Homocysteine metabolism is increasingly implicated in a diverse group of clinical disorders, including atheromatous vascular disease. We studied the disposition of homocysteine via the trans-sulphuration pathway, plasma glutathione peroxidase (GPx) activity and plasma levels of the sulphated hormone dehydro-epiandrosterone sulphate (DHEAS) in six vitamin B$_{12}$-deficient human subjects before and after 2 weeks of vitamin B$_{12}$ repletion, both in the fasting state and following an oral methionine load (0.1 g/kg body weight). Fasting plasma total homocysteine concentrations fell ($P < 0.03$) and total cysteine concentrations rose significantly ($P < 0.048$) after treatment for 2 weeks with vitamin B$_{12}$ injections. The magnitude of the mean fall in the fasting concentration of homocysteine (38.8 $\mu$mol/l) was similar to the mean rise in cysteine levels (36.0 $\mu$mol/l) following vitamin B$_{12}$ therapy. Circulating levels of homocysteine were increased at 4 h after a methionine load when compared with fasting levels, both before and after vitamin B$_{12}$ repletion ($P = 0.003$ for both). Total cysteinyl-glycine was lower post-methionine than in the fasting state following vitamin B$_{12}$ therapy ($P = 0.007$). Fasting plasma GPx fell significantly after 2 weeks of vitamin B$_{12}$ therapy ($P = 0.05$). The change in plasma GPx between the fasting state and 4 h after methionine loading was significantly different pre- and post-vitamin B$_{12}$ therapy ($P = 0.05$). The present study provides indirect support to the hypothesis that defects in the trans-sulphuration and remethylation of homocysteine produce hyperhomocysteinaemia in vitamin B$_{12}$ deficiency in human subjects. Elevated homocysteine levels directly or indirectly may up-regulate GPx. Sulphation status, as measured by plasma DHEAS, was unchanged.

INTRODUCTION

Deficiencies of folate and vitamin B$_{12}$ are not uncommon in the general population [1]. Approximately 1% of middle-aged and elderly subjects have vitamin B$_{12}$ deficiency, and folate deficiency is likewise common in states of poor nutrition [2]. These deficiencies may be subclinical and detected by plasma measurements only, or present as clinically recognizable disease states. The widely recognized clinical syndromes associated with these deficiency states are megaloblastic anaemia and neurological disease. It has long been known that vitamin B$_{12}$ and folate regulate homocysteine/methionine metabolism, with vitamin B$_{12}$ acting as cofactor to the enzyme methionine synthase and folate as the precursor of tetrahydrofolate (both part of the remethylation cycle of homocysteine), and that hyperhomocysteinaemia is a feature of these deficiency states [3]. Homocysteine is increasingly recognized as a risk factor for atheromatous vascular disease [4,5], thrombophilia [6] and neural tube...
defects [7]. Despite this potential for causing disease, classical descriptions of pernicious anaemia, known to be associated with raised homocysteine levels, do not include increased thrombogenicity or atherogenesis.

The molecular mechanisms underlying the damage due to homocysteine are not fully understood, although oxidative damage generated by high homocysteine levels has been implicated [8,9]. Since homocysteine can be eliminated by remethylation to form methionine, or converted into cysteine and glutathione by trans-sulphuration mechanisms [10], we investigated the disposition of homocysteine by the latter route, which may potentially generate molecules that are protective against oxidative injury. Cellular and plasma glutathione peroxidase (GPx) enzymes form part of the antioxidant defences, including those against peroxide-induced damage [11,12]. As hyperhomocysteinaemia has been shown to injure endothelial cells via the generation of hydrogen peroxide [12] and to decrease the activity of cellular GPx [13], we wished to study the effects of the hyperhomocysteinaemia of vitamin B12 deficiency on plasma GPx. Trans-sulphuration of homocysteine also generates cysteine, which is crucial for the eventual sulphation of endogenous and exogenous substances that is necessary for elimination from the body of endogenous as well as xenobiotic molecules [14].

The aims of the present study were: (1) to assess the disposition of homocysteine via the trans-sulphuration pathway in vitamin B12-deficient subjects, (2) to assess any relationship with plasma GPx, and (3) to evaluate sulphation status by measuring levels of an endogenous sulphated hormone, dehydro-epiandrosterone sulphate (DHEAS). All of these measurements were carried out both before and after 2 weeks of vitamin B12 repletion. Homocysteine metabolism was studied in the fasting state as well as after an oral methionine load in six subjects with low plasma levels of vitamin B12. Oral methionine administration transiently increases circulating levels of homocysteine and other thiol compounds, reaching a peak between 4 and 8 h in healthy subjects. Methionine administration allows an assessment of the activity of the trans-sulphuration pathway [15,16].

MATERIALS AND METHODS

Study protocol

The programme of investigation was approved by the Epsom General Hospital Medical Research Ethics Committee. Written consent was obtained from all participants.

Subjects (Table 1)

Two pre-menopausal female and four male subjects with low plasma vitamin B12 levels (but with plasma folate concentrations within the reference range) were chosen for study. All subjects had their plasma vitamin B12 measured by macrocytosis; levels of plasma vitamin B12 were less than the lower limit of the reference range (210–910 pg/ml) in all subjects. None of the subjects was a smoker or abused alcohol or had impairment of renal function. All subjects were Caucasian, on a Western diet, and were requested to follow their usual diet and other lifestyle factors for the duration of the study. All were explicitly advised to avoid taking any extra vitamin supplementation.

Experimental protocol

Subjects were seen between 07.30 and 09.00 hours on two separate occasions, each following an overnight fast of 12 h. After a basal 10 ml venous blood sample had been collected, methionine (0.1 g/kg body weight) flavoured with Modjul® flavour system (Scientific Hospital Supplies, Liverpool, U.K.) in 400 ml of water was given orally. A second 10 ml venous blood sample was obtained 4 h later [16]. All subjects received five intramuscular injections of hydroxycobalamin (1 mg) between the first and second visits, and plasma concentrations of vitamin B12 were greater than 2000 pg/ml at the second visit. None of the subjects had any adverse reaction to oral methionine.

Chemical analyses

Blood samples were collected into EDTA glass collection bottles (for thiol metabolites, GPx and DHEAS) and centrifuged at 2000 g for 10 min; separated plasma was frozen within 15 min of venesection. Total homocysteine, cysteine, cysteinyl-glycine and glutathione (the terms homocysteine, cysteine, cysteinyl-glycine and glutathione in the rest of the paper refer to sum of the reduced, oxidized and protein-bound compounds, unless stated otherwise) were measured by HPLC on a Drew DS30 analyser (Drew Scientific, Barrow-in-Furness, U.K.), in an adaptation of an HPLC method [17]; the within-run coefficients of variation for homocysteine, cysteine, cysteinyl-glycine and glutathione were 2.7%, 3.3%, 6.0% and 7.7% at concentrations of 16.9, 292, 13 and 3.7 μmol/l respectively (n = 10). DHEAS was measured by radioimmunoassay (DPC, Llanberis, U.K.), with a within-run coefficient of variation of <10% throughout the standard range. Plasma GPx was measured by the method of Paglia and Valentine [18] (Ransel; Randox Laboratories, Antrim, U.K.) on a Falcor 300 analyser (A. Menarini, Wokingham, U.K.), with a within-run coefficient of variation of 6.4% at a concentration of 625 units/l.

Statistical analyses

Experimental groups were compared using the paired Student’s t test. Pearson’s correlation coefficients were calculated to assess relationships between groups. Values of P < 0.05 were taken to be significant.
Table 1  Details of subjects and of fasting pre-therapy measurements
M, male; F, female.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subject no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td>45</td>
<td>57</td>
<td>68</td>
<td>70</td>
<td>86</td>
<td>47</td>
<td>–</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>–</td>
</tr>
<tr>
<td>Plasma vitamin B₁₂ (pg/ml)</td>
<td>121</td>
<td>204</td>
<td>139</td>
<td>87</td>
<td>149</td>
<td>126</td>
<td>210–910</td>
<td></td>
</tr>
<tr>
<td>Plasma folate (ng/ml)</td>
<td>4.4</td>
<td>2.1</td>
<td>5.7</td>
<td>8.1</td>
<td>5.2</td>
<td>4.0</td>
<td>2.8–20.0</td>
<td></td>
</tr>
<tr>
<td>Red cell folate (ng/ml)</td>
<td>156</td>
<td>194</td>
<td>206</td>
<td>261</td>
<td>180</td>
<td>355</td>
<td>145–1100</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.4</td>
<td>14.2</td>
<td>15.7</td>
<td>14.3</td>
<td>14.5</td>
<td>13.6</td>
<td>F, 11.8–14.8; M, 13.3–16.7</td>
<td></td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.39</td>
<td>0.43</td>
<td>0.48</td>
<td>0.43</td>
<td>0.42</td>
<td>0.39</td>
<td>0.36–0.44; M, 0.39–0.50</td>
<td></td>
</tr>
<tr>
<td>White blood cells (×10⁹/l)</td>
<td>12</td>
<td>18.5</td>
<td>6.2</td>
<td>5.9</td>
<td>7.5</td>
<td>13.5</td>
<td>3.5–11.0</td>
<td></td>
</tr>
<tr>
<td>Platelets (×10⁹/l)</td>
<td>287</td>
<td>266</td>
<td>278</td>
<td>214</td>
<td>219</td>
<td>344</td>
<td>150–400</td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular volume (fl)</td>
<td>103</td>
<td>105</td>
<td>103</td>
<td>117</td>
<td>113</td>
<td>102</td>
<td>80–100</td>
<td></td>
</tr>
<tr>
<td>Homocysteine (µmol/l)</td>
<td>17.1</td>
<td>31.3</td>
<td>12.5</td>
<td>142.5</td>
<td>79.0</td>
<td>9.0</td>
<td>5–15</td>
<td></td>
</tr>
<tr>
<td>Cysteine (µmol/l)</td>
<td>240</td>
<td>129</td>
<td>235</td>
<td>194</td>
<td>205</td>
<td>167</td>
<td>140–260</td>
<td></td>
</tr>
<tr>
<td>Cysteinyl-glycine (µmol/l)</td>
<td>36</td>
<td>45</td>
<td>34</td>
<td>26</td>
<td>36</td>
<td>33</td>
<td>55–75</td>
<td></td>
</tr>
<tr>
<td>Glutathione (µmol/l)</td>
<td>1.0</td>
<td>13</td>
<td>4.9</td>
<td>1.5</td>
<td>1.2</td>
<td>5.1</td>
<td>0.7–11.5</td>
<td></td>
</tr>
<tr>
<td>DHEAS (µmol/l)</td>
<td>436</td>
<td>379</td>
<td>380</td>
<td>526</td>
<td>380</td>
<td>531</td>
<td>300–450</td>
<td></td>
</tr>
</tbody>
</table>

* Data taken from [31].
† Data provided by Ransel, Randex Laboratories, Antrim, U.K.

RESULTS

Homocysteine and other thiol metabolites (Tables 1 and 2)
Fasting plasma homocysteine concentrations ranged from 9.0 to 142.5 µmol/l, and fell significantly after treatment for 2 weeks with vitamin B₁₂ injections (P = 0.03), with each subject showing a fall. Concentrations of homocysteine were increased at 4 h post-methionine compared with fasting levels, both before and after vitamin B₁₂ repletion (P = 0.003). The magnitude of the rise in plasma homocysteine after administration of methionine was similar before and after the vitamin B₁₂ therapy.

Table 2  Fasting and 4 h post-methionine plasma levels of thiol metabolites, DHEAS and GPx in six vitamin B₁₂-deficient patients before and after vitamin B₁₂ therapy
Values are means (S.E.M.). Significance of differences: *P < 0.05 for after compared with before vitamin B₁₂ therapy; †P < 0.01 for after a methionine load compared with the fasting state.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Before B₁₂ treatment</th>
<th>After B₁₂ treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td>Post-methionine</td>
</tr>
<tr>
<td>Homocysteine (µmol/l)</td>
<td>48.7 (21.6)</td>
<td>72.7 (19.2)*</td>
</tr>
<tr>
<td>Cysteine (µmol/l)</td>
<td>195 (17.2)</td>
<td>188 (15.7)</td>
</tr>
<tr>
<td>Cysteinyl-glycine (µmol/l)</td>
<td>35.0 (2.5)</td>
<td>32.0 (3.6)</td>
</tr>
<tr>
<td>Glutathione (µmol/l)</td>
<td>3.52 (0.4)</td>
<td>4.37 (0.9)</td>
</tr>
<tr>
<td>DHEAS (µmol/l)</td>
<td>2.50 (0.79)</td>
<td>2.73 (1.04)</td>
</tr>
<tr>
<td>GPx (units/l)</td>
<td>439 (30)</td>
<td>420 (35)</td>
</tr>
</tbody>
</table>

In contrast with homocysteine, fasting plasma cysteine concentrations increased following treatment for 2 weeks with vitamin B₁₂ injections (P = 0.048), with increases in five of the six subjects. The mean increase in fasting plasma cysteine after vitamin B₁₂ treatment (36.0 µmol/l) was similar to the mean fall in fasting homocysteine (38.8 µmol/l), suggesting a reciprocal relationship. There were no other significant changes in cysteine levels, although cysteine concentrations tended to be lower post-methionine. Cysteinyl-glycine levels post-methionine tended to be lower than fasting levels both before and after vitamin B₁₂ treatment, although only post-vitamin B₁₂ levels achieved statistical significance (P = 0.007). No significant differences were seen in plasma glutathione.
before or after vitamin B\textsubscript{12} therapy, either in the fasting state or following oral methionine loading.

**Plasma DHEAS (Tables 1 and 2)**
There were no significant differences in plasma DHEAS levels measured pre-vitamin B\textsubscript{12} fasting (2.50 ± 0.79 \mu mol/l), pre-vitamin B\textsubscript{12} post-methionine (2.73 ± 1.04 \mu mol/l), post-vitamin B\textsubscript{12} fasting (2.15 ± 0.65 \mu mol/l) and post-vitamin B\textsubscript{12} post-methionine (3.68 ± 1.26 \mu mol/l).

**Plasma GPx (Tables 1 and 2)**
Fasting plasma GPx fell significantly following 2 weeks of vitamin B\textsubscript{12} therapy (P = 0.05). The change in plasma GPx that occurred between the fasting state and 4 h after methionine loading was significantly different pre- (falling levels) and post- (rising levels) vitamin B\textsubscript{12} therapy (P = 0.05).

**DISCUSSION**
The range of fasting homocysteine concentrations before vitamin B\textsubscript{12} therapy was wide, and it is noteworthy that some vitamin B\textsubscript{12}-deficient subjects had plasma homocysteine concentrations comparable with those found in classical homocystinuria, i.e. fasting levels of approx. 100 \mu mol/l. It is difficult to be sure why there was such a wide range of plasma homocysteine levels in our vitamin B\textsubscript{12}-deficient subjects, although contributory factors could include the period of deficiency, vitamin B\textsubscript{6} status and tissue vitamin B\textsubscript{12} status, which were not assessed in the present study. Despite this variation in fasting homocysteine levels before vitamin B\textsubscript{12} therapy, all subjects showed a decrease in these levels after vitamin B\textsubscript{12} therapy, to very low levels, with a very narrow range (7.8–12.9 \mu mol/l; reference range < 15.0 \mu mol/l). This confirmed previous studies showing a significant fall in fasting plasma homocysteine following vitamin B\textsubscript{12} therapy [19]. The rise in circulating homocysteine levels and the fall in plasma cysteinyl-glycine at 4 h after a methionine load in the present study are also similar to previous findings in six healthy subjects studied by Mansoor and colleagues [15].

We did not find evidence for an increase in the plasma levels of cysteine and downstream thiol products (cysteinyl-glycine and glutathione) in hyperhomocystinaemia of vitamin B\textsubscript{12} deficiency, which may potentially be protective against oxidative damage due to homocysteine. Rather, plasma cysteine levels were relatively lower before treatment and increased after vitamin B\textsubscript{12} repletion, in a reciprocal molar manner with homocysteine. Although deficiencies of vitamin B\textsubscript{12} and folic acid are known to cause hyperhomocystinaemia due to impaired remethylation of homocysteine, the enzymes of the trans-sulphuration pathway are not known to be dependent on vitamin B\textsubscript{12} or folate as cofactors. Several possible explanations are considered here. First, the changes in homocysteine and cysteine levels noted here might suggest direct stimulation of the trans-sulphuration pathway by vitamin B\textsubscript{12}. Folic acid has been suggested to increase trans-sulphuration by an unknown mechanism [20]. Secondly, a change in the disposal of cysteine through alternative pathways, protein and peptide synthesis and provision of intracellular organic sulphate was a possibility [14]. However, no change in circulating DHEAS levels was seen in the present study, providing no support for an alteration in sulphation status (a surrogate marker of altered cysteine disposal). Thirdly, co-existing vitamin B\textsubscript{6} deficiency could have impaired the trans-sulphuration of homocysteine through inactivation of cystathionine synthase [21,22]. However, although vitamin B\textsubscript{6} was not measured in the present study, there is no reason to believe that vitamin B\textsubscript{6} status differed between the two visits, and all subjects were explicitly requested not to take vitamin supplementation or change their usual diet for the duration of the study. Fourthly, cysteine and homocysteine may form mixed-disulphide bridges [20], although such an occurrence in plasma is unlikely to account for our results, since total plasma homocysteine was measured; however, intracellular homocysteine–cysteine disulphide bonds may have been present during hyperhomocystinaemia before vitamin B\textsubscript{12} therapy, binding more cysteine in the intracellular compartment and thus accounting for the lower plasma cysteine levels. Unlike plasma homocysteine–cysteine disulphide bridges, intracellular disulphides are not measured in our assays. Support for this explanation comes from the fact that the rise in cysteine and the fall in homocysteine occuring after vitamin B\textsubscript{12} therapy were similar in molar terms. It is also notable that the rise in homocysteine levels after a methionine load was associated with a fall in cysteine levels. Finally, the hypothesis of Selhub and Miller [23], based on studies in rats and pigs, suggests that when remethylation of homocysteine is impaired, methionine salvage is impaired, and the S-adenosylmethionine concentration within the cells falls; a fall in intracellular S-adenosylmethionine inhibits trans-sulphuration [23–25]. Our studies in human vitamin B\textsubscript{12} deficiency, showing defects in remethylation and in trans-sulphuration of homocysteine, provide support for this hypothesis.

Although the pathogenic mechanisms of homocysteinaemia are not fully understood, oxidative damage may be important. Homocysteine is oxidized to homocystine, and in the process hydrogen peroxide is generated. Hydrogen peroxide is a powerful oxidant and causes injury to living cells. Among the defence mechanisms against oxidative damage that are available, GPx is an important component, and is present in the plasma as well as within cells. Homocysteine has been shown to...
inhibit intracellular GPx in vitro [12]. The present study showed that plasma GPx levels were higher before vitamin B$_{12}$ therapy, when homocysteine levels were high, and fell after vitamin B$_{12}$ therapy (in parallel with homocysteine), which was not consistent with inhibition by increased hydrogen peroxide generation. Rather, our results are consistent with an up-regulation of plasma GPx in response to high homocysteine concentrations and, possibly, increased hydrogen peroxide formation. Cellular GPx was not measured in the present study, as previous work has shown that changes in cellular activity occur over a much longer period than plasma changes, and would not be expected to change within the time scale of the present study [26]. Another potential mechanism that may account for the hyperhomocysteinaemia of vitamin B$_{12}$ deficiency is leakage from haemopoietic cells due to ineffective erythropoiesis. We do not have any direct evidence for such a mechanism, although leakage of homocysteine and GPx from the cells before vitamin B$_{12}$ therapy could explain their parallel temporal changes. Both pernicious anaemia [27] and ischaemic/hypoxic damage to organs such as the heart, liver and skeletal muscle have been associated with high levels of circulating enzymes due to their release into the plasma by leakage through damaged cell membranes [28–30]. Our GPx assay recognizes both plasma and cellular GPx activity equally.

In summary, the present study has shown that patients with vitamin B$_{12}$ deficiency had raised plasma homocysteine levels, which fell following vitamin B$_{12}$ therapy. Additionally, a lower plasma cysteine level pre-therapy, which increased post-therapy, was also seen in the present study; this suggests that a defect in trans-sulphuration may co-exist with a defect in the trans-methylation of homocysteine, as proposed by Miller and Selhub [23]. Plasma GPx activity was higher before vitamin B$_{12}$ therapy, which may indicate an up-regulation of the enzyme by higher homocysteine or hydrogen peroxide concentrations. Despite the lower plasma cysteine levels before vitamin B$_{12}$ therapy, sulphation status (as measured by plasma DHEAS), was similar before and after vitamin B$_{12}$ therapy.

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