Recent evidence suggests that an increased plasma concentration of the sulphur amino acid homocysteine is a risk factor for the development of vascular disease. The tissue(s) responsible for homocysteine production and export to the plasma are not well known. However, given the central role of the liver in amino acid metabolism, we developed a rat primary hepatocyte model in which homocysteine (and cysteine) production and export were examined. The dependence of homocysteine export from incubated hepatocytes on methionine concentration fitted well to a rectangular hyperbola, with half-maximal homocysteine export achieved at methionine concentrations of approx. 0.44 mM. Hepatocytes incubated with 1 mM methionine and 1 mM serine (a substrate for the transulphuration pathway of homocysteine removal) produced and exported significantly less homocysteine (25–40 %) compared with cells incubated with 1 mM methionine alone. The effects of dietary protein on homocysteine metabolism were also examined. Rats fed a 60 % protein diet had a significantly increased total plasma homocysteine level compared with rats fed a 20 % protein diet. In vitro effects of dietary protein were examined using hepatocytes isolated from animals maintained on these diets. When incubated with 1 mM methionine, hepatocytes from rats fed the high protein diet exported significantly more homocysteine compared with hepatocytes from rats fed the normal protein diet. Inclusion of serine significantly lowered homocysteine export in the normal protein group, but the effect was more marked in the high protein group. In vivo effects of serine were also examined. Rats fed a high protein diet enriched with serine had significantly lower total plasma homocysteine (25–30 %) compared with controls. These data indicate a significant role for the liver in the regulation of plasma homocysteine levels.

Key words: hepatocyte, cysteine, high protein diet, metabolic regulation, transulphuration.

INTRODUCTION

An increased plasma concentration of the sulphur amino acid homocysteine is recognized as an independent risk factor for cardiovascular disease (for review, see [1]). During the course of its metabolism, methionine is adenylated to form S-adenosylmethionine (SAM), the methyl donor in virtually all known biological methylation reactions. The end products of these methyltransferase reactions are a methylated substrate and S-adenosylhomocysteine (SAH), which is reversibly hydrolysed to homocysteine and adenosine. Homocysteine has several possible fates: (1) catabolism to cysteine via the pyridoxal phosphate-dependent transulfuration enzymes cystathionine β-synthase and cystathionine γ-lyase [2]; (2) remethylation to methionine via cobalamin-dependent methionine synthase or betaine:homocysteine methyltransferase [3]; (3) export to the extracellular space.

A number of studies identify genetic (for review, see [4]), pharmacological [5,6] and pathological factors [7] that cause elevations in plasma homocysteine, but greater understanding of the basic aspects of homocysteine homoeostasis is needed. Renal uptake and catabolism of homocysteine in the rat have been characterized in our laboratory [8,9]. However, the tissues responsible for homocysteine production and export to the plasma are not well known. Homocysteine export by human umbilical vein endothelial cells [10], murine lymphoma cells [11], human fibroblasts [12] and cultured rat liver cells [13] has been demonstrated, but to what extent these cells contribute to plasma homocysteine is debatable. Indeed, it is likely that an interplay of many tissues regulates the overall plasma level.

The liver is a central organ of amino acid metabolism and contains a full complement of enzymes involved in the methionine cycle and transulphuration pathway [14]. For these reasons we sought to develop a rat primary hepatocyte model whereby factors that acutely affect homocysteine and cysteine metabolism and export could be examined. Preliminary experiments suggested that an appreciable amount of the cysteine produced remained bound to protein. As we employ perchloric acid to terminate incubations we were, therefore, concerned that the precipitation of protein would result in a loss of thiols bound via a disulphide linkage to protein. We therefore quantified thiol loss by resuspending the precipitated protein pellets, and removing bound thiols from the protein by means of a reducing agent. This enabled us to measure both extracellular (exported) and intracellular homocysteine (total minus exported).

Our experiments indicate that the liver is indeed a key organ of homocysteine metabolism and potentially contributes to much of the plasma homocysteine level. The effects of dietary and of normal and high protein diets were examined.

MATERIALS AND METHODS

Animals and Diets

Male Sprague–Dawley rats, weighing 250–300 g, were used throughout the study. The animals were obtained from our institute’s breeding colony and were housed and treated in accordance with the Canadian Council on Animal Care’s guidelines [15]. All procedures were approved by Memorial University’s Institutional Animal Care Committee. All animals had free access to water and Purina rat chow. In some studies rats were fed either a 20 %, or 60 %, casein-based AIN-93 diet designed to meet the nutritional requirements for growth of laboratory animals, for one week prior to experimentation. In some cases additional serine (5 g/100 g of casein) or alanine (5 g/100 g of casein) was added to the diet, replacing 5 g of sucrose. Rats were

Abbreviations used: DMF, dimethylformamide; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SBDF, 7-fluoro-2-oxa-1,3-diazoole-4-sulphonic acid; TNB, tri-n-butylphosphine.

1 To whom correspondence should be addressed (e-mail jbrosnan@morgan.ucs.mun.ca).
housed at 22 °C and exposed to a 12 h light/12 h dark cycle, with the light cycle commencing at 08:00 h. All experiments were performed immediately following the end of the dark cycle.

**Chemicals**

All chemicals were purchased from Sigma (Oakville, ON, Canada), except where noted in the text.

**Preparation and incubation of isolated hepatocytes**

Hepatocytes were isolated as previously described [16] and viability was assessed by 0.2% Trypan Blue exclusion. Viability was at least 95% in all cases. Hepatocytes were preincubated for 20 min at 6–8 mg dry weight of cells/ml in Krebs–Henseleit medium (144 mM NaCl/6 mM KCl/1.3 mM CaCl2/1.2 mM MgCl2/126 mM Cl−/1.2 mM H2PO4−/1.2 mM SO4−/25 mM HCO3−) containing 1.25% (w/v) BSA, in a final volume of 1 ml. At the end of the preincubation, substrates were added and the incubation was allowed to continue for an additional 30 min, except in the case of time course experiments. Cells were gassed with O2/CO2 (19:1) at the beginning of the incubation and on the addition of substrates.

When homocysteine and cysteine export were measured, the cells plus the incubation medium were immediately centrifuged at 14000 g (Brinkman Instruments, Rexdale, ON, Canada) for 2 min to sediment the cells, and an aliquot of the supernatant was removed for exported thiols analysis. This involved reduction of all thiols with 10 mM tri-n-butylphosphine (TNB) in dimethylformamide (DMF), followed by deproteinization with perchloric acid to reprecipitate protein and the supernatants were then transferred to scintillation vials containing 10 ml of scintillation fluid (Omnifluor) and radioactivity was determined. To ensure that all 14CO2 was liberated from α-ketoacids, a new centre well was introduced to each flask and 0.3 ml of 30% (w/v) hydrogen peroxide was added through the septa with a syringe, as previously described [17]. 14CO2 was collected for 1 h and centre wells were then transferred to scintillation vials containing 10 ml of scintillation fluid (Omnifluor) and radioactivity was determined.

The presence of other amino acids and their effects on homocysteine and cysteine export were also examined. Krebs–Henseleit medium was supplemented with a mixture of amino acids to resemble rat plasma amino acid concentrations as follows: 204 μM alanine, 194 μM arginine, 64 μM asparagine, 32 μM aspartic acid, 123 μM glutamic acid, 643 μM glutamine, 295 μM glycine, 72 μM histidine, 99 μM isoleucine, 153 μM leucine, 72 μM phenylalanine, 226 μM proline, 61 μM tryptophan, 75 μM tyrosine, 130 μM threonine, 184 μM valine, 305 μM taurine, 88 μM citrulline and 72 μM ornithine [18]. Incubations were performed using amino acid-supplemented and non-supplemented Krebs–Henseleit medium, containing 1 mM methionine in the presence and absence of 1 mM serine. Homocysteine and cysteine export were measured as described in the Analyses section.

**Analyses**

Homocysteine and cysteine concentrations were determined by reverse-phase HPLC and fluorescence detection of ammonium SBDF thiol adducts, using the method of Vester and Rasmussen [19]. Plasma samples were taken into heparinized syringes from the abdominal aorta of anaesthesitized animals (Somnitol; MTC Pharmaceuticals, Cambridge, ON, Canada; 60 mg/kg of weight intraperitoneally). The blood was immediately centrifuged at 14000 g for 5 min, and the plasma removed for either immediate analysis or storage at −20 °C. For plasma methionine analysis the samples were deproteinized with 10% (w/v) sulphasalicylic acid, the protein was removed by centrifugation and the supernatant adjusted to pH 2.2. The samples were then analysed on a Beckman 121 MB Amino Acid Analyser using Benson D-X8, 25 Cation Exchange Resin and a single-column three-buffer lithium method according to the Beckman 121 MB-TB-O17 application notes. Results were quantified using a Hewlett Packard Computing Integrator Model 3395 A.

**Statistics**

Data were analysed by ANOVA and Neuman–Keuls multiple comparison post test, with P < 0.05 considered significant. All results are presented as means ± S.E.M.

**RESULTS**

**Characterization of the hepatocyte system**

**Linearity and folate supplementation studies**

In incubations with 1 mM methionine we found that homocysteine and cysteine export and production were linear with respect to time (up to 45 min) and cell dry weight (up to 10 mg) (n = 3 for both; results not shown). It is conceivable that the preparation of hepatocytes, which involves considerable washing of cells, could deplete them of folate coenzymes. We therefore sought to determine whether our incubation system required supplementation with folates to best reflect the in vivo situation. A series of experiments was performed in which incubations...
containing 1 mM methionine and 1 mM serine were supplemented with varying concentrations of folates. Cells were preincubated with each of the following for 20 min prior to the addition of substrates: 3 μM folic acid or 100 nM folinic acid (calcium salt) or 100 nM 5-methyltetrahydrofolate (barium salt). No effect on homocysteine or cysteine export or production was observed (n = 3; results not shown). It was therefore concluded that an adequate supply of folates exists within the cell for our incubation period and supplementation was not warranted.

Dependence of homocysteine export on methionine

The effects of methionine concentration on homocysteine export were investigated in a series of experiments in which methionine concentration in the incubation medium was varied from 0 to 5 mM. These data fitted well to a rectangular hyperbola ($r^2 = 0.85$) (Figure 1). A concentration of approx. 0.44 mM methionine yielded a half-maximal rate of homocysteine export. A methionine concentration of 1 mM was chosen for subsequent experiments.

Effects of serine, glycine and glucose

As previously mentioned serine condenses with homocysteine via cystathionine β-synthase to form cystathionine, thereby removing homocysteine from the methionine cycle. We undertook a series of experiments to determine whether inclusion of serine in the incubation medium would alter homocysteine export from hepatocytes by facilitating flux through the transulfuration pathway. Hepatocytes were incubated in the presence of 1 mM methionine and various serine concentrations (Figure 2). The lowest concentration of serine, 0.2 mM, significantly lowered homocysteine export, from 2.10 ± 0.18 nmoles/mg per h to 1.62 ± 0.29 nmoles/mg per h, a decrease of approx. 23%. Although further increases in serine appeared to reduce homocysteine export further, in fact the rates for 0.2–5 mM serine were not significantly different. We chose 1 mM serine for use in subsequent experiments.

A series of experiments was performed with glycine and glucose to assess the effects of these substances on homocysteine export. Glycine is a substrate for SAM-dependent glycine N-methyltransferase and its provision may permit more rapid methionine metabolism via this route. Therefore glycine was included to ensure that the conversion of SAM into SAH was not limited by an inadequate supply of methyl acceptors. It is thought that conversion of glycine into sarcosine via glycine N-methyltransferase acts in such an overflow capacity in vitro [14]. Glucose was provided as a potential source of NADPH via the pentose phosphate pathway, since NADPH is required by N,N'-methylene tetrahydrofolate reductase. Table 1 depicts the means of four experiments. Again, serine decreased homocysteine export (by approx. 40%). However, provision of glycine and glucose had no effect.

Table 1 Hepatocyte incubations with glycine, serine and glucose

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Homocysteine export (nmoles/mg per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>2.40 ± 0.17*</td>
</tr>
<tr>
<td>Methionine + glycine</td>
<td>2.21 ± 0.25*</td>
</tr>
<tr>
<td>Methionine + serine</td>
<td>1.47 ± 0.03†</td>
</tr>
<tr>
<td>Methionine + glycine + serine</td>
<td>1.44 ± 0.11†</td>
</tr>
<tr>
<td>Methionine + glycine + serine + glucose</td>
<td>1.45 ± 0.07†</td>
</tr>
</tbody>
</table>

© 2000 Biochemical Society

Hepatic homocysteine metabolism

Figure 1 Dependence of homocysteine export on methionine

Hepatocytes were preincubated, gassed and centrifuged as described in Table 1 and the Materials and methods section. Incubations were performed with 1 mM methionine and various serine concentrations for 30 min. Means ± S.E.M. are shown for 3 experiments.

Figure 2 Effect of serine on homocysteine export

Hepatocytes were preincubated, gassed and centrifuged as described in Table 1 and the Materials and methods section. Incubations were carried out with 0, 0.2, 0.5, 1, 3 and 5 mM methionine for a period of 30 min at 6–8 mg dry weight of cells/ml. Means ± S.E.M. are shown. * indicates statistically significant differences from incubations with 1 mM methionine alone (P < 0.05).

Homocysteine and cysteine export and production

Since thiols such as cysteine and homocysteine can form dithiols with protein thiol (cysteine) groups, and since deproteinizing agents will precipitate these protein-bound thiols, it is apparent that measurements of the total production of these amino acids...
Table 2 Homocysteine and cysteine export and production

Hepatocyte incubations were carried out as described in Table 1 and the Materials and methods section. All concentrations are 1 mM. Means ± S.E.M. for 7 experiments are shown. Data were analysed using ANOVA followed by Newman–Keuls multiple comparisons post test, with P < 0.05 taken to indicate a significant difference. * indicates a significant difference from the methionine export value; † indicates a significant difference from the methionine + serine production values (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Homocysteine (nmoles/mg per h)</th>
<th>Cysteine (nmoles/mg per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Export</td>
<td>Production</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.17 ± 0.06</td>
<td>2.80 ± 0.06†</td>
</tr>
<tr>
<td>Methionine + serine</td>
<td>1.29 ± 0.09†</td>
<td>1.60 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3 14CO2 release from isolated rat hepatocytes incubated with L-[1-14C]methionine ± serine

Hepatocytes were incubated as described in Table 1 and the Materials and methods section. All concentrations are 1 mM. 'Acid' refers to 14CO2 production following termination of the incubation with perchloric acid. 'H2O2' refers to additional 14CO2 release from α-ketobutyrate following the addition of hydrogen peroxide as described in the Materials and methods section. 'Total production' is the sum of these numbers. Means ± S.E.M. for 4 experiments are shown. Data were analysed using ANOVA followed by Newman–Keuls multiple comparisons post test. † indicates a statistically significant difference from the respective methionine-alone value (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>14CO2 production (nmoles/mg of dry weight per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid</td>
</tr>
<tr>
<td>L-[1-14C]Methionine</td>
<td>2.11 ± 0.11</td>
</tr>
<tr>
<td>L-[1-14C]Methionine + serine</td>
<td>3.02 ± 0.15*</td>
</tr>
</tbody>
</table>

Table 2 shows homocysteine and cysteine export and production by hepatocytes incubated in the presence of 1 mM methionine ± 1 mM serine. Homocysteine production was significantly greater than export for both incubation conditions. Cysteine values were more variable and no statistically significant differences were noted. This variability probably results from the numerous metabolic routes available to cysteine, such as protein synthesis, glutathione synthesis, taurine synthesis and catabolism to sulphate. However, the mean cysteine export and production tended to increase in the presence of serine, as might be expected.

Since cysteine accumulation alone cannot be used as a measure of total flux through the transulphuration pathway, another means of determining this flux was needed. To this end we incubated hepatocytes with L-[1-14C]methionine (1 mM) in the presence and absence of 1 mM serine as described in the Materials and methods section. Table 3 shows the results of these experiments. Total 14CO2 production was significantly higher in hepatocytes incubated with L-[1-14C]methionine and 1 mM serine as compared with hepatocytes incubated with L-[1-14C]methionine alone (2.79 ± 0.12 nmoles/mg of dry weight per h versus 3.81 ± 0.13 nmoles/mg of dry weight per h). The increase in 14CO2 production observed on addition of serine was roughly equal to the decrease in homocysteine export and production seen under the same conditions (Table 2). Together these results strongly suggest that the inclusion of serine, in our system, acts at the level of cystathionine β-synthase to remove homocysteine.

(a opposed to their export) presents an analytical challenge. Simple measurement of cysteine and homocysteine in the supernatant after deproteinization of cell incubations and removal of the precipitated protein by centrifugation will be an underestimate. We experimented with various methods that did not employ acid to obtain total homocysteine and cysteine. These included sonication, freeze-thawing, digitonin disruption, and combinations of these, but none proved satisfactory. We found that the incubations with perchloric acid followed by resuspension of the acid-precipitated protein pellet was the most reliable method. The resuspended protein was exposed to the reducing agent TNB (details are given in the Materials and methods section to reduce and remove bound thiols. The protein was reprecipitated and the released thiols were measured in the supernatant. Experiments were performed to ensure that all homocysteine and cysteine residues were removed from the pellet during the first resuspension. Figure 3 illustrates that this was the case, since repetition of the procedure released no additional cysteine or homocysteine (column D, Figure 3). Figure 3 shows that, without our method for releasing thiols from the precipitated protein, cysteine loss due to protein binding is appreciable (3.91 ± 0.82 nmoles/mg per h), whereas homocysteine loss is minor (0.28 nmoles/mg per h). Figure 3 also shows the difference between exported thiols (column A) and total thiol production (the sum of columns B and C). In these experiments total homocysteine production was approx. 3 nmol/mg per h, as opposed to an export of approx. 2 nmol/mg per h. The corresponding values for cysteine were approx. 11.2 and 7.2 nmol/mg per h.
Table 4 Effects of amino acid supplementation on homocysteine and cysteine export

Hepatocyte incubations were carried out as described in Table 1 and the Materials and methods section. Methionine and serine concentrations were both 1 mM. ‡ indicates that amino acid-supplemented Krebs–Henseleit medium was used. Amino acids and their final concentrations are listed in the Materials and methods section. Means ± S.E.M. are shown for 4 experiments. Data were analysed using ANOVA followed by Newman–Keuls multiple comparisons post test, with P < 0.05 taken to indicate a significant difference. * indicates a significant difference from the methionine alone values.

<table>
<thead>
<tr>
<th></th>
<th>Homocysteine export (nmols/mg of dry weight per h)</th>
<th>Cysteine export (nmols/mg of dry weight per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>2.61 ± 0.16</td>
<td>6.40 ± 0.78</td>
</tr>
<tr>
<td>Methionine + AA</td>
<td>2.86 ± 0.28</td>
<td>6.55 ± 0.84</td>
</tr>
<tr>
<td>Methionine + serine</td>
<td>1.64 ± 0.08*</td>
<td>10.62 ± 0.69*</td>
</tr>
<tr>
<td>Methionine + serine + AA</td>
<td>1.69 ± 0.07*</td>
<td>9.34 ± 0.49*</td>
</tr>
</tbody>
</table>

Figure 5 Extracellular, intracellular and total production of homocysteine from hepatocytes isolated from animals maintained on 20% and 60% casein diets

Means ± S.E.M. for 4 experiments are shown. Open and hatched bars represent data from 20% and 60% casein diets respectively. Hepatocytes were preincubated, gassed and centrifuged as described in Table 1 and the Materials and methods section, and pellet resuspensions were carried out as described in Table 2. Intracellular values were obtained by subtracting extracellular values from the total. Animals were maintained on the indicated diets for 7 days. Methionine and serine concentrations were both 1 mM. The letters apply to comparisons within each panel. Bars with different letters are significantly different from each other (P < 0.05).

Figure 4 Total plasma homocysteine (A) and methionine (B) in rats fed 20% and 60% casein diets

Animals were fed a 20% or 60% casein-based diet for 7 days. Homocysteine and methionine were assayed as described in the Materials and methods section. Open and hatched bars represent 20% and 60% casein diets respectively. Means ± S.E.M. for 4 animals are shown. * indicates a statistically significant difference from the rats fed the 20% protein diet (P < 0.05).

Effect of dietary protein

We examined the effects of different levels of protein intake on homocysteine metabolism, since an increased protein intake will oblige the liver to catabolise more methionine. Similar induction of methionine metabolism has been observed by Finkelstein and Martin [20], who showed that methionine addition to a normal diet activates methionine metabolism. Rats were, therefore, fed purified diets containing 20% (normal) or 60% (high) protein.

Those fed the 60% protein diet had a significantly increased total plasma homocysteine level (Figure 4) compared with those fed the 20% protein diet (15.24 ± 0.28 μM compared with 10.35 ± 0.34 μM; P < 0.05). Plasma methionine was unchanged (Figure 4).

In vitro studies were performed using hepatocytes isolated from animals maintained as above. Hepatocytes were incubated with 1 mM methionine ± 1 mM serine, and extracellular, intracellular and total production of homocysteine (Figure 5) and cysteine (Figure 6) were measured. In the presence of 1 mM methionine, hepatocytes from rats fed the high protein diet exported (extracellular) significantly more homocysteine compared with hepatocytes from rats fed the normal protein diet.

© 2000 Biochemical Society
(4.19 ± 0.12 nmol/mg per h versus 2.62 ± 0.13 nmol/mg per h). Total homocysteine production was significantly greater in the high protein group in the presence of 1 mM methionine compared with the hepatocytes from rats fed the normal protein diet (5.02 ± 0.27 nmol/mg per h versus 3.69 ± 0.12 nmol/mg per h). However, no significant change in export was observed between the two groups.

Inclusion of serine had an interesting effect. In the normal protein group, 1 mM serine significantly lowered both components (extra- and intra-cellular) of homocysteine production from hepatocytes (Figure 5). Total production was decreased from 3.24 ± 0.11 nmol/mg per h to 2.13 ± 0.06 nmol/mg per h. Similarly, exported (extracellular) homocysteine was lowered from 2.62 ± 0.13 nmol/mg per h to 2.05 ± 0.07 nmol/mg per h. However, there was a much more marked effect of serine in hepatocytes prepared from rats fed the high protein diet. Again, serine significantly lowered both components (extra- and intra-cellular) of homocysteine production. Homocysteine production was decreased from 5.02 ± 0.06 nmol/mg per h to 1.34 ± 0.08 nmol/mg per h. Similarly, homocysteine export was significantly decreased from 4.19 ± 0.12 nmol/mg per h to 1.08 ± 0.09 nmol/mg per h in hepatocytes prepared from animals fed the high protein diet. Thus the addition of serine to hepatocytes prepared from rats fed the high protein diet reduced homocysteine export and production below the levels seen in hepatocytes from rats fed the normal protein diet.

Serine also affected cysteine accumulation (Figure 6). In the normal protein group cysteine production significantly increased when serine was included (from 3.69 ± 0.12 nmol/mg per h to 5.36 ± 0.13 nmol/mg per h) due to an increase in the intracellular component. However, no difference in cysteine export was observed. It was in the high protein group, serine significantly increased both cysteine production (from 5.02 ± 0.27 nmol/mg per h to 7.61 ± 0.60 nmol/mg per h) and export (3.47 ± 0.33 nmol/mg per h versus 5.34 ± 0.38 nmol/mg per h) compared with methionine alone. Between-group comparisons show that hepatocytes from the high protein-fed rats both exported (3.14 ± 0.46 nmol/mg per h versus 5.34 ± 0.38 nmol/mg per h) and produced (5.36 ± 0.135 nmol/mg per h versus 7.61 ± 0.605 nmol/mg per h) more cysteine than hepatocytes from the normal protein-fed group in the presence of 1 mM methionine and 1 mM serine.

**In vivo effect of dietary serine on plasma homocysteine levels**

The data in Figure 5 showed that the inclusion of serine in hepatocyte incubations could reduce homocysteine production and export more profoundly in cells from rats fed the 60% casein diet compared with the 20% casein diet while, in the absence of serine, the opposite result was found. Since, in vivo, rats fed the high protein diet had elevated plasma homocysteine (Figure 4) we investigated whether provision of extra serine to these animals could reduce plasma homocysteine. Therefore rats were fed the 60% casein diets for 7 days, supplemented with additional serine (5 g/100 g of casein) or alanine (5 g/100 g of casein) as a control. This level of supplementation roughly doubled the total serine and alanine intake of these animals. The results of these experiments are shown in Table 5. Total plasma homocysteine was significantly lowered by 25–30% in the rats fed the serine-enriched diet versus controls. Plasma methionine values were also lowered in the rats fed the serine-enriched diet, but this significance is lost when compared with the alanine-supplemented group. No significant differences were noted in the total plasma values of cysteine.

**DISCUSSION**

The importance of the liver in homocysteine metabolism

The objective of the present study was to establish experimental conditions whereby factors that affect homocysteine and cysteine production in, and export from, hepatocytes could be examined and compared with the situation in vivo. It should be noted, of course, that in these studies the terms ‘cysteine’ and ‘homocysteine’ are used generically (and encompass such species as cysteine, cystine, homocysteine, homocystine and mixed disulphides), since the analytical methodology reduces all of the oxidized species to thiol.

We have shown that hepatocytes can make a major contribution to plasma homocysteine. The dependence of homocysteine export on methionine concentration was established (Figure 1), with half-maximal effects evident at a methionine concentration of 0.44 mM. This is much higher than circulating methionine concentrations (60 μM in the rat, as shown in Figure 4, and 20–50 μM in humans [21]) and is, therefore, entirely consistent with the elevations in plasma homocysteine that occur upon methionine loading, in rats [22] and man [23].

The liver is the major site of methionine catabolism [20,24]. It is also the exclusive site of guanidinoacetate methyltransferase, which synthesizes creatine and is, by far, the dominant methylating action in mammals [25]. It is to be expected, therefore, that the liver would play a major role in determining circulating homocysteine levels. This is reflected in the high rates of homocysteine export relative to those of other cells. This has been reported by Christensen et al. [13] in studies of homocysteine export by cultured human and rat cells. It was found that homocysteine export from non-transformed cultured hepatocytes was much greater than from other cell lines (lymphoma cells, fibroblasts and hepatoma cells) and very much dependent on extracellular methionine. We compared cultured-hepatocyte homocysteine export rates in these experiments with our own. With 1 mM methionine, homocysteine export was approx. 11 nmol/mg of dry weight per h, after approximating their cell expression to mg of dry weight according to Krebs et al. [26], compared with our rate of 2–3 nmol/mg of dry weight per h.

Table 5  Selected plasma amino acids from rats fed a high protein diet further enriched with serine or alanine

<table>
<thead>
<tr>
<th>Plasma amino acid (μM)</th>
<th>Control diet</th>
<th>Serine-enriched diet</th>
<th>Alanine-enriched diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>13.37 ± 0.96*</td>
<td>9.58 ± 1.07*</td>
<td>12.61 ± 0.52*</td>
</tr>
<tr>
<td>Methionine</td>
<td>56.00 ± 1.70*</td>
<td>50.11 ± 1.71*</td>
<td>53.20 ± 1.33*</td>
</tr>
<tr>
<td>Cysteine</td>
<td>362.0 ± 3.26</td>
<td>368.9 ± 6.50</td>
<td>351.1 ± 5.24</td>
</tr>
</tbody>
</table>

Animals were fed a 60% casein-based diet for 7 days. The serine- and alanine-enriched diets were supplemented with the indicated amino acid at a level of 5 g/100 g of casein. The control diet was without supplementation. Homocysteine, cysteine and methionine were assayed as described in the Materials and methods section. Means ± S.E.M. are shown; n = 4 for the control and alanine-enriched diets; n = 3 for the serine-enriched diet. Data were analysed using ANOVA followed by Newman–Keuls multiple comparisons post test, with P < 0.05 taken to indicate a significant difference. Columns with differing superscripts are significantly different (P < 0.05).
another study, Svardal et al. [27] incubated rat primary hepatocytes in a manner similar to our own and obtained homocysteine export rates of approx. 1.6 nmol/mg of dry weight per h, again after converting their cell expression into mg of dry weight. It is possible that the different media present in the cell culture experiments may account for the differences in homocysteine export.

It is possible to relate the rate of homocysteine export found in our experiments to the situation in vivo. The rate of 2.4 nmol/mg of dry weight per h (Table 1) with 1 mM methionine as substrate converts into approximately 480 nmol/g of liver per h, or 5 μmol/h in a 250 g rat with a 10 g liver. However, these values must be corrected to reflect the physiological plasma methionine concentration, approx. 60 μM, as given in Figure 4. Since homocysteine export exhibits Michaelis– Menten-type kinetics with respect to methionine concentration, with a $K_m$ of 0.44 mM (Figure 1), it may be calculated that hepatocyte homocysteine export at 60 μM methionine corresponds to a total hepatic export of approx. 40 μmoles/day in a 250 g rat. Thus hepatic export of homocysteine could account for as much as 5% of the total methionine ingested in animals ingesting a 20% protein diet. It compares with a metabolic removal of plasma homocysteine by rat kidneys of approx. 11 μmoles/day in a 250 g rat [9].

**Measurement of total homocysteine and cysteine**

Hepatocyte incubations are routinely terminated with acid, as this is convenient, quick and facilitates accurate timing of the incubations. Such methodology is quite straightforward when the metabolites of interest are stable and soluble in the acid. However, occasionally a metabolite is precipitated by the acid and supplementary techniques must be employed to extract such a metabolite from the precipitate, e.g. long-chain acyl-CoA [28].

A similar situation obtained with these thiols became evident to us when we obtained the impossible result that cysteine export from hepatocytes exceeded production. Such a problem was not unexpected, since acid-precipitated plasma protein contains bound thiols [27], and there is no reason why this should not apply to cellular proteins. Clearly such errors in determining the rates of thiol accumulation and metabolism unless we could introduce a technique for liberating these bound thiols that was compatible with our hepatocyte incubation methodologies. We accomplished this by re-suspending the precipitated protein and displacing the bound thiols with a reducing agent. We showed that a single cycle of this procedure could remove all of the bound homocysteine and cysteine. It is clear from the data in Figure 3 that protein-bound homocysteine is only a minor component of its total production (< 9%). Binding of homocysteine to hepatic proteins has been observed by Svardal et al. [27], who also showed that the principal binding sites were located in the microsomal fraction. The physiological and pathological relevance of this homocysteine binding has yet to be determined. However, Stumm and Reynolds [29] have argued that intracellular homocysteine, rather than total plasma homocysteine, may be more important in determining pathological outcomes.

In contrast, it is clear that the protein-bound fraction of cysteine is a major component of total production. Hence application of a method such as ours is necessary for accurate measurement of cysteine production in hepatocytes.

**Effect of high protein and the role of serine**

Perhaps the most important finding in the present study is the effect of a high protein diet and the role of serine in modifying it. Several studies have examined the short- and long-term effects of dietary protein intake on total homocysteine levels in humans. A single high-protein meal has been shown to significantly increase both plasma methionine and total homocysteine levels [21]. The elevation in plasma homocysteine was still evident in samples taken after an overnight fast the following day. Conversely, a study of retired American schoolteachers found that increased dietary protein intake (long-term) was associated with lower fasting serum total homocysteine [30]. Such a result appears counter-intuitive, since oral methionine loading increases homocysteine [23] and dietary methionine is correlated with dietary protein [30]. However, increased dietary protein and methionine are known to induce methionine-catabolizing enzymes, allowing for efficient removal of homocysteine. Sustained high protein and methionine intakes can, therefore, be accommodated. Homocysteine removal, however, can only be effective in the presence of adequate serine.

Figures 4–6 summarize our in vivo and in vitro dietary studies. Figure 4 shows that total plasma homocysteine in rats maintained on a 60% casein diet was approx. 30% higher than that of rats maintained on a 20% casein diet (15.24 ± 0.28 μM versus 10.35 ± 0.34 μM), while plasma methionine remained unchanged. It might be thought that plasma homocysteine would remain unchanged if induction of the transulfuration enzymes by diet would compensate for an increased availability of methionine [20]. Such a homoeostatic mechanism should therefore maintain plasma homocysteine concentrations within a narrow range. However, this was not observed.

Our hepatocyte studies mirrored these results. Hepatocytes isolated from animals maintained on 60% casein diets and incubated with 1 mM methionine exported and produced significantly more homocysteine compared with those maintained on 20% casein diets. Similarly cysteine production was significantly greater in the 60% casein group under the same conditions. When serine was included in these incubations both the production and export of homocysteine were reduced, the effect being very pronounced in hepatocytes from the 60% casein-fed rats, such that these cells now produced (and exported) less homocysteine than the cells from the 20% casein-fed rats. This result was intriguing. It has been shown by Fafournoux et al. [31] that both plasma and hepatic serine levels are decreased in rats fed a high protein diet compared with a moderate protein diet, possibly due to induction of serine dehydratase. As previously mentioned, it is known that the enzymes of the transulfuration pathway are induced under these dietary conditions. The $K_m$ of cystathionine $\beta$-synthase for serine is 2–4 mM [32], however, so that at low hepatic serine concentrations, flux through the transulfuration pathway may be limited by serum availability. In light of both the effects of high protein diet on serine levels as outlined above and our plasma values, we suggest that serine may become limiting under high protein conditions and thus reduce the effectiveness of the homoeostatic increase in trans sulphuration enzymes. Under these conditions, steps leading to the synthesis of homocysteine may outpace those that remove it.

The effects of serine on plasma homocysteine were further characterized by supplementing high protein diets with serine. It was found that total homocysteine was significantly reduced in the plasma of rats fed the serine-supplemented diet compared with rats receiving control diets (9.58 ± 1.07 μM versus 13.67 ± 0.96 μM). Thus under high protein conditions serine does indeed become limiting and this limitation affects homocysteine levels. Finally, we believe the data we have presented here indicate a significant role for the liver in the regulation of plasma homocysteine and cysteine levels.
This study was supported by grants from the Canadian Diabetes Association (CDA) and the Medical Research Council (MRC) of Canada. L.M.S. was the recipient of a Natural Sciences and Engineering Research Council (NSERC) Graduate Fellowship. We acknowledge the invaluable assistance of B. Hall.

REFERENCES


Received 10 May 2000; accepted 16 June 2000

© 2000 Biochemical Society