.2. The structures of the oxidized forms of homocysteine, cysteine, and glutathione: homocystine, cystine, and oxidized glutathione.

Fig. 2.3. The structures of homocysteine-cysteine mixed disulfide and homocysteine-glutathione mixed disulfide.



Homocysteine-cysteine mixed disulfide

MW 254.33



Homocysteine-glutathione mixed disulfide

MW 439.49

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