Characterization of homocysteine metabolism in the rat liver

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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 transsulphuration 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, transsulphuration.

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 transsulphuration 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 homeostasis 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 transsulphuration 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 serine 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-diazole-4-sulphonic acid; TNB, tri-*n*-butylphosphine.

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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 Na⁺/6 mM K⁺/1.3 mM Ca²⁺/1.2 mM Mg²⁺/126 mM Cl⁻/1.2 mM H₂PO₄⁻/1.2 mM SO₄²⁻/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 thiol analysis. This involved reduction of all thiols with 10% (v/v) tri-*n*-butylphosphine (TNB) in dimethylformamide (DMF), followed by deproteinization with perchloric acid and derivatization with 7-fluoro-2-oxa-1,3-diazole-4-sulphonic acid (SBDF; see the Analyses section). Total homocysteine and cysteine production were measured after adding perchloric acid to stop the incubation [final concentration of 4.5% (w/v)]. After centrifugation to remove the precipitated protein (14000 g for 2 min) the supernatant was removed for assay as above, and then thiol bound to the precipitated protein was quantified as follows: the precipitated protein was resuspended in 2 M potassium borate buffer, pH 10.5, containing 5 mM EDTA. Thiols were subsequently reduced with 10% TNB in DMF for 45 min at 4 °C. The samples were then re-acidified with perchloric acid to reprecipitate protein and the supernatants were assayed for homocysteine and cysteine as described below. Experiments described in the Results section demonstrate that all of the bound homocysteine was liberated by this procedure.

In a series of experiments hepatocytes were incubated with L-[1-¹⁴C]methionine (American Radiolabeled Chemicals Ltd., St. Louis, MO, U.S.A.), in the presence and absence of 1 mM serine. The second step in the transsulphuration pathway involves production of α -ketobutyrate. In the presence of L-[1-¹⁴C]methionine this gives rise to [1-¹⁴C] α -ketobutyrate. [1-¹⁴C] α -Ketobutyrate can be metabolized via pyruvate dehydrogenase, which will produce ¹⁴CO₂. Label in unmetabolized [1-¹⁴C] α -ketobutyrate can be released with hydrogen peroxide. The sum of these numbers is the total ¹⁴CO₂ production and this can be used as a measure of flux through the transsulphuration pathway. We carried out such experiments to define the role of production of α -ketobutyrate via the transsulphuration pathway. This procedure involved the trapping and counting of ¹⁴CO₂ after incubation of the cells with L-[1-¹⁴C]methionine (1 mM). Incubations were performed as described above, except that incubation flasks were equipped with rubber septa in which plastic centre wells containing NCS-II Tissue Solubilizer (Amersham International, Oakville, Ontario, Canada) were suspended. Incubations were terminated with 0.3 ml of 30% (w/v) perchloric acid added through the septa with a syringe, and ¹⁴CO₂ was collected for 1 h. Centre

wells 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 μ M alanine, 194 μ M arginine, 64 μ M asparagine, 32 μ M aspartic acid, 123 μ M glutamic acid, 643 μ M glutamine, 295 μ M glycine, 72 μ M histidine, 99 μ M isoleucine, 153 μ M leucine, 72 μ M phenylalanine, 226 μ M proline, 61 μ M tryptophan, 75 μ M tyrosine, 130 μ M threonine, 184 μ M valine, 303 μ M taurine, 88 μ M citrulline and 72 μ M 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 anaesthetized 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) sulphosalicylic 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 Xchange 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 $\pm$ 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

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 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 $\pm$ 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 performed with 1 mM methionine and various serine concentrations for 30 min. Means $\pm$ S.E.M. for 4 experiments are shown. * indicates statistically significant differences from incubations with 1 mM methionine alone ($P < 0.05$).

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 μ M 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 ($r^2 =$

Table 1 Hepatocyte incubations with glycine, serine and glucose

Hepatocytes were preincubated for 20 min at 6–8 mg dry weight of cells/ml in a final volume of 1 ml in Krebs–Henseleit medium containing 1.25% BSA. At the end of the preincubation period, 1 mM methionine, 1 mM glycine, 1 mM serine and 5 mM glucose were added as shown and the incubation was allowed to continue for an additional 30 min. Cells were gassed with O_2/CO_2 (19:1) at the beginning of the incubation and on the addition of substrates. At the end of the incubation, cells and incubation medium were centrifuged at 14 000 g for 2 min and the supernatant was removed for analysis as described in the Materials and methods section. Means $\pm$ S.E.M. for 4 experiments are shown. Results with differing superscripts were significantly different from each other ($P < 0.05$).

Incubation	Homocysteine export (nmol/mg per h)
Methionine	2.40 $\pm$ 0.17*
Methionine + glycine	2.21 $\pm$ 0.25*
Methionine + serine	1.47 $\pm$ 0.03†
Methionine + glycine + serine	1.44 $\pm$ 0.11†
Methionine + glycine + serine + glucose	1.45 $\pm$ 0.07†

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 transsulphuration 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 nmol/mg per h to 1.62 ± 0.29 nmol/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 vivo* [14]. Glucose was provided as a potential source of NADPH via the pentose phosphate pathway, since NADPH is required by N^5,N^{10} -methylenetetrahydrofolate 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.

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

Figure 3 Reduction and removal of bound thiols from perchloric acid-precipitated protein

Hepatocytes were preincubated, gassed and centrifuged as described in Table 1 and the Materials and methods section. Homocysteine values (top panel) are for incubations which contained 1 mM methionine. Cysteine data (bottom panel) are for incubations which contained 1 mM methionine and 1 mM serine. Pellet resuspensions were carried out as described in Table 2 and in the Materials and methods section. Letters refer to homocysteine or cysteine as follows: A, exported homocysteine or cysteine; B, homocysteine or cysteine in the acid supernatant after the cell incubations were terminated by perchloric acid; C, homocysteine or cysteine removed from the pellet produced in B by treatment with TNB; D, homocysteine or cysteine removed from the pellet produced in C by treatment with TNB. Means $\pm$ S.E.M. for 3 experiments are shown.

(as 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 termination of 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 nmol/mg per h), whereas homocysteine loss is minor (0.28 nmol/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 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 $\pm$ 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 ($P < 0.05$).

	Homocysteine (nmol/mg per h)		Cysteine (nmol/mg per h)	
	Export	Production	Export	Production
Methionine	2.17 ± 0.06	$2.80 \pm 0.06^{*†}$	3.73 ± 0.84	4.90 ± 0.85
Methionine + serine	$1.29 \pm 0.09^{*†}$	1.60 ± 0.05	5.37 ± 1.28	7.51 ± 0.84

Table 3 $^{14}\text{CO}_2$ release from isolated rat hepatocytes incubated with L-[1- ^{14}C]methionine $\pm$ serine

Hepatocytes were incubated as described in Table 1 and the Materials and methods section. All concentrations are 1 mM. 'Acid' refers to $^{14}\text{CO}_2$ production following termination of the incubation with perchloric acid. ' H_2O_2 ' refers to additional $^{14}\text{CO}_2$ 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 $\pm$ 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 ($P < 0.05$).

	$^{14}\text{CO}_2$ production (nmol/mg of dry weight per h)		
	Acid	H_2O_2	Total production
L-[1- ^{14}C]Methionine	2.11 ± 0.11	0.69 ± 0.05	2.79 ± 0.12
L-[1- ^{14}C]Methionine + serine	$3.02 \pm 0.15^*$	0.78 ± 0.08	$3.81 \pm 0.13^*$

Table 2 shows homocysteine and cysteine export and production by hepatocytes incubated in the presence of 1 mM methionine $\pm$ 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 transsulphuration pathway, another means of determining this flux was needed. To this end we incubated hepatocytes with L-[1- ^{14}C]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 $^{14}\text{CO}_2$ production was significantly higher in hepatocytes incubated with L-[1- ^{14}C]methionine and 1 mM serine as compared with hepatocytes incubated with L-[1- ^{14}C]methionine alone (2.79 ± 0.12 nmol/mg of dry weight per h versus 3.81 ± 0.13 nmol/mg of dry weight per h). The increase in $^{14}\text{CO}_2$ 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.

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. 'AA' 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 $\pm$ 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.

	Homocysteine export (nmoles/mg of dry weight per h)	Cysteine export (nmoles/mg of dry weight per h)
Methionine	2.81 ± 0.16	6.40 ± 0.78
Methionine + AA	2.86 ± 0.28	6.55 ± 0.84
Methionine + serine	$1.64 \pm 0.08^*$	$10.62 \pm 0.69^*$
Methionine + serine + AA	$1.69 \pm 0.07^*$	$9.34 \pm 0.49^*$

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 $\pm$ S.E.M. for 4 animals are shown. * indicates a statistically significant difference from the rats fed the 20% protein diet ($P < 0.05$).

The effects of other amino acids on our system were examined using amino acid-supplemented Krebs–Henseleit medium as described in the Materials and methods section. No significant differences in either homocysteine or cysteine export were observed in amino acid-supplemented incubations when compared with non-supplemented controls (Table 4). We therefore felt that further supplementation was not warranted. Inclusion of serine, however, significantly increased cysteine export in both the supplemented and non-supplemented groups, as compared with cells incubated with methionine in the absence of serine.

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 catabolize 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.

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

Means $\pm$ 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 6 Extracellular, intracellular and total production of cysteine from hepatocytes isolated from animals maintained on 20% and 60% casein diets

Hepatocytes were preincubated, gassed and centrifuged as 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. Means $\pm$ S.E.M. for 4 experiments are shown. Open and hatched bars represent data from 20% and 60% casein diets respectively. 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 ($P < 0.05$).

Those fed the 60% protein diet had a significantly increased total plasma homocysteine level (Figure 4) compared with those fed the 20% protein diet ($15.24 \pm 0.28 \mu\text{M}$ compared with $10.35 \pm 0.34 \mu\text{M}$; $P < 0.05$). 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 $\pm$ 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

(4.19 ± 0.12 nmoles/mg per h versus 2.62 ± 0.13 nmoles/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 (5.62 ± 0.06 nmoles/mg per h versus 3.24 ± 0.11 nmoles/mg per h).

Cysteine production (Figure 6) 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 nmoles/mg per h versus 3.69 ± 0.12 nmoles/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 nmoles/mg per h to 2.13 ± 0.06 nmoles/mg per h. Similarly, exported (extracellular) homocysteine was lowered from 2.62 ± 0.13 nmoles/mg per h to 2.05 ± 0.07 nmoles/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 5.62 ± 0.06 nmoles/mg per h to 1.34 ± 0.08 nmoles/mg per h. Similarly, homocysteine export was significantly decreased from 4.19 ± 0.12 nmoles/mg per h to 1.08 ± 0.09 nmoles/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 nmoles/mg per h to 5.36 ± 0.13 nmoles/mg per h) due to an increase in the intra-cellular component. However, no difference in cysteine export was observed. In the high protein group, serine significantly increased both cysteine production (from 5.02 ± 0.27 nmoles/mg per h to 7.61 ± 0.60 nmoles/mg per h) and export (3.47 ± 0.33 nmoles/mg per h versus 5.34 ± 0.38 nmoles/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 nmoles/mg per h versus 5.34 ± 0.38 nmoles/mg per h) and produced (5.36 ± 0.135 nmoles/mg per h versus 7.61 ± 0.605 nmoles/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

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

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 $\pm$ 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 ($P < 0.05$).

Plasma amino acid (μ M)	Control diet	Serine-enriched diet	Alanine-enriched diet
Homocysteine	$13.37 \pm 0.96^*$	$9.58 \pm 1.07^\dagger$	$12.61 \pm 0.52^*$
Methionine	$56.00 \pm 1.70^*$	$50.11 \pm 1.71^\dagger$	$53.20 \pm 1.33^*\dagger$
Cysteine	362.0 ± 3.26	368.9 ± 6.50	351.1 ± 5.24

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 thiols.

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 μ M in the rat, as shown in Figure 4, and 20–50 μ M 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 methylation reaction 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. In

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 μ mol/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 μ M, 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 μ M methionine corresponds to a total hepatic export of approx. 40 μ moles/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 μ moles/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 there could be appreciable 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, Stamm 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 ($15.24 \pm 0.28 \mu$ M versus $10.35 \pm 0.34 \mu$ M), while plasma methionine remained unchanged. It might be thought that plasma homocysteine would remain unchanged if induction of the transulphuration enzymes by diet would compensate for an increased availability of methionine [20]. Such a homeostatic 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 transulphuration pathway are induced under these dietary conditions. The K_m of cystathionine β -synthase for serine is 2–4 mM [32], however, so that at low hepatic serine concentrations, flux through the transulphuration pathway may be limited by serine 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 homeostatic increase in transulphuration 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 ($9.58 \pm 1.07 \mu$ M versus $13.67 \pm 0.96 \mu$ M). 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.

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