

PLASMA HOMOCYSTEINE, A RISK  
FACTOR FOR VASCULAR DISEASE:

PLASMA LEVELS IN HEALTH,  
DISEASE, AND DRUG THERAPY

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Reprinted from

THE JOURNAL OF LABORATORY AND CLINICAL  
MEDICINE,  
St. Louis

Vol. 114, No. 5, pp. 473-501, Nov., 1989

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(Printed in the U.S.A.)

## Plasma homocysteine, a risk factor for vascular disease: Plasma levels in health, disease, and drug therapy

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**Abbreviations:** AdoHcy = S-adenosylhomocysteine, AUC = area under the curve

**H**omocysteine was discovered in 1932 by DeVigneaud<sup>1</sup> as a product of demethylation of methionine. In the years to follow, the role of homocysteine in the conversion of methionine to cystathionine and cysteine, that is, the transsulfuration pathway, was studied. It was soon discovered that homocysteine could support the growth of animals fed diets deficient in cysteine, methionine, or choline.

In 1962, Carson and Neill<sup>2</sup> surveyed mentally retarded persons in Northern Ireland and reported on two siblings, aged 4 and 6 years, with homocystinuria. At the same time Gerritsen and Waisman<sup>3</sup> identified homocystine in the urine of a mentally retarded infant with congenital anomalies. These pioneering discoveries initiated numerous studies into the clinical features and biochemical basis of homocystinuria. In 1964 Mudd et al.<sup>4</sup> demonstrated lack of the enzyme cystathionine  $\beta$ -synthase in a liver biopsy specimen from a typical patient. Later, other rare enzyme defects causing homocystinuria were identified.<sup>5</sup>

The clinical condition homocystinuria has stimulated research of the role of homocysteine in cellular functions. Predominating symptoms in such patients

are thromboembolism, premature arteriosclerosis, and mental retardation,<sup>6</sup> and the ability of homocysteine to provoke vascular lesions<sup>7</sup> and its role in nervous functions and epilepsy<sup>8-11</sup> have been widely studied. Since 1974 altered homocysteine metabolism in transformed cells has become an expanding field of research.<sup>12</sup>

During the last decade there have been major improvements of the techniques used for the determination of homocysteine in biologic material. Homocysteine, which once was regarded as nonexistent in human plasma and tissues under physiologic conditions,<sup>13</sup> has now been quantified in plasma from normal individuals and from patients with various diseases other than homocystinuria, in various deficiency states, and during exposure to various drugs.<sup>14</sup> One possible consequence of moderately elevated levels of plasma homocysteine, which has attracted much attention during the last years, is an increased risk of premature vascular disease.<sup>7</sup> Knowledge of the pharmacologic modulation of plasma homocysteine may form the basis for interventions with the prospect to predict and prevent such consequences.

The present article reviews the central features of homocysteine metabolism, and the evidence that moderate homocystinemia may be a risk factor for arteriosclerosis. A survey of the literature of homocysteine in various pathologic conditions is presented, with emphasis on disease states other than homocystinuria, which has been covered recently in excellent review articles.<sup>5,6,15</sup> Finally, agents that modulate plasma homocysteine level are reviewed.

### HOMOCYSTEINE METABOLISM

AdoHcy is a product formed from the versatile methyl donor, S-adenosylmethionine, on transfer of the

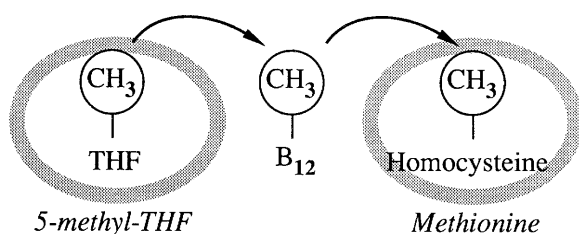
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Supported in part by The Norwegian Cancer Society and the Norwegian Society for Science and the Humanities.

Submitted for publication Jan. 9, 1989; revision submitted April 3, 1989; accepted May 24, 1989.

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**Fig. 1.** The methyl transfer between the reduced folates, cobalamin, and homocysteine in the 5-methyltetrahydrofolate-homocysteine methyltransferase reaction. THF, tetrahydrofolate.

methyl group to cellular acceptors. It functions as a feedback inhibitor of *S*-adenosylmethionine-dependent transmethylation reactions. Cleavage of AdoHcy to homocysteine and adenosine is the only known metabolic pathway leading to formation of homocysteine in vertebrates. The reaction is catalyzed by the ubiquitous enzyme AdoHcy hydrolase (EC 3.3.1.1). The reaction favors synthesis of AdoHcy, but because both products are removed by further metabolism, the metabolic flux is directed toward hydrolysis.<sup>16,17</sup>

Intracellular homocysteine is either remethylated to methionine or is condensed with serine to form cystathionine. The latter reaction is catalyzed by the enzyme cystathionine  $\beta$ -synthase (EC 4.2.1.22), which is widely distributed in mammalian tissues. Notably, this enzyme requires pyridoxal 5'-phosphate, the biologic active form of vitamin B<sub>6</sub>, as cofactor.<sup>5</sup> The physiologic significance of cystathionine synthesis may be caused by the irreversibility of this reaction, which implies that beyond this step homocysteine can no longer serve as a methionine precursor.<sup>13</sup> Increased synthesis of cystathionine seems to be a metabolic adaptation to methionine excess.<sup>18,19</sup>

Cystathionine is cleaved by the enzyme  $\gamma$ -cystathionase (EC 4.4.1.1) to cysteine and  $\alpha$ -ketobutyrate. Also this enzyme requires pyridoxal 5'-phosphate for activity.  $\gamma$ -Cystathionase completes the conversion of methionine to cysteine, that is, the so-called transsulfuration pathway.<sup>19</sup>

The salvage of homocysteine to methionine is either catalyzed by betaine-homocysteine methyltransferase (EC 2.1.1.5) or 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase, EC 2.1.1.13). The former is confined to the liver, except for an occasional finding of minor activity in the kidney, whereas the latter enzyme is widely distributed in animal tissue. Methionine synthase requires 5-methyltetrahydrofolate as methyl donor and vitamin B<sub>12</sub> as cofactor.<sup>5</sup> Studies<sup>20</sup> with the bacterial enzyme suggest that methylcobalamin methylates homocysteine, and the methyl group of 5-methyltetrahydrofolate remethylates cobalamin. 5-Methyltetrahydrofolate is

converted to tetrahydrofolate in this reaction (Fig. 1). 5-Methyltetrahydrofolate-homocysteine methyltransferase links homocysteine metabolism to folates and vitamin B<sub>12</sub>, and together with L-methylmalonyl-CoA mutase, they represent the only cobalamin-dependent enzymes in mammals.<sup>21</sup>

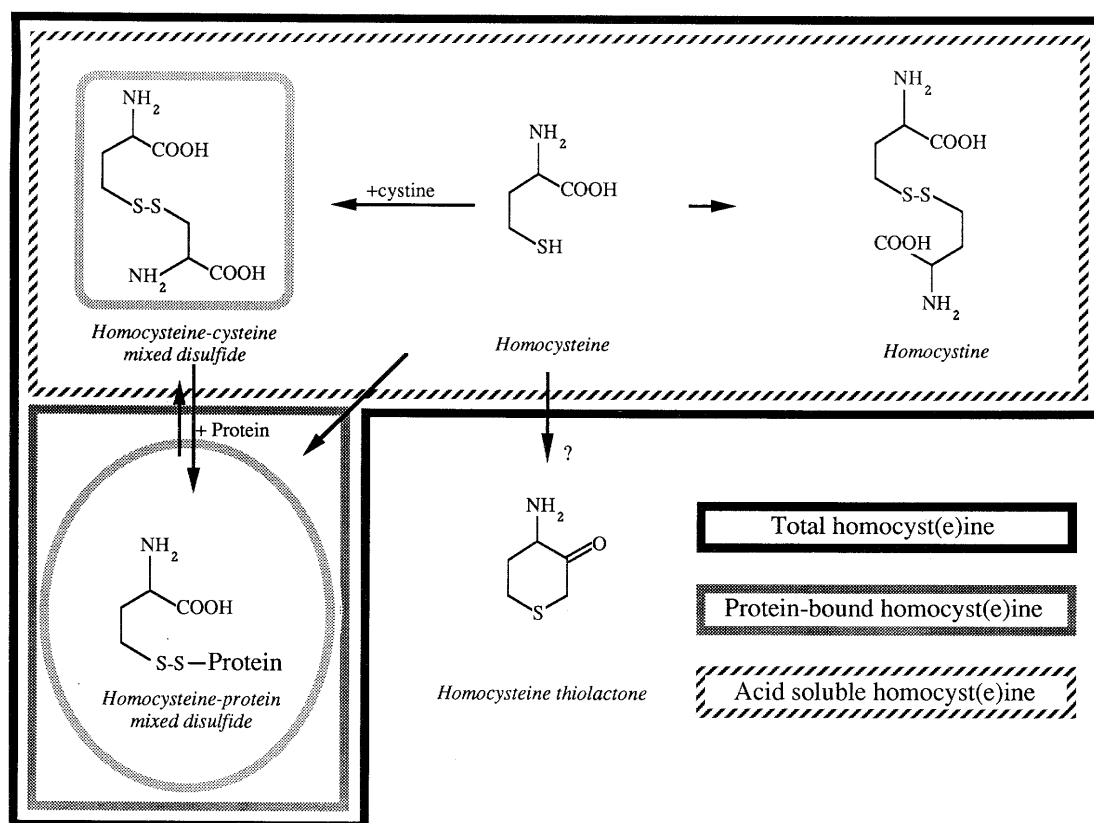
The folate and vitamin B<sub>12</sub>-dependent remethylation of homocysteine explains the clinically well-established relation between vitamin B<sub>12</sub> and folates. Deficiency of either results in changes in the blood and bone marrow; that is, megaloblastosis, and supplementing folates can partly relieve the bone marrow changes induced by vitamin B<sub>12</sub> deficiency.<sup>21,22</sup>

These metabolic relations are encompassed in the so-called methyl folate trap hypothesis, which was presented 25 years ago.<sup>23</sup> This theory states that when the methionine synthase reaction is inhibited because of either lack of homocysteine or vitamin B<sub>12</sub> deficiency, the conversion of 5-methyltetrahydrofolate to tetrahydrofolate is blocked, and the reduced folates are trapped as 5-methyltetrahydrofolate.<sup>23</sup> This diverts reduced folates away from the purine and pyrimidine synthesis and thereby inhibits deoxyribonucleic acid synthesis and cell proliferation.<sup>24</sup> Low intracellular *S*-adenosylmethionine resulting from insufficient supply of methionine has a similar effect on the distribution of reduced folates through regulatory effects on 5,10-methylenetetrahydrofolate reductase, the enzyme forming 5-methyltetrahydrofolate, and on methionine synthase.<sup>25,26</sup> The methyl folate trap process may represent a favorable adaptation to methionine deficiency but may cause lethal effects during vitamin B<sub>12</sub> deficiency.<sup>27</sup>

Conversion of homocysteine to methionine is also catalyzed by the enzyme betaine-homocysteine methyltransferase (EC 2.1.1.5), which requires betaine as a methyl donor.<sup>28</sup> This enzyme is confined to the liver and possibly kidney.<sup>29</sup> It was long believed that betaine-homocysteine methyltransferase is responsible for choline catabolism and homocysteine removal, but recent evidence suggests that also this enzyme is involved in the methionine homeostasis in mammals.<sup>29-31</sup>

It has recently been possible to determine small (1 to 5 nmol/gm) but stable amounts of homocysteine in tissues,<sup>32</sup> a fraction of which is associated with proteins.<sup>33</sup> Studies with isolated cells show that both fractions of intracellular homocysteine have a short half-life, and homocysteine is efficiently exported into the extracellular medium. Such export is enhanced during increased production of homocysteine<sup>34</sup> and decreased during inhibition of production,<sup>34,35</sup> suggesting that homocysteine egress plays a role in keeping the intracellular level within certain limits. These observations with isolated cells are important, since they support the possibility<sup>14</sup> that the amount of homocysteine in extra-





**Fig. 3.** Hypothetical scheme for the dynamic relation between various species of homocysteine in human plasma and their inclusion by various assay procedures. The encircled areas indicate the relative size of protein-bound and homocysteine-cystine mixed disulfide fractions in human plasma under physiologic conditions. The *black border* defines the homocysteine species, which represent total homocysteine; the species within the *cross-hatched border* are free homocysteine as determined in plasma deproteinized with acid. The protein-bound homocysteine probably exists as homocysteine-protein mixed disulfide, which is symbolized as an oval area located within the *shaded border*. These graphic symbols are also defined by the boxes in the lower, right corner of the figure.

homocysteine-cystine mixed disulfide<sup>37,39,40</sup> have been elaborated in studies including protein-bound homocysteine. Higher total plasma homocysteine levels (free plus bound) in men than in women have been reported.<sup>45,46</sup> Sex differences in plasma homocysteine could be masked by the fact that the age of the subjects is not taken into account.<sup>42</sup>

Kang et al.<sup>47</sup> found an age-dependent increase in total homocysteine in women, determined as protein-bound homocysteine in stored plasma, with a marked increase at approximately the age of 50 years. Above this age the amount approaches or even exceeds the level found in men.<sup>47</sup> Others<sup>48,49</sup> have found levels of total plasma homocysteine to be higher in postmenopausal (aged 58 years) than premenopausal women (aged 45 years), but the difference was not significant.

**Relation between the amount of various species of homocysteine in plasma.** The prevailing form of ho-

mocysteine in vivo in plasma from healthy subjects is unknown. Perry et al.<sup>50</sup> demonstrated more than 20 years ago that in subjects with homocystinuria a major fraction of homocysteine in plasma in vivo exists in the reduced form. They obtained this evidence by allowing freshly collected whole blood to react with iodoacetic acid, thereby converting homocysteine to the stable derivative *S*-carboxymethylhomocysteine.<sup>50</sup> This observation suggests that homocysteine may circulate as free thiol under physiologic conditions, but this possibility and the degree of protein binding in vivo remain to be investigated.

In the freshly prepared plasma, homocysteine exists as a combination of mixed disulfides, trace amounts of homocysteine, possibly free thiol,<sup>46</sup> and a major fraction is complexed with serum albumin, probably via a disulfide bond.<sup>42</sup> When plasma is deproteinized with acid, the protein-bound homocysteine coprecipitates. The ho-

**Table I.** Normal values for homocysteine in human plasma/serum

	Age (yr)	n	Homocysteine species	Value*	Author(s), yr	Ref. no.
Male and female	(23-50)	20	MDS† (free‡)	3.25 ± 0.85	Gupta and Wilcken (1978)	37
Male	(21-50)	24	MDS (free)	3.3 ± 0.8	Wicken and Gupta (1979)	39
Female	(21-50)	24		2.4 ± 0.7		
Male	(45-61)	10	MDS (free)	3.1 ± 0.3	Boers et al. (1983)	40
Male	(22-35)	10		3.5 ± 0.5		
Female (postm.)	(45-59)	10		2.6 ± 0.4		
Female (prem.)	(14-42)	10		0.9 ± 0.3		
Male	(25-55)	18	Free	2.27 ± 0.48	Refsum et al. (1985)	42
			Bound	6.51 ± 1.35		
Female	(25-55)	16	Free	1.95 ± 0.56		
			Bound	7.29 ± 2.62		
Male	(<30)	5	Bound = total§	6.82 ± 1.28	Kang et al. (1986)	47
	(30-39)	14		8.92 ± 2.32		
	(40-49)	25		9.44 ± 2.00		
	(50-59)	26		8.84 ± 2.02		
	(60-69)	23		8.06 ± 2.32		
Female	(<30)	9	Bound = total	7.50 ± 2.02		
	(30-39)	8		7.26 ± 1.64		
	(40-49)	24		7.00 ± 1.94		
	(50-59)	30		8.82 ± 3.82		
	(60-69)	38		9.20 ± 3.62		
Male and female	(18-65)	50	Total	13.0 (7.2-21.7)	Stabler et al. (1987)	45
Male				(20% higher than in female)		
Male	(19-39)	20	Free	2.06 ± 0.44	Araki and Sako (1987)	46
			Bound	4.47 ± 0.78		
			Total	6.53 ± 1.08		
Female	(19-39)	15	Free	1.79 ± 0.47		
			Bound	3.92 ± 0.86		
			Total	5.71 ± 1.20		
Male	(51.5 ± 6.7)	22	Total	12.1 ± 4.0	Brattström et al. (1989)	49
Female (prem.)	(45.0 ± 0)	10		8.9 ± 1.0		
Female (postm.)	(58.3 ± 2.1)	14		10.2 ± 2.5		

Postm., postmenopausal; prem., premenopausal.

\*Values are given as homocysteine equivalents, mean ± standard deviation.

†MDS, homocysteine-cystein mixed disulfide.

‡MDS accounts for most free (i.e. acid soluble) homocysteine in plasma.

§Bound = total homocysteine in stored samples, because free homocysteine becomes associated with plasma protein(s).

||Range, skew distribution of values.

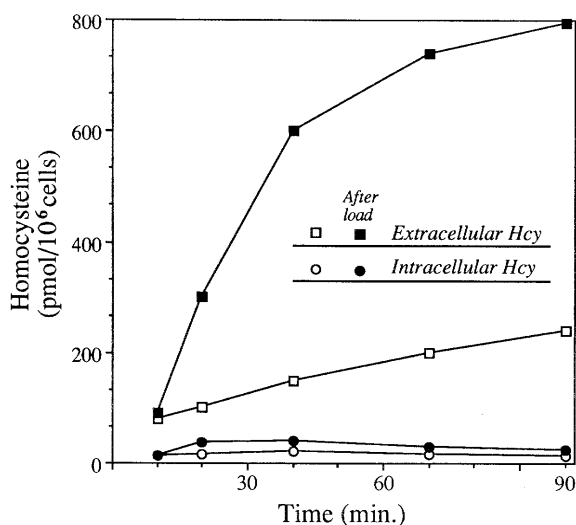
homocysteine mixed disulfide, homocystine, and homocysteine remains in solution. The sum of these soluble species is referred to as free homocysteine. Free plus protein-bound homocysteine is total homocysteine.

A marked redistribution between free and bound homocysteine takes place after preparation of plasma. At a high temperature there is a rapid association of free homocysteine with plasma protein, and when stored at -20° C for some weeks, most homocysteine becomes protein-bound and only traces of free can be detected.<sup>42</sup> Similar observations have been done for cystine, homocystine, and homocysteine-cystein mixed disulfide in plasma from subjects with homocystinuria.<sup>51</sup> This implies that, without cautious sample handling and storage, total plasma homocysteine should be determined. Free (acid soluble) homocysteine may become arti-

cially low, and the possibility that displacement<sup>43,52</sup> may affect the distribution between bound and free homocysteine should be considered.

The relation between these various forms of homocysteine in plasma is shown in Fig. 3, and normal values for fasting homocysteine in plasma obtained in various laboratories are listed in Table I.

**Urine.** Determination of homocysteine in urine from normal subjects<sup>42,45</sup> or experimental animals<sup>53</sup> has recently been accomplished. Renal clearance of homocysteine is about 0.3% of creatinine clearance<sup>45</sup> and amounts to 3.5 to 10 µmol/24 hr,<sup>42</sup> which is about 1:1000 of the turnover of labile methyl groups in humans.<sup>38</sup> Thus renal excretion of homocysteine seems to account for a minor portion of total homocysteine clearance under physiologic conditions.



**Fig. 4.** Methionine loading of isolated rat hepatocytes. At time 0 minute the concentration of methionine in the medium was increased from 1  $\mu\text{mol/L}$  to 200  $\mu\text{mol/L}$ . Intracellular homocysteine and homocysteine in the medium were determined for both these cells (closed symbols) and control cells exposed to 1  $\mu\text{mol/L}$  methionine (open symbols). (From Svardal A, Refsum H, Ueland PM. *J Biol Chem* 1986;261:3156-63.)

**Men versus women.** There are consistent reports, which are described in detail elsewhere in this article, that sex differences exist in levels of plasma homocysteine. During fasting<sup>37,39,42,45-47</sup> and after methionine loading,<sup>40</sup> the amount of various species of plasma homocysteine in plasma is lower in premenopausal women than in young men. In postmenopausal women the levels approach or even exceed those seen in men.<sup>40,47-49</sup> Urinary homocysteine excretion is higher in men than in women.<sup>54</sup> It is conceivable that these differences may be mediated by sex hormones, but as will be outlined below, the possible role of estrogen as a modulator of plasma homocysteine seems complex and has not been settled.

The transsulfuration pathway has been regarded as the main route of methionine degradation in humans, but there is evidence suggesting an additional catabolic pathway by means of transamination.<sup>55,56</sup> Blom et al.<sup>57</sup> recently reported that two transamination metabolites 4-methylthio-2-oxo-butyrate and methanethiol mixed disulfides in serum were higher in premenopausal women than in young men, both during fasting and after methionine loading. In addition, the urinary excretion of these compounds after loading was highest in premenopausal women. The serum profiles for homocysteine-cysteine mixed disulfides in this study confirmed an earlier report.<sup>40</sup> Because the transamination pathway seems quantitatively not important in normal subjects,<sup>56</sup> it is uncertain to what extent the higher

transamination capacity in premenopausal women may contribute to lower serum homocysteine levels compared with young men.<sup>57</sup>

**Sample handling and analytic methods.** The somewhat inconsistent data on the level of free and protein-bound homocysteine in plasma and its relation to age and sex may reflect the fact that analysis of plasma homocysteine is influenced by sample handling, redistribution between the free and bound fraction during sample storage, and different derivatization techniques and chromatographic procedures. Knowledge on such factors is required to obtain reproducible data on plasma homocysteine in the clinical setting, and data related to methodologies are considered in detail below.

Accurate determinations require rapid sample processing. Rapidly prepared plasma contains somewhat less (10% to 20%) homocysteine (free plus bound) than serum. This can be explained by slow release of homocysteine from cells in whole blood left at room temperature.<sup>14,42</sup> Total homocysteine in serum is not affected by incubating whole blood at room temperature for 1 hour, whereas incubation for 4 hours increased the serum concentration by about 35%.<sup>45</sup> Because only trace amounts of homocysteine occur intracellularly (Refsum H, unpublished data, 1987), the artificial increase in plasma level is far less pronounced for homocysteine than for glutathione, where release from blood cells rich in this thiol may give a marked increase in plasma content.<sup>58</sup> The release of homocysteine from cells into plasma is retarded when the blood sample is left on ice. Under these conditions there is no change in free or protein-bound homocysteine within 0.5 hours, and total homocysteine remains stable for at least 12 hours.<sup>14</sup>

To obtain both free and protein-bound homocysteine in plasma caution must be taken to prevent redistribution between these forms. The plasma sample should be immediately cooled on ice and then deproteinized with acid. Samples so treated can be stored below  $-20^{\circ}\text{C}$  for months.<sup>42</sup>

The first studies<sup>39,40</sup> on homocysteine-cysteine mixed disulfides in plasma from healthy subjects, including the pioneering work of Gupta and Wilcken,<sup>37</sup> were based on determination of disulfides in deproteinized plasma with second generation amino acid analyzers. Others<sup>59</sup> have determined acid soluble homocysteine in plasma with high-performance liquid chromatography and electrochemical detection.<sup>59</sup>

In assays for total or protein-bound homocysteine in plasma, the thiol is liberated from protein-mixed disulfides and acid soluble mixed disulfides in the presence of a reducing agent, that is, dithioerythritol,<sup>42</sup> 2-mercaptoethanol,<sup>47</sup> tri-n-butylphosphine,<sup>46</sup> or sodium borohydride.<sup>60</sup> Homocysteine is readily reoxidized and may form various species of mixed disulfides before or

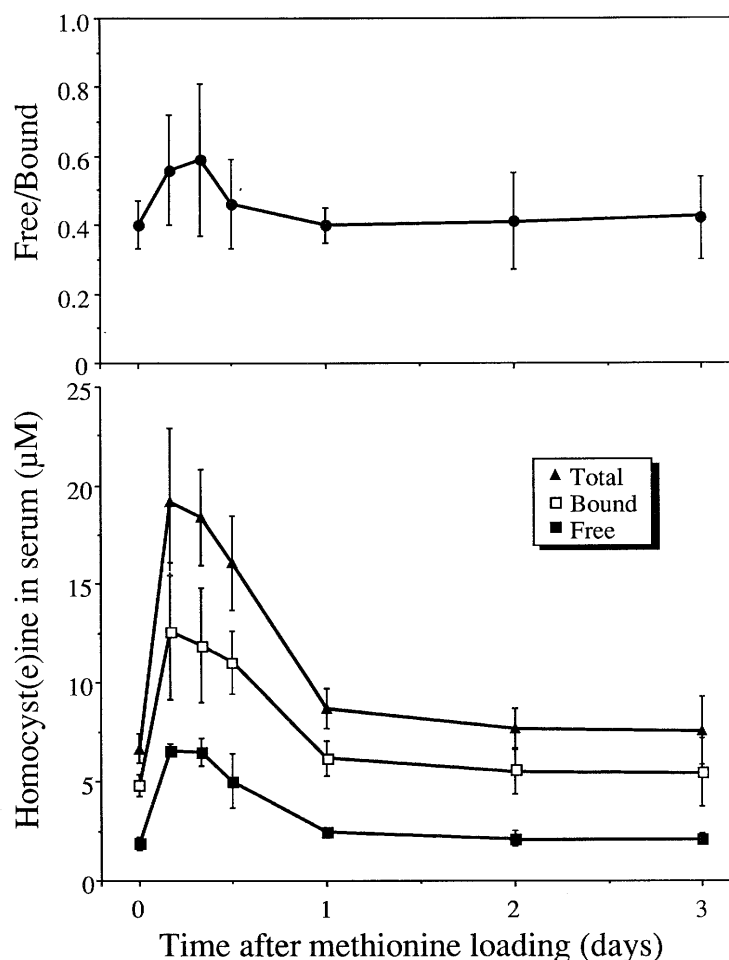


Fig. 5. Free and bound homocysteine in plasma after methionine loading. Seven healthy premenopausal women were given peroral methionine (0.1 gm/kg). Data are given as mean  $\pm$  SD.

during analysis.<sup>45,61</sup> This must be corrected for,<sup>45</sup> or homocysteine must be derivatized and thereby trapped in the presence of a reducing agent<sup>41,47</sup> to obtain reproducible data.

Consistent values for protein-bound and free homocysteine in human plasma from normal subjects have been obtained by different methods, including a radioenzymic assay,<sup>42,62</sup> ion-exchange chromatography of *S*-carboxymethylated sulfhydryl compounds,<sup>47</sup> reversed-phase chromatography of fluorescent compounds obtained after precolumn derivation with ammonium 7-fluorobenzo-2-oxa-1,3-diazolo-4-sulfonate<sup>46</sup> or monobromobimane,<sup>60</sup> and by gas chromatography-mass spectrometry.<sup>45</sup>

#### METHIONINE LOADING

Superfluous intracellular homocysteine is effectively exported into the extracellular medium, and extracellular homocysteine may therefore be a convenient mea-

sure of the balance between intracellular homocysteine production and utilization.<sup>14</sup> Homocysteine production can be enhanced by exposing cells to high concentrations of methionine. Methionine loading of liver cells in vitro results in a marked increase in homocysteine egress, whereas the level of intracellular homocysteine is only moderately elevated<sup>33</sup> (Fig. 4).

A condition resembling that observed with isolated cells can be obtained in humans. A standard dose of methionine (usually 0.1 gm/kg) is taken orally, and plasma homocysteine, which reflects homocysteine egress, is determined after a fixed time period, usually 4 hours. Methionine ingestion induces a transient increase in plasma homocysteine. Postloading plasma homocysteine, determined as homocysteine mixed disulfide,<sup>40,63-67</sup> homocystine,<sup>40,64,65,67</sup> or total homocysteine<sup>49,68</sup> has been widely used to reveal possible defects in homocysteine metabolism.

The time course for the postloading plasma homo-



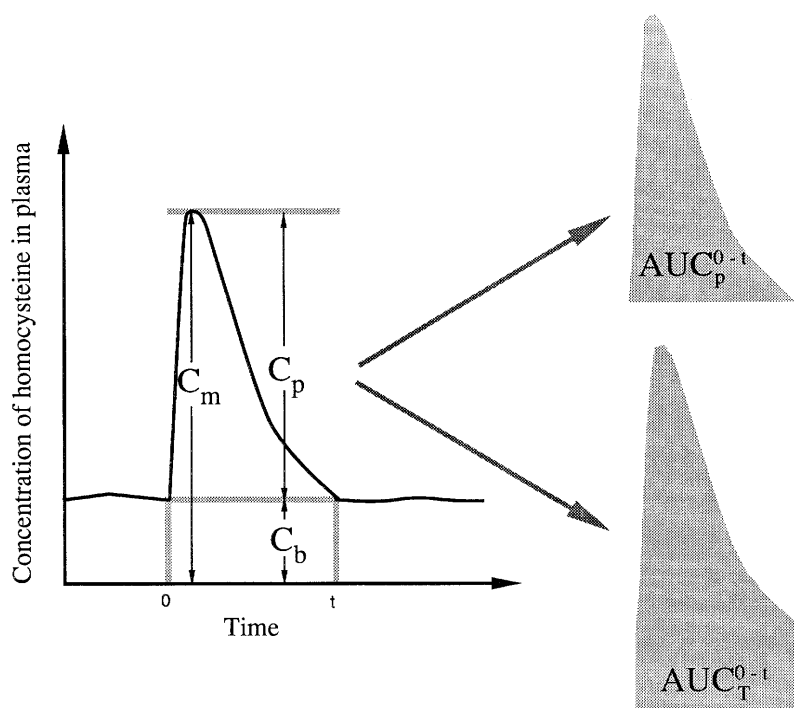


Fig. 6. Terms for the description of the plasma homocysteine response after methionine loading.

cysteine in normal individuals and the relation between the free and protein-bound fraction are shown in Fig. 5. There is a rapid increase in both fractions with a peak concentration at about 4 to 8 hours. The percent increase for free homocysteine is somewhat higher than for bound homocysteine. Both fractions approach pre-loading values within 2 to 4 days. Thus, although free homocysteine is somewhat more responsive after methionine loading, measurement of either free or total homocysteine seems to be adequate. Determination of total homocysteine is not obscured by redistribution of plasma homocysteine during storage of samples and should not be affected by possible differences in protein-binding characteristics.

The plasma profile for homocysteine obtained after methionine loading can be described in various terms, some of which are depicted in Fig. 6. Most clinical studies, including postloading plasma homocysteine, report this parameter as maximum concentration ( $C_m$ , the maximum plasma concentration) obtained a few hours after loading. This value and basal level of homocysteine ( $C_b$ , the basal plasma concentration) seem to increase in patients with defects in the transsulfuration pathway.<sup>63,65</sup> It has been found that  $C_b$  but not the increase in plasma level above  $C_b$  (i.e.,  $C_p$ , the increase in plasma concentration) is correlated to erythrocyte folate<sup>49</sup> and serum vitamin B<sub>12</sub>.<sup>68</sup> This suggests that methionine loading may selectively

stress a pathway not dependent on folates (i.e., the transsulfuration pathway), whereas folate available to the methionine synthase reaction is a determinant of fasting homocysteine in plasma. Alternatively, erythrocyte folate may not reflect body folate status<sup>69</sup> or methionine loading may stress the methionine synthase reaction in tissues, such as the liver, not deficient in folate.

Together with plasma methionine, which may be high in transsulfuration block<sup>5</sup> and low in remethylation defects,<sup>70</sup>  $C_b$  and  $C_p$  values may provide information on the role of various homocysteine pathways under methionine intake and inherited or acquired metabolic defects.

The single point registrations before ( $C_b$ ) and after ( $C_m$ ,  $C_p$ ) methionine loading are convenient for studies involving a large number of subjects, but in smaller studies the whole plasma profile for homocysteine after methionine loading can be obtained. Pharmacokinetics after single-dose ingestion are described in terms of AUC, which is the area under the plasma concentration curve. This is a measure of total drug exposure.<sup>71</sup> We use this term to describe the plasma homocysteine response after methionine loading. Since the response is superimposed on basal plasma level, the peak area ( $AUC_p^{0-t}$ ) rather than the total area ( $AUC_T^{0-t}$ ) seems the most useful term. It is conceivable that in analogy with metabolism of xenobiotics, alteration of metabolic

and renal clearance may affect peak concentration as well as  $AUC_p^{0-t}$ . This approach for describing impaired homocysteine metabolism should be evaluated.

## HOMOCYSTINURIA

Homocystinuria is an inborn error of metabolism, which is either caused by defect transsulfuration or 5-methyltetrahydrofolate-dependent remethylation of homocysteine to methionine. Details of this class of disease are comprehensively reviewed elsewhere.<sup>5,6,15,72</sup> The metabolic basis and some central clinical features, including vascular lesions, are briefly described herein.

Subjects with homocystinuria usually have fasting plasma homocysteine levels of 0.2 mmol/L, and the nonfasting levels may be even higher. Large amounts are excreted into the urine.<sup>5</sup> Such high concentrations can be detected by qualitative screening with the sodium nitroprusside reaction after reduction of disulfides,<sup>73</sup> but its confirmation requires analysis of blood and urine by column chromatography.

The most common form of homocystinuria is associated with cystathionine  $\beta$ -synthase (Fig. 2) deficiency.<sup>4</sup> This state is inherited in an autosomal recessive manner, but there seems to be some genetic heterogeneity. Some patients have residual enzyme activity, whereas in others no activity can be demonstrated. About 50% of the patients respond to pyridoxine therapy, and the patients who respond are among those with residual activity.<sup>5</sup>

The incidence of homocystinuria is about 1:200,000 worldwide, but markedly higher (1:60,000) in some areas like New South Wales and Ireland.<sup>5,74</sup> Approximately 630 patients have been reported to this date.<sup>6</sup>

Rare forms of homocystinuria are associated with impaired remethylation of homocysteine to methionine catalyzed by methionine synthase (Fig. 2). One form is due to 5,10-methylenetetrahydrofolate reductase (Fig. 2) deficiency, which causes lack of 5-methyltetrahydrofolate,<sup>75</sup> whereas others include various derangements in cobalamin uptake, transport, or use.<sup>70,76-79</sup>

Hereditary defects of cobalamin metabolism may stem from block in the synthesis of 5'-deoxyadenosyl-transferase serving as a coenzyme in the enzyme methylmalonyl-CoA mutase. These patients (cblA and cblB mutations) have methylmalonic aciduria but no megaloblastosis and homocystinuria. Impaired formation of methyl cobalamin, the cofactor in the methionine synthase reaction, results in homocystinuria but no methylmalonic aciduria. Notably, these patients (cblE and cblG mutations) have megaloblastic anemia. Patients with defects in the formation of both coenzymes (cblC, cblD and cblF mutations) have methylmalonic aciduria, homocystinuria, and some have megaloblastic anemia.<sup>70</sup> These observations strongly suggest that in-

hibition of the methionine synthase reaction is important for the development of megaloblastic anemia in these inherited disorders. Furthermore, neurologic disorders occur in patients classified as cblG mutation, suggesting that the nervous dysfunctions are mediated by impaired function of the methionine synthase, and possibly by the homocysteinemia or methionine deficiency.<sup>80</sup>

The most prominent biochemical feature of cystathionine  $\beta$ -synthase deficiency is elevated homocysteine and homocysteine-mixed disulfide in plasma and excretion of large amounts of these compounds into the urine. Plasma methionine is also elevated. The clinical phenotype is characterized by several defects including dislocation of the optic lens, skeletal anomalies, mental retardation, and thromboembolism. The severity of clinical manifestations seems to be related to the plasma homocysteine level.<sup>15</sup>

There are seemingly conflicting data on platelet survival in subjects with homocystinuria. Harker et al.<sup>81,82</sup> observed a marked decrease in mean platelet survival time in four patients with homocysteinemia, whereas others have reported on normal mean platelet survival times in six<sup>38</sup> and 12<sup>84</sup> patients.

Thromboembolism occurs in the arteries and large veins<sup>85-88</sup> and is the major cause of the high mortality (between 20%<sup>15</sup> and 75%<sup>89</sup> at age of 30 years) of individuals deficient in cystathionine  $\beta$ -synthase. Vascular occlusion may occur at any age and even in childhood.<sup>6</sup> Lethal thromboembolic disease has been reported by Dunn et al.<sup>87</sup> in 1966 in a boy aged 10.5 months. The rate of occurrence of thromboembolic events is about 25% by the age of 16 years and 50% by the age of 30 years.<sup>15</sup>

Thromboembolism occurs in both the extra- and intracranial cerebral arteries and veins, the sinuses, in the coronary arteries, and the peripheral arteries and veins. Occlusion of peripheral vessels often results in renovascular hypertension, intermittent claudication, and mesenteric ischemia.<sup>90</sup> Peripheral venous thrombosis is often complicated by pulmonary embolism, and there are reports of lethal thrombosis of renal veins and the portal vein in very young patients.<sup>85-87,90</sup> The most frequent localization of thromboembolism was the peripheral veins (51%), less frequent in the cerebral vessels (32%) and peripheral arteries (11%), and seldom in the coronary arteries (4%).<sup>15</sup> On postmortem examination mild to severe arteriosclerotic lesions have been found in most large and medium sized arteries.<sup>90</sup>

The clinical picture in all forms of homocystinuria include thromboembolism and arteriosclerosis,<sup>91,92</sup> and prominent vascular changes have been demonstrated on autopsy in seven of 10 children (aged 7.5 weeks to 10 years) afflicted with defect in the remethylation of homocysteine.<sup>91-94</sup> Thus, vascular lesions develop in homocystinuria with homocysteinemia as a result of dif-

ferent metabolic defects. As pointed out by McCully<sup>7</sup> this suggests that the vascular changes are induced by homocysteine itself and not by a particular metabolic deletion or some remote metabolic, epigenic, or phenotypic effect. Thus homocystinuria, like homozygous familial hyperlipoproteinemia, may be a model for premature atherosclerosis and has stimulated research for the role of homocysteine in atherogenesis.

### THERAPEUTIC INTERVENTIONS IN HOMOCYSTINURIA

Approximately 50% of patients with cystathionine  $\beta$ -synthase deficiency respond to large doses of pyridoxine, a precursor of pyridoxal 5'-phosphate, both by reduction of plasma and urinary homocysteine and decreased incidence of cardiovascular disease.<sup>15</sup> Whereas such treatment nearly attenuates the fasting homocysteinemia, there is only a slight reduction in the homocysteinemia after methionine loading. Thus the impaired capacity to handle postloading homocysteinemia persists.<sup>64</sup>

Patients not responsive to pyridoxine have been treated with variable outcome with folic acid and betaine to improve homocysteine remethylation.<sup>5</sup> Betaine efficiently normalizes the plasma homocysteine level without significant side effects.<sup>95,96</sup> Betaine has also been shown to reduce the elevation of plasma homocysteine in patients responsive to B<sub>6</sub> after methionine loading.<sup>97</sup>

Patients with homocystinuria as a result of lack of 5,10-methylenetetrahydrofolate reductase respond to folate<sup>98</sup> or betaine administration<sup>99</sup> or a combination of vitamin B<sub>6</sub>, B<sub>12</sub>, and folinic acid.<sup>100</sup> These patients show both clinical improvement and normalization of the plasma amino acids.

Homocystinuria with reduced methionine synthase activity caused by various congenital defects in cobalamin metabolism and transport<sup>79</sup> may respond to hydroxycobalamin administration, alone<sup>70,78,101</sup> or in combination with betaine.<sup>102</sup>

Plasma serine was below normal in two patients deficient in cystathionine  $\beta$ -synthase on folate therapy but normal in two patients with defective remethylation of homocysteine.<sup>103</sup> Betaine treatment reduced plasma homocysteine in both types of patients but increased plasma serine only in the patients deficient in cystathionine  $\beta$ -synthase. One possible explanation is that increased tissue burden of homocysteine in the patients deficient in cystathionine  $\beta$ -synthase may increase the 5,10-methylenetetrahydrofolate-reductase reaction to provide 5-methyltetrahydrofolate required for enhanced homocysteine remethylation. Serine is consumed in the serine hydroxymethylase reaction, which forms 5,10-methylenetetrahydrofolate (Fig. 2). It is conceivable that this mechanism may not operate in patients with remethylation defects.

The biochemical and clinical response to compounds like betaine and folic acid in various forms of congenital homocystinuria points to the possibility that these factors may reduce the homocysteine level by enhancement of its remethylation. This therapeutic success suggests that such intervention may also work under conditions characterized by moderate elevation of plasma homocysteine.

### HOMOCYSTEINE, EXPERIMENTAL THROMBOEMBOLISM AND ARTERIOSCLEROSIS

The potential atherogenic properties of homocysteine have been evaluated by both in vivo and in vitro experiments, and two different approaches have been used. Homocysteine is either directly tested in the experimental system or indirectly by inducing metabolic defects assumed to elevate homocysteine. Such defects can be obtained by depletion of cofactors or metabolites involved in homocysteine or one-carbon metabolism.

**In vivo experiments.** In 1952 Hartroft et al.<sup>104</sup> described atheromatous changes in aorta, carotid, and coronary arteries of rats deficient in choline. Choline deficiency has recently been shown to increase the homocysteine content in tissues and plasma in this species.<sup>105</sup>

In 1949 Rinehart and Greenberg<sup>106</sup> reported on arteriosclerosis in monkeys with chronic pyridoxine deficiency. In pigs, which have a cardiovascular system resembling that of man,<sup>107</sup> vitamin B<sub>6</sub> deficiency induced limited arterial lesions within 12 weeks.<sup>108</sup> Since pyridoxal 5'-phosphate is a cofactor of cystathionine  $\beta$ -synthase (Fig. 2) inhibition of homocysteine catabolism may be responsible for the cardiovascular effects. Pyridoxine-deficiency enhances homocysteine excretion in rabbits<sup>109</sup> and man<sup>110,111</sup> and induces increased plasma homocysteine in the pig<sup>108</sup> and rat.<sup>112</sup> It is been suggested that vitamin B<sub>6</sub> deficiency early in life may predispose to arteriosclerosis by interfering with elastin metabolism.<sup>113</sup> This is supported by an experimental study in rat pups deficient in vitamin B<sub>6</sub>. Changes in elastin cross-linking were observed, resembling those observed in penicillamine toxicity and homocystinuria, and there was an increase in plasma homocysteine.<sup>114</sup> However, one should not rush to conclusion that the vascular effects of vitamin B<sub>6</sub> deficiency are induced by elevated homocysteine, since vitamin B<sub>16</sub> is a coenzyme for more than 50 enzymes.<sup>115</sup>

There is one recent report on cardiovascular lesions in experimental animals induced by vitamin B<sub>12</sub> deficiency. Hemorrhagic inflammation of certain blood vessels accompanied by necrosis in the heart were observed in five out of six sheep after a period of 34 weeks of vitamin B<sub>12</sub> deficiency. These changes resembled those observed in early arteriosclerosis.<sup>116</sup>

Arteriosclerosis has been produced by parenteral administration of homocysteine thiolactone to rabbits<sup>117</sup>

and by feeding rabbits sulfur amino acids.<sup>118</sup> Short-term injection of homocystine caused patchy loss of arterial endothelium and shortening of platelet survival in baboons, whereas sustained treatment induced arterial damage resembling those observed in early human arteriosclerosis.<sup>81</sup> These chronic prearteriosclerotic lesions, but not the acute endothelial damage from homocystinemia, were prevented by the antiplatelet drug dipyridamole. This may suggest that homocysteine produces arteriosclerosis through platelet-mediated intimal proliferation of smooth muscle cells.<sup>119</sup> Sulfinpyrazone, which is also regarded as a platelet function inhibitor, decreased aortic endothelial injury and normalized platelet survival time in chronic homocystinemic baboons, but the drug was ineffective *in vitro* in an assay for endothelial cell detachment in the presence of homocysteine. The authors suggest that *in vivo* sulfinpyrazone may have a protective effect on endothelial integrity by an indirect mechanism.<sup>120</sup>

Endothelial damage, platelet sequestration, and venous thrombosis were also observed in rats given a single homocystine injection.<sup>121</sup> The endothelium after methionine administration to rats was reduced by pyridoxine, acetylsalicylic acid,<sup>122</sup> and the antiarteriosclerotic drugs pyridinolcarbamate and phthalazinole II<sup>123</sup> and increased by estrogen.<sup>124</sup> Endothelial damage by homocystinemia could not be produced in pigs.<sup>125</sup>

For unknown reasons the experimentally induced arteriosclerosis produced *in vivo* by homocysteine thiolactone in the rabbit, or vitamin B<sub>6</sub> deficiency in the monkey could not always be reproduced.<sup>126-128</sup>

Some objections have been made to the use of homocysteine thiolactone in experimental arteriosclerosis. This compound is readily converted to diketopiperazine and is slowly and spontaneously hydrolyzed to homocysteine at neutral pH (7.4) of plasma and probably efficiently hydrolyzed by esterase present in liver and other tissues. The fraction not hydrolyzed may acylate amino groups of proteins, peptides, and amino acids, and thereby alter their structure and function.<sup>129</sup>

***In vitro* experiments.** Pyridoxal 5'-phosphate is an inhibitor of platelet aggregation<sup>130-132</sup> and blood coagulation<sup>130</sup> and inhibits many enzymes. However, it does not readily cross the cell membrane, suggesting a site of action at the cell surface.<sup>132</sup> The mechanism of the platelet effect, and its relation to homocysteine metabolism is not established.

In cystathionine  $\beta$ -synthase-deficient cells in culture, homocysteine thiolactone increased the amount of aggregated proteoglycan matrix with altered structure and function.<sup>133</sup> Factors that may contribute to vascular disease in homocystinuria include inhibition of cross-linking during synthesis of elastin and collagen,<sup>134</sup> increased prostaglandin formation in platelets and blood vessels<sup>135</sup> possibly mediated by hydrogen peroxide,<sup>136</sup>

activation of coagulation factors XII<sup>137</sup> and V,<sup>138</sup> which may initiate blood coagulation, and a direct cytotoxic effect on the endothelial cells.<sup>139,140</sup> Endothelial cell injury was decreased by penicillamine, which probably acts by blocking the free thiol group through the formation of a mixed disulfide.<sup>140</sup> Lysis of endothelial cells may be induced by hydrogen peroxide, which is formed during oxidation of homocysteine in the presence of copper.<sup>141</sup> Notably, patients with homocystinuria have increased plasma copper,<sup>142</sup> and endothelial cells from such patients seem to be even more sensitive to homocysteine than do normal cells.<sup>143</sup> Homocysteine and to a lesser degree cysteine promote detachment of human endothelial cells from tissue culture dishes, suggesting that sulfhydryl-containing amino acids may contribute to atherogenesis by diminishing cell adhesions.<sup>144</sup>

There are two reports suggesting a link between homocysteine and plasma lipids. Various thiols, including homocysteine, oxidize low-density lipoproteins in the presence of redox metals.<sup>145</sup> Modified low-density lipoproteins may not be recognized by the low-density lipoproteins receptor but seems to interact with the scavenger receptor.<sup>146</sup> It is conceivable that such interaction is a process involved in the accumulation of cholesterol esters in foam cells that are often found in early atherosclerotic lesions.

#### **HETEROZYGOUS HOMOCYSTINURIA AND MODERATE HOMOCYSTEINEMIA AS RISK FACTORS FOR VASCULAR DISEASE**

The pioneering work of Gupta and Wilcken<sup>37</sup> shows that men have higher level of plasma homocysteine-cysteine mixed disulfide than women, and the plasma concentration in women increases after the age of 50 years, and in postmenopausal women it equals levels found in men.<sup>147</sup> Plasma homocysteine after methionine loading has been reported to be higher in postmenopausal women than in men.<sup>40</sup> Based on these findings Boers et al.<sup>40</sup> suggested that efficient methionine metabolism protects women against vascular disease in their reproductive years. The sex- and age-related variations in plasma homocysteine,<sup>37,40</sup> which have been contested in one report,<sup>48</sup> but recently confirmed in studies based on protein-bound<sup>47</sup> or total homocysteine,<sup>68</sup> parallel well-established<sup>148</sup> age- and sex-related risk factors of atherosclerotic disease.

The frequency of heterozygosity for cystathionine  $\beta$ -synthase deficiency in the general population is 1/70 at most, and probably 1/100 to 1/290.<sup>5,72</sup> This may offer an opportunity to investigate the relation between mild homocystinemia and arteriosclerosis. In an epidemiologic study based on questionnaires from about 800 obligately heterozygous subjects, Mudd et al.<sup>149</sup> could not detect an increased incidence of heart attacks or strokes. However, in a reevaluation of this study,

Swift and Morrell<sup>150</sup> questioned the validity of some methods and the conclusion of Mudd et al., and suggested that the data indicate a relation between death from cardiovascular disease and heterozygosity for cystathionine  $\beta$ -synthase deficiency.

Individuals who are obligatory heterozygotes for cystathionine  $\beta$ -synthase deficiency have more than 50% reduction in enzyme activity,<sup>151</sup> and an increased amount of sulfur amino acids including homocysteine (mixed) disulfide in plasma after methionine loading. This response has been used for the identification of such individuals.<sup>63</sup> Such criteria for identification are not exclusive, since there is a considerable overlap in plasma homocysteine between heterozygous and control subjects,<sup>152</sup> and normal postmenopausal women may be erroneously classified as heterozygous.<sup>153</sup> Simultaneous measurement of cystathionine  $\beta$ -synthase in cultured fibroblasts may identify most heterozygous subjects.<sup>154</sup>

Recent reports suggest that protein-bound homocysteine may be a simple and particularly useful measurement to identify individuals with heterozygous homocystinuria.<sup>44,61</sup> When total homocysteine, which includes both free and protein-bound fraction, was determined during fasting and postmethionine loading, significantly higher levels were found in both premenopausal and postmenopausal women heterozygous for cystathionine  $\beta$ -synthase deficiency compared with their respective controls. In men who were heterozygous only the postloading values were found to be significantly higher than controls.<sup>68</sup>

The possible relation between mild homocysteinemia and coronary heart disease was first investigated by Wilcken and Wilcken<sup>36</sup> in 1976. The acid-soluble homocysteine mixed disulfide in plasma after methionine loading increased more in such patients compared with controls, suggesting impaired homocysteine metabolism. This finding was later contested by a study from the same laboratory including additional patients.<sup>152</sup> When the methionine dose was corrected for body weight, the investigators found elevated acid-soluble plasma homocysteine derivatives in only two of 20 patients.

Boers et al.<sup>65</sup> found a predominance for heterozygosity for homocystinuria among patients with premature peripheral and cerebral vascular disease, but not among patients with cardiac infarction. The authors related this finding to the observation of a similar distribution of vascular disease in coronary versus cerebral or peripheral arteries in homozygous patients. Brattström et al.<sup>49</sup> demonstrated methionine intolerance, defined as a certain increase in total plasma homocysteine above basal level and therefore consistent with heterozygosity for cystathionine  $\beta$ -synthase deficiency, in 28% of patients

with early-onset occlusive arterial disease. In one study plasma homocysteine mixed disulfide both before and after methionine loading was significantly elevated in patients ( $n = 19$ ) with arteriosclerotic cerebrovascular disease compared with controls.<sup>68</sup>

The somewhat inconsistent data on the role of mild homocysteinemia in coronary vascular disease should be considered in the light of the small number of patients in some early studies, the inaccuracy in recording disease incidence by questionnaire,<sup>150</sup> and last, but not the least, the determination of only acid-soluble homocysteine derivatives in plasma, which thereby excludes the major protein-bound fraction.

Kang et al.<sup>47</sup> recently studied the relation between coronary heart disease and protein-bound homocysteine in stored plasma in 241 patients. Factors like age and sex were taken into account. The difference in plasma level between patient and controls was highly significant.

Similar conclusions have been made from several other studies. In a preliminary report on 108 patients Freedman et al.<sup>155</sup> found plasma homocysteine (probably bound plus free fraction<sup>156</sup>) after methionine loading to be related to coronary artery occlusion. In a small study where homocysteine, folate, vitamin B<sub>6</sub> and vitamin B<sub>12</sub> in plasma were measured, free plasma homocysteine was found to be elevated in five men out of 20 at high risk for coronary heart disease relative to controls.<sup>157</sup> Murphy-Chutorian et al.<sup>67</sup> reported that 16% of 99 patients with coronary artery disease had abnormal methionine loading test defined as postloading homocysteine above the 95th percentile. Such response was observed in only 2% of angiographically normal controls. The prevailing acid-soluble homocysteine species in plasma after methionine loading, homocysteine-cysteine mixed disulfide, was also measured in this study, but a similar difference between coronary patients and control subjects was present but less pronounced.

In a recent, carefully conducted study, Israelsson et al.<sup>158</sup> determined total homocysteine in plasma during fasting and postmethionine loading in 21 men who had their first myocardial infarction before the age of 55 years. These patients were selected from a health screening to have low incidence of conventional risk factors like hypertension, smoking, serum cholesterol, but had a family burden of cardiovascular disease. These patients had a significantly higher fasting total homocysteine in plasma compared with matched controls, but there was a prominent overlap between the groups, and the difference was due to a marked elevation in five individuals. Among these five patients three had postloading homocysteine values comparable to those of heterozygous individuals. This study gives

support to the hypothesis that moderate elevation of plasma homocysteine is a risk factor for coronary heart disease.

Two recent reports dealt with homocysteinemia and peripheral arterial disease.<sup>68,159</sup> Total plasma homocysteine was significantly increased during fasting and after methionine loading in the patients with arteriosclerotic disease in the aortoiliac vessels, compared with controls. Most patients had normal serum vitamin B<sub>12</sub> and erythrocyte folate.<sup>68</sup> In another study, basal total homocysteine in plasma was significantly increased in patients with peripheral arterial occlusive disease, and analyses of data suggested that elevated plasma homocysteine was a risk factor independent of established factors like cigarette smoking, hypertension, and diabetes.<sup>159</sup>

These studies suggest that arteriosclerotic lesions induced by homocysteinemia are not confined to the coronary or cerebral vessels, but that homocysteine may be a risk factor for generalized arteriosclerotic disease.

Heterozygosity for cystathionine  $\beta$ -synthase has long been considered as a cause of moderate homocysteinemia and arterial disease, but there are indications that other enzymic defects are involved as well. Kang et al.<sup>160</sup> have recently demonstrated the association of thermolabile lymphocyte methylenetetrahydrofolate reductase with premature coronary artery disease, suggesting the presence of a mutant enzyme. It is conceivable that low methylenetetrahydrofolate reductase may cause lack of 5-methyltetrahydrofolate, which in turn may produce homocysteinemia. However, only two out of six patients with this enzymic defect had homocysteinemia.<sup>160</sup> Kang et al.<sup>161</sup> also report on two patients with intermediate homocysteinemia, one with coronary artery disease. Both had thermolabile lymphocyte methylenetetrahydrofolate reductase, subnormal serum folate, and low or subnormal vitamin B<sub>12</sub>. The role of homocysteine in the development of arterial disease in these patients is not defined.

There are recent, but unconfirmed reports that plasma homocysteine may exist in forms not detected by ordinary procedures and may accumulate as such in large amounts in patients with coronary disease. McCully and Vezeridis<sup>162</sup> described incredibly high concentration of homocysteine thiolactone (in the millimolar range) in human serum, and the level was higher in patients with coronary heart disease compared with controls. Two other reports describe remarkably high amounts (average of about 1000  $\mu$ mol/L) of homocysteine in acid hydrolyzates of plasma proteins from patients with ischemic heart disease. This level was 25 times the amount found in controls. In addition to homocysteine, cystathionine and  $\alpha$ -amino adipic acid were also reported to be markedly elevated in these patients, whereas the

amount of other amino acids was normal.<sup>163</sup> When these patients were given a mixture of pyridoxine, folate, cobalamin, choline, riboflavin, and troloxerutin, agents which may enhance homocysteine metabolism, there was a reduction in homocysteine in acid hydrolyzates of plasma as well as reduction in plasma lipids.<sup>164</sup>

The recognition of moderate homocysteinemia as an independent risk factor of premature atherosclerosis points to the possibility that conditions characterized by reduced plasma homocysteine should have a low incidence of cardiovascular disease. Down's syndrome may represent such a condition. Murdoch et al.<sup>165</sup> noted the absence of arteriosclerosis in five patients aged 40 to 66 years, whereas patients who were mental defectives without Down's syndrome in the same institution had mild to severe atheromatosis. The authors suggested that Down's syndrome should be considered an atheroma-free model. These patients have elevated level (166%) of cystathionine  $\beta$ -synthase,<sup>166</sup> which has been assigned to chromosome 21.<sup>6</sup> Increased gene dosage of the enzyme may explain the increased enzyme activity. Brattström et al.<sup>167</sup> first suggested that these patients may be protected against arteriosclerosis by low plasma homocysteine level as a result of efficient homocysteine metabolism. Interestingly, low fasting and postloading plasma homocysteine compared to controls have recently been demonstrated in patients with Down's syndrome.<sup>166</sup>

In conclusion, the present stage of knowledge strongly suggests a role of mild homocysteinemia in the development of atherosclerotic lesions, both in cerebral, coronary, and peripheral vessels. Obviously, it is of utmost importance to uncover such a relation. This will require large prospective studies taking into account factors like age, sex, serum folate, and vitamin B<sub>12</sub>, and other factors, which may modulate plasma homocysteine level. The interaction with other coronary risk factors should be considered as well. When assaying plasma homocysteine precaution should be taken to avoid redistribution between free and bound homocysteine, and in studies based on stored plasma samples total homocysteine should be measured.

#### **HOMOCYSTEINE IN PLASMA IN DEFICIENCY OR DISEASE STATES OTHER THAN HOMOCYSTINURIA**

**Vitamin B<sub>12</sub> deficiency.** It has been well documented that inborn errors of cobalamin metabolism resulting in decreased methionine synthase activity, lead to homocysteinemia (and homocystinuria) (see the paragraph herein entitled "Homocystinuria"). Recently, data have been published on homocysteine in cobalamin deficiency caused by the much more common conditions characterized by reduced bioavailability for vitamin B<sub>12</sub>.<sup>22</sup> In one small study, free plasma homocysteine

was negatively related to plasma vitamin B<sub>12</sub>.<sup>157</sup> Bratts-tröm et al.<sup>168</sup> recently reported that fasting total homocysteine in plasma of 20 patients deficient in vitamin B<sub>12</sub> was significantly higher ( $23.8 \pm 17 \mu\text{mol/L}$ ) than in healthy controls ( $11.5 \pm 4.2 \mu\text{mol/L}$ ) and heterozygotes for cystathionine  $\beta$ -synthase deficiency ( $13.8 \pm 5.8 \mu\text{mol/L}$ ). The plasma homocysteine was normalized 14 days after administration of hydroxycobalamin.<sup>168</sup> In a recent study including 78 patients deficient in vitamin B<sub>12</sub>, Stabler et al.<sup>169</sup> reported that all except one had basal total homocysteine in serum above normal (range 11 to 476  $\mu\text{mol/L}$ ), and extremely high levels (up to 476  $\mu\text{mol/L}$ ) were recorded in some of these patients. Total homocysteine showed a negative correlation with serum vitamin B<sub>12</sub>, and correlated with the hematologic abnormalities. The authors suggest that homocysteine in serum is a useful parameter for both diagnosis and follow-up during treatment of patients deficient in vitamin B<sub>12</sub>.<sup>170</sup> The soundness of this proposal has been thoroughly documented in a clinical study on 41 patients with neuropsychiatric disorders and cobalamin deficiency.<sup>171</sup> A significant portion (about 20%) of these patients revealed no hematologic abnormalities, but total homocysteine in plasma was markedly elevated. They benefited from cobalamin treatment, which also reduced plasma homocysteine. Thus, total homocysteine is important for the diagnosis of cobalamin deficiency in patients with unexplained neuropsychiatric disorders,<sup>171</sup> and may also become particularly useful in the cases, where the expected findings of very low serum cobalamin levels, anemia, and macrocytosis are lacking.<sup>172</sup>

Since total homocysteine in plasma is a function of a biologic effect of vitamin B<sub>12</sub>, this parameter may be particularly helpful in the diagnosis of congenital defects of vitamin B<sub>12</sub> metabolism. A defect characterized by the absence of the R-binder results in low serum vitamin B<sub>12</sub> level but no tissue deficiency, and such patients seem to have normal serum homocysteine.<sup>62</sup> In patients with a defect in methylcobalamin synthesis there is no total body vitamin B<sub>12</sub> deficiency and normal serum level, but total homocysteine in plasma is elevated.<sup>62</sup> One such adult patient has recently been described. She was 21 years old at the time of diagnosis, but had for years suffered from neurologic disorders, which had been misdiagnosed as multiple sclerosis. The macrocytosis was subtle.<sup>80</sup> Hereditary cobalamin disorders should be considered in young adults with mental or neurologic disorders of unknown cause. Monitoring plasma homocysteine may be a particularly helpful diagnostic tool.<sup>80</sup>

Low serum vitamin B<sub>12</sub> has been found in clinical studies on homocysteinemia in arteriosclerotic disease in patients with elevated plasma homocysteine<sup>152</sup> or with

premature arteriosclerotic disease alone or in combination with low folate or elevated plasma homocysteine.<sup>68</sup> However, apart from experimental study in sheep,<sup>116</sup> no direct evidence exists that nutritional vitamin B<sub>12</sub> deficiency causes premature vascular disease. Lack of such evidence may be related to the fact that this deficiency state usually develops at advanced age,<sup>22</sup> and the thrombocytopenia<sup>169</sup> may antagonize the development of arterial lesions.<sup>173</sup>

**Folate deficiency.** Folate deficiency is a common condition that is usually caused by low intake relative to the metabolic demand or interference of folate metabolism by drugs.<sup>174</sup> Folate is required for remethylation of homocysteine to methionine, which may account for a significant portion of intracellular homocysteine consumption (see paragraph "Homocysteine metabolism"). It is therefore conceivable that folate deficiency may cause homocysteinemia.

Stabler et al.<sup>169</sup> found elevated serum total homocysteine in 18 of 19 patients deficient in folate (range 17 to 185  $\mu\text{mol/L}$ ). Kang et al.<sup>175</sup> studied 239 subjects with subnormal (<2 ng/ml), low normal (2 to 3.9 ng/ml) or normal (4 to 17.9 ng/ml) serum folate. Protein-bound homocysteine in stored blood was determined, and errors related to redistribution of homocysteine in plasma were avoided. Folate deficiency was associated with marked elevation of homocysteine (up to 70  $\mu\text{mol/L}$ ), which was negatively correlated with serum folate level. Notably, more than 50% of the patients with subnormal serum folate had elevated serum homocysteine, suggesting that folic acid intake resulting in serum folate level defined as normal may not be sufficient for a efficient remethylation of homocysteine.<sup>175</sup> This possibility is supported by an experimental study in the rat given a folic acid deficient diet, showing that serum homocysteine was elevated to the same extent in rats having low normal and subnormal serum folate.<sup>176</sup>

Some patients receiving folic acid had high serum folate but also high protein-bound homocysteine.<sup>175</sup> This may be related to tissue depletion of reduced folates before folic acid administration. Thus, plasma homocysteine probably reflects the intracellular folate status. This possibility is strongly supported by the observation that small doses of the antifolate, methotrexate, gives transient increase in both free and bound homocysteine in plasma.<sup>177</sup>

It is noteworthy that the high plasma homocysteine level in some patients with folate deficiency equals the level associated with high incidence of arteriosclerotic disease. In a study of plasma homocysteine in patients with coronary heart disease, a negative correlation was observed between plasma homocysteine values and both erythrocyte folate and serum vitamin B<sub>12</sub>.<sup>158</sup> This

suggests that prolonged folate deficiency may play a role in the development of vascular disorders.<sup>175</sup>

**Vitamin B<sub>6</sub> deficiency.** The enzyme cystathionine  $\beta$ -synthase requires pyridoxal 5'-phosphate as a coenzyme.<sup>5</sup> In vitamin B<sub>6</sub>-deficient rats there is a decreased enzyme activity,<sup>178</sup> which leads to accumulation of homocysteine in tissues.<sup>179</sup> A marked increase of both free and protein-bound homocysteine in plasma has been observed in the rat within a few weeks,<sup>112,114</sup> and the homocysteine concentrations were similar to those observed in subjects with homocystinuria. These high values were normalized within 2 days after supplementing adequate amounts of vitamin B<sub>6</sub>. In pigs, vitamin B<sub>6</sub> deficiency induced an even greater increase in free and protein-bound plasma homocysteine than in rat.<sup>107</sup> After 12 weeks limited arterial lesions were revealed by light microscopy in the deficient pigs.<sup>108</sup>

Data on vitamin B<sub>6</sub> deficiency, homocysteine, and arteriosclerosis in humans are sparse, but the relation between the deficiency state and arteriosclerosis in man has been documented in several epidemiologic studies.<sup>49,113,180-182</sup> Furthermore, smoking<sup>183</sup> and pollutants such as carbon monoxide and carbon disulfide<sup>184</sup> induce vitamin B<sub>6</sub> deficiency. Low plasma pyridoxal 5'-phosphate has been reported in women taking oral contraceptives.<sup>115</sup> These are factors known to enhance the development of arteriosclerosis or thromboembolism. The possibility that such effects from lack of vitamin B<sub>6</sub> are mediated by homocysteine is in accordance with the finding of elevated plasma homocysteine in subjects deficient in pyridoxine.<sup>110</sup> However, the fact that more than 50 vitamin B<sub>6</sub>-dependent enzymes exist<sup>115</sup> is a serious objection to this conclusion.

One study on the relation between vitamin B<sub>6</sub> deficiency, homocysteinemia, and cardiovascular disease in humans has recently been published.<sup>49</sup> About 30% of the patients with premature peripheral or cerebral arterial disease had basal homocysteinemia or methionine intolerance determined as marked postloading increase in plasma homocysteine, and the basal homocysteinemia was strongly negatively correlated to serum vitamin B<sub>12</sub> and folate. Notably, 60% of the patients had subnormal plasma vitamin B<sub>6</sub> levels, which showed no correlation with plasma homocysteine levels. This suggests that vitamin B<sub>6</sub> deficiency independently of homocysteine may be associated with vascular disease.

**Zinc deficiency.** Zinc deficiency causes manifestations from various organs, that is, central nervous system, skin, gastrointestinal tract, endocrine glands, and affects growth and development. Such diverse symptoms are related to the fact that zinc is an essential constituent in more than 200 enzymes, the activities of which may become altered during zinc deficiency.<sup>185</sup>

Defects in amino acid metabolism, especially sulfur amino acids,<sup>186</sup> protein synthesis,<sup>187</sup> and the nucleic acid metabolism<sup>188,189</sup> have been reported. Moreover, zinc deficiency affects folate metabolism.<sup>190</sup> No information exists on serum homocysteine levels during zinc deficiency in humans, but there are some indications that lack of zinc may affect homocysteine metabolism.

The use of S-adenosylmethionine for methylation of various compounds<sup>191</sup> and methionine for protein synthesis<sup>192</sup> seems to be impaired in isolated liver from zinc-deficient rats. Homocysteine is diverted from remethylation to increased flux through the transsulfuration pathway.<sup>192</sup> The finding of increased amount of catabolic products of methionine, like cysteine or taurine in the urine from zinc-deficient rats<sup>186,193,194</sup> is in accordance with increased net flux through this pathway. No enzymes involved in the recycling of homocysteine back to methionine were adversely affected,<sup>192</sup> but there was an increased methionine synthase activity in the liver from zinc-deficient rats.<sup>190</sup> The increase in this enzyme may be related to low hepatic 5-methyltetrahydrofolate and low plasma folate levels.<sup>190</sup> Notably, increased egress of homocysteine has in fact been observed from the isolated liver from the zinc-deficient rat,<sup>192</sup> which could be explained by impaired remethylation due to lack of 5-methyltetrahydrofolate.

The knowledge on impaired metabolism of sulfur amino acids and folates during zinc deficiency make it pertinent to study plasma homocysteine levels in this deficiency state in man.

**Chronic renal failure.** Wilcken et al.<sup>195,196</sup> first demonstrated that plasma homocysteine, determined as the acid-soluble homocysteine-cysteine mixed disulfide, is increased (about twofold) in patients with chronic renal insufficiency including those regularly treated with hemodialysis. After dialysis the amount was markedly reduced to concentrations less than those of controls. The amount of plasma homocysteine-cysteine mixed disulfide in patients with reduced renal function<sup>195</sup> and renal transplant recipients<sup>197</sup> showed a positive correlation with serum creatinine. It has been suggested that reduced excretion may contribute to elevated plasma homocysteine-cysteine mixed disulfide in renal failure,<sup>198</sup> but this explanation is difficult to reconcile with the finding that renal excretion of homocysteine represents less than 0.1% of total homocysteine production.<sup>38,45</sup>

Increased plasma homocysteine in renal insufficiency has been confirmed and elaborated by others. Both total<sup>169</sup> and protein-bound homocysteine<sup>199</sup> in plasma are elevated in this condition. Protein-bound homocysteine in plasma was markedly increased (3.7 fold above normal) in patients requiring chronic hemodialysis, but the protein-bound fraction was only moderately (23%) re-



duced after dialysis, suggesting that lowering of the protein-bound homocysteine may be limited by the rate of dissociation of this form into free, dialysable homocysteine species.<sup>199</sup>

The homocysteinemia in patients with chronic renal failure is associated with reduced plasma serine.<sup>103,198</sup> Increased homocysteine burden may stimulate homocysteine consumption by increased flux through the 5-methyltetrahydrofolate-dependent remethylation of homocysteine and the cystathionine  $\beta$ -synthase reaction. Both the formation of 5-methyltetrahydrofolate from tetrahydrofolate and formation of cystathionine consume serine (Fig. 2). Thus reduced plasma serine in renal insufficiency brings support to the idea that homocysteinemia is not due to impaired homocysteine metabolism.<sup>198</sup>

Supplementing folic acid to patients with chronic renal failure reduced the elevated plasma homocysteine-cysteine mixed disulfide,<sup>197</sup> and the reduction was linearly related to the pretreatment level.<sup>198</sup> Vitamin B<sub>6</sub> or B<sub>12</sub> were without effect.<sup>197</sup> Folic acid also lowered plasma serine and increased plasma glycine suggesting further enhancement of remethylation of homocysteine to methionine.<sup>197</sup> Folate deficiency may occur in severe azotemia, but serum folate was normal in most of the patients studied.<sup>97</sup> The folic acid response may suggest a relative shortage of intracellular folates.

One may speculate whether elevated plasma homocysteine during chronic renal failure may contribute to the high incidence of occlusive arterial disease in such patients.<sup>200</sup> If so, folic acid treatment may reduce the risk for cardiovascular disease in chronic renal insufficiency.<sup>199</sup>

**Liver disease.** Data on plasma homocysteine in hepatic insufficiency have not been published, but consistent reports on impaired methionine metabolism in chronic liver disease<sup>201-203</sup> suggest that such investigations are warranted. In addition, there is reduced methionine clearance after a methionine loading in cirrhotic patients. The reduction was associated with reduced urinary excretion of inorganic sulfur, but no detectable accumulation of cystathionine or homocysteine in plasma or urine. This suggests decreased flux through the transsulfuration pathway as a result of a metabolic block proximal to homocysteine.<sup>204</sup> Plasma homocysteine in hepatic failure should be further investigated.

**Cancer.** There is ample evidence that homocysteine metabolism may become altered on malignant transformation of cells, but data on homocysteine in patients with cancer are sparse.

Most, but not all malignant cells are methionine-dependent, that is, they do not grow when methionine in the culture medium is replaced with homocysteine. This has been demonstrated with numerous cell lines

both of animal and human origin. Nontransformed cells thrive under these conditions. The metabolic basis for the inability of transformed cells to use homocysteine for growth has been extensively studied. Methionine-dependent cancer cells are able to synthesize methionine from homocysteine, but endogenous methionine does not meet the increased or altered metabolic demand of these cells. Methionine dependence of cancer cells is the subject of recent review articles.<sup>12,205,206</sup>

Only preliminary data exist on homocysteine metabolism in cancer patients. Total plasma homocysteine was found to be markedly increased (up to 70  $\mu\text{mol/L}$ ) in three out of six patients with acute lymphoblastic leukemia. Plasma methionine levels were normal.<sup>207</sup> We found normal values for total homocysteine in six patients with solid tumors treated with chemotherapy,<sup>208</sup> but high total homocysteine (15 to 25  $\mu\text{mol/L}$ ) in seven children with acute lymphatic leukemia before initiation of treatment. In the patients with leukemia plasma homocysteine fell drastically (to 4 to 5  $\mu\text{mol/L}$ ) within a few days after administration of cytotoxic drugs (Refsum H, et al., unpublished data, 1989). Thus plasma homocysteine seems related to the total burden of proliferating leukemic cells. Similar conclusion has been made by others.<sup>207</sup>

There may be some relation between methionine dependence of leukemic cells and homocysteinemia in patients with acute leukemia, but this hypothesis has not been sustained by experimental evidence. Most, if not all, leukemic cells are methionine-dependent,<sup>205</sup> and such cells have an insufficient capacity for the salvage of exogenous as well as endogenous homocysteine. Insufficient methionine synthesis could result from relative lack of reduced folate as cofactor in the homocysteine 5-methyl-tetrahydrofolate methyltransferase reaction, because reduced folates may be drained into other pathways that are enhanced in rapidly proliferating cells, such as the synthesis of purines and pyrimidines.<sup>24</sup> Low remethylation capacity together with enhanced transmethylation rate and thereby increased production of homocysteine in proliferating cells<sup>209</sup> result in a pronounced egress of homocysteine from such cells. Thus plasma homocysteine may reflect the burden of rapidly dividing leukemic cells, which are unable to metabolize endogenous homocysteine. Homocysteinemia may therefore be a measure for leukemic activity during remission and relapse. This hypothesis should be investigated.

**Psoriasis.** We have investigated the metabolic response measured as plasma homocysteine, with and without methionine loading, in patients with severe psoriasis receiving low-dose methotrexate.<sup>177</sup> During this study we observed that the basal homocysteine level in these patients before methotrexate treatment was significantly above that of matched controls. In addition,

some of the patients showed an increased homocysteine response after methionine loading.<sup>177</sup> Notably, a retrospective study has demonstrated an increased incidence of occlusive vascular disease in patients with psoriasis as compared with appropriate control subjects.<sup>210</sup>

It is conceivable that the mechanism behind the homocysteinemia in severe psoriasis resembles that proposed for malignant disease. These patients have a large burden of rapidly proliferating cells.<sup>211</sup> The germinative cell population has been estimated to be increased nine-fold, and a much larger fraction of these cells are in S-phase compared with normal skin. Furthermore, the total cell cycle was computed to be 37.5 hours which probably is the fastest cell cycle in human tissue *in vivo*.<sup>211</sup> In analogy with the malignant cells, rapidly proliferating germinative cells in the skin may export large amounts of homocysteine. Cellular homocysteine egress determined as homocysteinemia should be evaluated as a measure of the severity of psoriasis.

#### PHARMACOLOGIC AGENTS ESTABLISHED AS MODULATORS OF PLASMA HOMOCYSTEINE

**Folic acid.** Oral treatment with high doses of folic acid corrects within a few days elevated total plasma homocysteine in patients deficient in folate.<sup>169</sup> In patients with renal insufficiency<sup>198</sup> and renal transplant recipients with stable but reduced renal function<sup>197</sup> plasma homocysteine-cysteine mixed disulfide was markedly reduced after 2 to 4 weeks of oral folic acid intake, even in those patients without overt folate deficiency. The reduction was largest in the patients with high homocysteine level before folic acid administration.<sup>198</sup> A homocysteine lowering effect of folic acid has also been observed in patients with vascular disease, including patients with normal serum folates.<sup>49,152</sup> Administration of cofactors for homocysteine metabolism, like vitamin B<sub>6</sub> and vitamin B<sub>12</sub>, were without effect.<sup>49,197</sup>

Brattström et al.<sup>147</sup> first demonstrated that oral folic acid administration reduced plasma homocysteine, determined as the homocysteine-cysteine mixed disulfide, in healthy men and women not deficient in folate. Such intervention reduced both the basal fasting as well as postloading levels. These findings were later confirmed and extended in a study on total plasma homocysteine in normal subjects. Folic acid at doses of 5 mg daily for 14 days markedly reduced (mean 52%) plasma homocysteine, especially in subjects with high pretreatment values. The concentrations in plasma of other amino acids linked to homocysteine metabolism were only marginally affected. Again, vitamin B<sub>6</sub> and vitamin B<sub>12</sub> were not effective.<sup>212</sup>

Folic acid probably reduces plasma homocysteine by increasing the rate of its intracellular metabo-

lism. It is conceivable that folic acid administration may increase the intracellular pool of 5-methyltetrahydrofolate which in turn may serve as a methyl-donor in the methionine synthase reaction. Increased 5-methyltetrahydrofolate has been suggested to enhance this transmethylation reaction by so called "mass action effect."<sup>186</sup> Reduced plasma serine after folic acid administration<sup>147,212</sup> is in agreement with such a mechanism since serine may be consumed to supply 5-methyltetrahydrofolate to the methionine synthase reaction. No effect from administration of either vitamin B<sub>6</sub> or vitamin B<sub>12</sub> to subjects not deficient in these compounds<sup>212</sup> may be related to the fact that they function as cofactors in homocysteine metabolism and are not consumed in these reactions (Fig. 2).

Since there is evidence that moderate homocysteinemia is a risk factor for arteriosclerotic vascular disease, folic acid administration may represent a useful pharmacologic intervention. There are few side effects, mostly urticarial in nature.<sup>174</sup>

**Methotrexate.** Methotrexate is an antifolate drug that has been widely used in the cytostatic therapy of acute leukemia and several solid tumors,<sup>213</sup> and recently in low doses in the management of psoriasis,<sup>214</sup> rheumatoid arthritis,<sup>215</sup> and some other nonmalignant disorders.<sup>216</sup>

This drug is metabolized intracellular to polyglutamates, and both the parent drug and these anabolites inhibit dihydrofolate reductase, the enzyme responsible for the regeneration of tetrahydrofolate from dihydrofolate. Methotrexate reduces the amount of reduced folates and thereby inhibits several pathways dependent on these species, including the thymidylate synthase reaction required for deoxyribonucleic acid synthesis. These and other aspects of the mechanism of action of methotrexate have been recently reviewed.<sup>213,217,218</sup>

Methotrexate enhanced the homocysteine egress from both nontransformed and malignant fibroblasts in culture.<sup>219</sup> These cells have the ability to grow on homocysteine instead of methionine,<sup>220</sup> suggesting that the 5-methyltetrahydrofolate-homocysteine methyltransferase activity is sufficiently active to provide methionine for cell growth. A murine leukemic cell line, which is completely methionine-dependent, exports large amounts of homocysteine, but the egress is only slightly enhanced during methotrexate exposure (Refsum H, et al., unpublished data, 1989). Thus, some but not all cell types show an increase homocysteine export in the presence of methotrexate. The possibility that the homocysteine response may be related to the efficiency of the methionine synthase reaction and so-called methionine dependence of some cell types, is an intriguing but unsolved question.

The results from the *in vitro* experiments were pursued by investigating the amount of homocysteine in

plasma and urine from patients with solid tumors receiving methotrexate infusion in doses of 1 to 13 gm. This caused within few hours a transient increase in free and total plasma homocysteine, which was reversed at the time of administration of leucovorin "rescue."<sup>208</sup> Notably, this homocysteine response as well as the basal homocysteine level were progressively decreased after each methotrexate dose, suggesting development of an adaptive process.<sup>208</sup> Alternatively, this reduction in plasma homocysteine after each methotrexate infusion may be induced by the leucovorin administrations, or may be related to kill of cancer cells exporting homocysteine.

In children with acute leukemia and high pretreatment levels of plasma homocysteine, chemotherapy with methotrexate as well as other cytotoxic agents reduced plasma homocysteine parallel to reduction in leukemic cell count (Refsum H, et al., unpublished data, 1989). In these patients the effect of methotrexate on homocysteine metabolism may be masked by the fall in plasma homocysteine related to the eradication of proliferating cells.

The limited data on folate status in patients receiving long-term, low-dose methotrexate<sup>221-223</sup> suggest that treatment for years<sup>221</sup> but not for months<sup>222,224</sup> induces a cumulative effect on folate status. However, the antifolate effect of methotrexate seems to be responsible for the therapeutic effect at least in rheumatoid arthritis, since leucovorin antagonizes this effect as well as the side effects of methotrexate.<sup>224</sup>

Administration of low-dose methotrexate (25 mg) to patients with psoriasis induced a transient increase in plasma homocysteine. Plasma homocysteine returned to normal within 6 days, but the basal level between treatments remained stable. The response was observed through eight consecutive treatments over a time period of 2 months in some patients.<sup>177</sup> This suggests that low-dose methotrexate treatment induced a short-term impairment of some folate-dependent processes, but had no cumulative effect on tissue folates within the time period of the investigation. These findings also add support to the hypothesis<sup>14</sup> that plasma homocysteine is a sensitive measure of antifolate effect.

The plasma homocysteine after standard methionine loading (peak area  $AUC_p^{0-t}$  and plasma level) was determined in patients with psoriasis before and during methotrexate treatment. Notably, methotrexate did not affect the postloading homocysteine,<sup>177</sup> which should be related to the observation that basal plasma homocysteine but not postloading level was related to erythrocyte folate.<sup>49</sup>

The source of plasma homocysteine is an important question. It seems likely that the increased basal homocysteine in some patients with cancer or psoriasis

stems from a large burden of rapidly proliferating cells. However, the homocysteine response to methotrexate exposure obviously is not confined to such cells, because in vitro at least, not all cancer cells show increased homocysteine export in the presence of methotrexate (Refsum et al, unpublished). Furthermore, in the normal rat with no tumor burden, methotrexate administration increased total serum homocysteine, and the serum level paralleled the response of the liver.<sup>105</sup> This suggests that the homocysteine metabolism in the liver has a major impact on the plasma level.

The possible mechanism behind the moderate homocysteinemia induced by methotrexate is rather straightforward. Methotrexate decreases intracellular level of reduced folates. Notably, among the different species, 5-methyltetrahydrofolate is most efficiently depleted.<sup>225,226</sup> Therefore, among the reactions requiring reduced folates, the 5-methyltetrahydrofolate-dependent salvage of homocysteine to methionine should be particularly impaired in the presence of methotrexate. Since the balance between homocysteine production and utilization and thereby level of intracellular homocysteine seems tightly coupled to homocysteine egress,<sup>34,35</sup> homocysteine in extracellular media like plasma may reflect the intracellular folate status.

There are some direct implications of the increased plasma homocysteine after methotrexate exposure. First, it is possible that increased basal homocysteine level may be a useful measure of the immediate metabolic effect and that a decrease reflects the eradication of proliferating cells. The latter effect may be shared by other cytostatic agents. Secondly, elevated plasma homocysteine may by itself be innocuous. It has recently been shown that anticancer regimens including methotrexate have thrombogenic effect that can be distinguished from that caused by the malignant disease.<sup>227</sup> Plasma homocysteine should be considered as a thrombogenic factor in patients receiving such treatment.<sup>228</sup>

**Nitrous oxide.** The anesthetic agent nitrous oxide, once considered to be chemically and biologically inert, reacts with transition metal complexes in solution, including cobalt in vitamin B<sub>12</sub>. Nitrous oxide oxidizes cob(I)alamin to cob(II)alamin, which can no longer function as a methyl carrier. There are two vitamin B<sub>12</sub>-dependent enzymes but vitamin B<sub>12</sub> functions as a methyl donor only in the homocysteine-5-methyltetrahydrofolate methyltransferase reaction (Fig. 1). This enzyme is prone to inactivation in the presence of nitrous oxide. In man there is a slow onset of inactivation with about 50% reduction in activity after 2 hours. A reduction in plasma methionine follows, which occurs between 8 and 24 hours of exposure. Interference with the folate-dependent step in thymidylate synthesis required for cell proliferation occurs after variable expo-

sure, and long-term effects like megaloblastic anemia and myelopathy have been reported.<sup>229</sup>

The biochemical and clinical effects of nitrous oxide are the subject of recent excellent review articles.<sup>229,230</sup>

In sheep exposed to nitrous oxide there is a pronounced increase in urinary excretion of homocysteine, which may reflect increased plasma level. Plasma homocysteine was not determined.<sup>231</sup> In the fruit bats, a species in which chronic nitrous oxide exposure leads to neurologic impairment, nitrous oxide markedly increases plasma homocysteine.<sup>232</sup>

In man nitrous oxide reduces plasma methionine, consistent with inactivation of methionine synthase. However, the plasma level of this amino acid is influenced by nutritional intake. It is also reduced after exposure to anesthetics other than nitrous oxide,<sup>229</sup> which may be related to the preoperative starvation. Preliminary data show that nitrous oxide may increase plasma homocysteine several times within 8 hours of exposure, and there is no concomitant reduction in serum cobalamin (Refsum H, et al., Ermens AMM, et al., unpublished data, 1989). These exciting results strongly indicate that plasma homocysteine is an early and sensitive measure of vitamin B<sub>12</sub> oxidation in tissues. Further studies should be carried out to characterize the homocysteine response after nitrous oxide exposure to evaluate whether it may be useful to predict interference with vitamin B<sub>12</sub> metabolism and function and thereby long-term effects.

**6-Azauridine triacetate.** 6-Azauridine triacetate (azauridine) is an antimetabolite interfering with de novo synthesis of uridine-5'-monophosphate. This drug has proved effective against refractory cases of psoriasis but was prohibited by the Food and Drug Administration in 1976 because its use was associated with increased incidence (about 1.6%) of thromboembolism. Treatment with 6-azauridine triacetate caused thromboembolism within a short period of 6 weeks, and both arterial and venous episodes were reported. This suggested a role of this drug in the development of vascular disease.<sup>233</sup>

6-Azauridine triacetate increases the urinary excretion and plasma level of several amino acids including homocysteine.<sup>234</sup> Shupack et al.<sup>235</sup> suggested and brought support to the idea of a role of homocysteinemia in the development of thrombosis in patients with psoriasis receiving 6-azauridine triacetate. In their study, five of 13 patients had homocystinuria and homocysteinemia, and one patient had a thromboembolic episode. The thrombosis resolved and the homocysteinemia subsided when the drug was withdrawn.<sup>235</sup>

A unifying hypothesis to explain the altered metabolism of homocysteine and other amino acids in patients taking 6-azauridine triacetate is its interference with the

functions of pyridoxal 5'-phosphate. This possibility was investigated in rabbits given the drug alone or in combination with pyridoxine.<sup>236</sup> 6-Azauridine triacetate significantly reduced the level of pyridoxal 5'-phosphate in serum, and there was a marked homocysteinemia. Both effects were absent in animals given the combination of 6-azauridine triacetate and pyridoxine. These data indicate that the homocysteinemia induced by 6-azauridine triacetate is due to depletion of pyridoxal 5'-phosphate.<sup>236</sup>

The implication of the possible role of pyridoxal 5'-phosphate deficiency in the thromboembolism in patients receiving 6-azauridine triacetate is twofold. The restoration of the clinical use of this drug should be considered because administration of pyridoxine may prevent thromboembolism.<sup>233</sup> Second, the monitoring of plasma homocysteine during such treatment may identify those at risk of thrombosis.

**Penicillamine.** Penicillamine (D-β,β-dimethylcysteine) is a metabolically stable cysteine analogue with chelating properties. It is used for the treatment of heavy metal poisoning, Wilson's disease, and in the management of cystinuria and rheumatoid arthritis.<sup>237</sup>

Penicillamine drastically reduces the amount of free as well as homocysteine-protein mixed disulfide in patients with homocystinuria.<sup>238</sup> Thus, this compound should be considered as a therapeutic means in patients with homocystinuria not responsive to pyridoxine and as an adjunct therapeutic agent in patients responsive to pyridoxine. Penicillamine also reduced the amount of plasma protein-bound homocysteine by about 50% in patients with rheumatoid arthritis.<sup>52</sup> Homocysteine in plasma of patients with rheumatoid arthritis not receiving penicillamine is normal, and the reduction by penicillamine suggests that this drug is effective in reducing moderately elevated plasma homocysteine.<sup>52</sup>

The potential usefulness of penicillamine to reduce elevated plasma homocysteine is indicated by the observation that this drug, in contrast to some other thiols, is not cytotoxic against endothelial cells in vitro.<sup>140</sup>

Penicillamine probably reduces plasma homocysteine by forming homocysteine-penicillamine mixed disulfide. This disulfide was not detected in plasma suggesting that its high renal clearance may contribute to efficient elimination of homocysteine from plasma.<sup>238</sup>

**Anticonvulsants.** From a chemical viewpoint antiepileptic drugs are a heterogeneous class of drugs. They have in common the ability to cause folate deficiency. Phenytoin is the drug most often linked to folate deficiency, but it has also been demonstrated for phenobarbital, primidone, carbamazepine, and valproic acid.<sup>239</sup> The antifolate effect of these drugs may be linked to their anticonvulsant action,<sup>239</sup> but folate sup-

plementation does not reduce the efficacy of these drugs.<sup>240</sup>

The proposed mechanisms for the antifolate effects include reduced intestinal absorption of folic acid, increased metabolism of folates in liver, and an altered activity of some enzymes involved in one-carbon transfer.<sup>239</sup> Notably, among these enzymes, methylenetetrahydrofolate reductase, which is responsible for the production of 5-methyltetrahydrofolate (Fig. 2), is altered during anticonvulsant therapy.<sup>241,242</sup> It is conceivable that this may cause secondary effects on homocysteine metabolism.

There are only preliminary data on plasma homocysteine in patients taking anticonvulsants. Total homocysteine was significantly elevated in patients treated with phenytoin (Brattström LE, unpublished data, 1988). Carbamazepine seems to exert a similar effect. The increase was not associated with overt folate deficiency (Refsum H et al., unpublished data, 1989).

The effect of anticonvulsants on homocysteine metabolism and homocysteine level in plasma should be further studied.

#### PHARMACOLOGIC AGENTS AS POTENTIAL MODULATORS OF PLASMA HOMOCYSTEINE

**Nucleoside analogues inhibiting AdoHcy catabolism.** Several nucleoside analogues block the AdoHcy hydrolase reaction, which is the only known source of homocysteine in vertebrates (Fig. 2).<sup>17</sup> These analogues may serve either as inhibitor, inactivator, or substrate for this enzyme, and most of these agents are active in the intact cell or in vivo, as judged by their ability to increase the cellular level of AdoHcy. Inhibitors of AdoHcy hydrolase have been reviewed in several articles.<sup>16,17,243</sup>

Homocysteine depletion may be an important consequence of inhibition of AdoHcy degradation, but data on this subject are sparse. We investigated the disposition of homocysteine by various cell types exposed to nucleoside analogues and found that AdoHcy build-up was closely linked to inhibition of homocysteine egress.<sup>34,35</sup> Thus such agents should efficiently reduce plasma homocysteine levels.

The nucleoside analogues that block AdoHcy catabolism, are potential antiviral agents,<sup>244</sup> and some may exert an immunosuppressive<sup>245</sup> or cytostatic effect.<sup>246,247</sup> Although these agents may become useful drugs in the future, few are in clinical use at present. Therefore their ability to reduce plasma homocysteine in humans has not been widely documented.

Hershfield et al.<sup>248</sup> demonstrated that the antiviral nucleoside ara-A inactivated AdoHcy hydrolase and increased AdoHcy in circulating lymphocytes in a patient with T-cell leukemia. 2'-Deoxycoformycin is not an inhibitor of isolated AdoHcy hydrolase but functions as a tight binding inhibitor of adenosine deami-

nase. This leads to accumulation of some purine metabolites among which 2'-deoxyadenosine interacts with AdoHcy hydrolase thereby inactivating the enzyme.<sup>249</sup> This drug is lymphocytotoxic, has anticancer activity against some forms of lymphoma and leukemia, and is especially promising in the treatment of hairy cell leukemias.<sup>250</sup> It has been demonstrated<sup>207</sup> that 2'-deoxycoformycin, given to six patients with acute lymphoblastic leukemia reduced plasma homocysteine in all patients within 4 to 48 hours, and before or concurrent with a decrease in peripheral or marrow lymphoblasts. Notably, the decrease was observed irrespective of the pretreatment level. The authors concluded that nonspecific effects of lymphoblast lysis may explain the decrease in plasma homocysteine in patients with high levels, but do not account for the effect seen in three patients with low homocysteine levels in whom 2'-deoxycoformycin decreased plasma homocysteine levels below that of controls. These findings may be explained by inhibition of the conversion of AdoHcy to homocysteine.<sup>207</sup>

The role of depletion of plasma and cellular homocysteine in the mechanism of action of 2'-deoxycoformycin should be studied.

**Estrogens and agents affecting estrogen status.** There are consistent reports that plasma homocysteine, determined either as the total amount or the acid-soluble homocysteine-cysteine mixed disulfide is lower in premenopausal women compared with postmenopausal women and men (Table I), and urinary homocysteine excretion is higher in men than in women.<sup>54</sup> This suggests that homocysteine metabolism may be influenced by the female sex hormones. This explanation is supported by the reduction of total plasma homocysteine (mean 1.99  $\mu\text{mol/L}$ ) in women during pregnancy,<sup>251</sup> a state characterized by a high level of circulating estrogens.<sup>252</sup> The reduction may be related to altered flux through one or more pathways leading to homocysteine formation<sup>57</sup> or utilization.

Women taking peroral contraceptives have an increased incidence of thromboembolism,<sup>253</sup> which has been related to the fact that these formulations may affect the metabolism of sulfur-containing amino acids.<sup>254</sup> There is one report that an estrogen containing contraceptive produced increased endothelium in methionine-loaded young females, whereas methionine intake alone was without effect.<sup>124</sup> This may indicate that estrogen decreases the tolerance against the ability of methionine or a metabolite thereof to induce endothelial detachment.

Thomson et al.<sup>255,256</sup> evaluated the component in peroral contraceptives by measuring urinary homocysteine excretion in rats injected with ethinyl estradiol, alone or in combination with a gestagen. The estrogen was responsible for a marked increase in the urinary excretion of homocysteine in the rat.<sup>53,255,256</sup> In contrast, the

same authors could not demonstrate any effect of oral contraceptives on urinary homocysteine excretion in young females.<sup>54</sup>

In women taking oral contraceptives containing estrogens or in men with prostatic cancer treated with estrogens, there was no significant alteration in plasma homocysteine (Brattström LE, et al., unpublished data, 1988). However, preliminary data have been published<sup>257</sup> suggesting that some women taking oral contraceptives have elevated plasma homocysteine, whereas contraceptives reduce the level to those observed during pregnancy<sup>251</sup> in most subjects.<sup>257</sup>

We investigated total plasma homocysteine in premenopausal and postmenopausal women treated with tamoxifen, and in premenopausal women receiving an analogue of the luteinizing hormone-releasing hormone (LHRH) (Lien E, et al., unpublished data, 1989). Such treatment was part of the management of breast cancer. Tamoxifen is a nonsteroidal antiestrogen, which functions as an agonist in some in vitro systems,<sup>258</sup> whereas the LHRH hormone induces "medical oophorectomy" in premenopausal women and thereby an estrogen deficiency state.<sup>259</sup> The effect of treatment on estrogen and other endocrine parameters was continuously monitored throughout treatment, and some patients were followed for up to 600 days. The LHRH caused a transient increase in plasma estrogen followed by a permanent estrogen deficiency, and tamoxifen induced large oscillations in plasma estrogen. The LHRH-induced increase in estrogen level was not associated with consistent alteration in plasma homocysteine. Whether the chronic deficiency state induces alterations is under study. The response to tamoxifen seems to be dependent on the pretreatment homocysteine level. In subjects with high plasma homocysteine prolonged tamoxifen treatment gradually reduced plasma homocysteine, whereas the opposite response was observed in patients with low pretreatment level.

No marked increase in plasma homocysteine in women under chronic tamoxifen treatment seems advantageous in the light of the fact that this drug has been suggested as preventive intervention in healthy women at high risk of breast cancer.<sup>260</sup>

The diverse information on homocysteine and estrogens does not lead to a unifying hypothesis. The relation between estrogen status and plasma homocysteine seems to be complex, and marked interindividual differences in response probably exist. Furthermore, the observations in cancer patients treated with tamoxifen are complicated by the fact some of these patients may have elevated plasma homocysteine as a result of the burden of malignant cells. The possible effects of steroids on plasma homocysteine is an important area of future research.

**Ethanol.** It was discovered 40 years ago that ethanol increases the choline requirements in rats,<sup>261</sup> and inter-

ference with folate metabolism was first suggested by Sullivan and Herbert in 1964.<sup>262</sup>

Chronic ethanol treatment of the rat induces metabolic effects in the liver such as accumulation of folates as 5-methyltetrahydrofolate, reduction in the betaine content, increased betaine-homocysteine methyl-transferase activity, and reduction in 5-methyltetrahydrofolate-homocysteine methyltransferase activity.<sup>261,263</sup>

These findings have been interpreted as inhibition of 5-methyltetrahydrofolate-homocysteine methyltransferase by ethanol or a product thereof, leading to a build-up of 5-methyltetrahydrofolate. Increased betaine-homocysteine methyltransferase and betaine consumption may be adaptive changes. *S*-Adenosylmethionine is regarded as a measure of available endogenous methionine, and maintenance of normal amounts of this metabolite in the liver during ethanol feeding<sup>264</sup> suggests that increased betaine-dependent synthesis of methionine may compensate for suppression of the methionine synthase activity. In the rat, which has high activity of the betaine forming enzyme choline oxidase in the liver, the compensation may efficiently protect the liver against injury. In man, who is more prone to liver damage, the activity of this enzyme is low.<sup>261</sup>

If 5-methyltetrahydrofolate-homocysteine methyltransferase is a primary molecular target for ethanol or its metabolite(s), then plasma homocysteine may be both an early, sensitive, and practical measure of chronic ethanol intake. This possibility has not been investigated.

## CONCLUSION

Homocysteine is a branch-point metabolite, the biologic fate of which is linked to vitamin B<sub>12</sub>, reduced folates, and vitamin B<sub>6</sub>. Various inborn defects in homocysteine metabolism, among which cystathionine β-synthase deficiency is most common, lead to the clinical condition homocystinuria. A central feature of this clinical state is thromboembolism and premature arteriosclerosis. These patients benefit from agents like folates, betaine, or vitamin B<sub>6</sub>, which reduce both the homocysteine levels in plasma and urine by improving homocysteine metabolism and the incidence of vascular episodes. Experimental data point to homocysteine as an arteriosclerotic agent.

Homocysteine in plasma forms mixed disulfides with other sulfur compounds and albumin, and the latter accounts for about 70% of homocysteine in normal plasma. Total plasma homocysteine includes all these species, and plasma homocysteine should be determined as such because errors related to redistribution between different forms are avoided. Plasma homocysteine shows a transient increase after methionine intake, and postloading homocysteine seems to be a

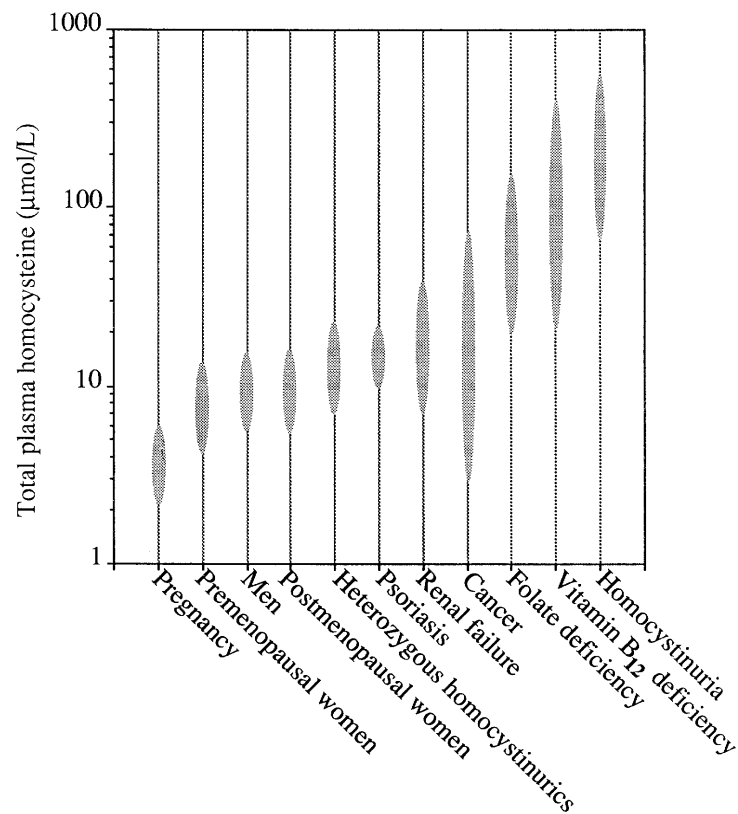


Fig. 7. Conditions causing elevated plasma homocysteine.

Table II. Agents modulating the amount of plasma homocysteine in nonhomocystinurics

Agent	Effect	Possible mechanism	Comments	Ref. no.
Folic acid	Reduction	Enhanced remethylation	Effective with and without overt folate deficiency	49, 147, 152, 169, 197, 198, 212,
Methotrexate	Increase	Depletion of 5-methyl-THF	Effective in doses from 10 mg to 13 gm	177, 208,
Nitrous oxide	Increase	Inactivation of vitamin B <sub>12</sub>	Marked increase within hours	231, 232 and unpublished
6-Azauridine triacetate	Increase	Vitamin B <sub>9</sub> antagonist		233, 235, 236
2-Deoxycoformycin	Reduction	Indirect inactivation of AdoHcy hydrolase		207
D-Pencillamine	Reduction	Formation of mixed disulfide	Reduce elevated and normal plasma homocysteine	52, 238
Anticonvulsants	Increase	Interference with folate metabolism or function		Unpublished

measure of the efficiency of homocysteine metabolism, and has been used for the diagnosis of subjects heterozygous for homocystinuria.

New analytic techniques allow the determination of plasma concentration of homocysteine in healthy sub-

jects, and normal values for this parameter have been established. Plasma homocysteine is higher in men and postmenopausal women than in young women. A moderate increase in plasma homocysteine above normal, that is, intermediate homocysteinemia, is statistically

associated with premature arteriosclerosis, both in the coronary, cerebral, and peripheral arteries. Thus intermediate homocysteinemia seems to be an arteriosclerotic risk factor independent of conventional risk factors like plasma cholesterol, hypertension, smoking, and diabetes.

Elevated plasma homocysteine has been demonstrated in patients with vitamin B<sub>12</sub> deficiency, folate deficiency, chronic renal failure, malignant disease such as acute leukemia, and in psoriasis. Among these conditions vitamin B<sub>12</sub> deficiency and to a lesser degree folate deficiency cause extreme elevation of plasma homocysteine. Plasma homocysteine seems to be a better parameter for impaired vitamin B<sub>12</sub>-dependent functions than serum vitamin B<sub>12</sub> itself and has been suggested to be particularly useful for the diagnosis and follow up of patients with vitamin B<sub>12</sub> deficiency.

Plasma homocysteine concentrations in various clinical conditions are summarized in Fig. 7.

Plasma homocysteine level is decreased by high doses of folic acid. Since such treatment results in minor side effects, it may be an innocuous mean to reduce plasma homocysteine, thereby reducing the risk for vascular disease. Plasma homocysteine is also decreased by penicillamine, but is increased by methotrexate, the antimetabolite 6-azauridine triacetate and some anti-convulsants. Notably, nitrous oxide, known to inactivate vitamin B<sub>12</sub>, may induce within hours high plasma homocysteine levels, comparable to those observed during chronic vitamin B<sub>12</sub> deficiency (Table II).

There are several clinical conditions or drugs which possibly alter plasma homocysteine. These include zinc deficiency, hepatic failure, and agents like some nucleoside analogues, sex hormones, drugs affecting estrogen status, and ethanol. Such relations have not been documented, and these are important areas for future research.

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