

## Distribution of methionine between cells and incubation medium in suspension of rat hepatocytes

Tatyana K. Korendyaseva · Michael V. Martinov ·  
Alexander M. Dudchenko · Victor M. Vitvitsky

Received: 13 December 2009 / Accepted: 10 March 2010 / Published online: 24 March 2010  
© Springer-Verlag 2010

**Abstract** Methionine is an essential amino acid involved in many significant intracellular processes. Aberrations in methionine metabolism are associated with a number of complex pathologies. Liver plays a key role in regulation of blood methionine level. Investigation of methionine distribution between hepatocytes and medium is crucial for understanding the mechanisms of this regulation. For the first time, we analyzed the distribution of methionine between hepatocytes and incubation medium using direct measurements of methionine concentrations. Our results revealed a fast and reversible transport of methionine through the cell membrane that provides almost uniform distribution of methionine between hepatocytes and incubation medium. The steady-state ratio between intracellular and extracellular methionine concentrations was established within a few minutes. This ratio was found to be  $1.06 \pm 0.38$ ,  $0.89 \pm 0.26$ ,  $0.67 \pm 0.16$  and  $0.82 \pm 0.06$  at methionine concentrations in the medium of  $64 \pm 19$ ,  $152 \pm 39$ ,  $413 \pm 55$ , and  $1,204 \pm 104$   $\mu\text{mol/L}$ , respectively. The fast and uniform distribution of methionine between hepatocytes and extracellular compartments provides a possibility for effective regulation of blood methionine levels due to methionine metabolism in hepatocytes.

**Keywords** Methionine transport ·  
Methionine distribution · Hepatocytes · Rat · Liver

T. K. Korendyaseva · M. V. Martinov · V. M. Vitvitsky (✉)  
National Research Center for Hematology,  
Novozykovsky proezd, 4a, Moscow 125167, Russia  
e-mail: victor\_vitvitsky@yahoo.com

A. M. Dudchenko  
Institute of General Pathology and Pathophysiology,  
Moscow 125315, Russia

### Introduction

Methionine is an essential amino acid which plays a significant role in many intracellular processes. Methionine functions as an initiator for the synthesis of protein molecules, as a substrate for synthesis of *S*-adenosylmethionine, the main intracellular methylating agent, and as a precursor for polyamine synthesis. In many cells and tissues, methionine can be converted to cysteine, a substrate for synthesis of the main intracellular antioxidant, glutathione. Aberrations in methionine metabolism are associated with a number of complex pathologies, such as neural tube defects (Beaudin and Stover 2009), cancer (Mato et al. 2008), cardiovascular disorders (Yamada et al. 2008), and neurodegenerative diseases (Martignoni et al. 2007; Tchanchou and Shea 2008). Study of methionine metabolism is very significant for understanding of normal regulation of the level of this essential amino acid in different organs and tissues as well as for understanding molecular mechanisms of many pathologies.

It has been shown that a tenfold increase in dietary methionine barely increases the methionine concentration in liver, while levels of its metabolite *S*-adenosylmethionine increase several-fold (Finkelstein et al. 1982; Finkelstein and Martin 1986). This implies that liver activates methionine metabolism in accordance with methionine intake in order to prevent an increase in methionine concentration. A several-fold increase in liver methionine concentration can be obtained after an intraperitoneal injection of methionine (Finkelstein et al. 1982). This is accompanied by a corresponding increase in the *S*-adenosylmethionine level and followed by a rapid drop of methionine to its initial values. In peripheral blood, methionine levels increase 2–3 times after food consumption but return rapidly to normal values (Forslund et al.

2000; Guttormsen et al. 2004). An excess of methionine is disposed in liver cells via transsulfuration pathway *in vitro* (Rao et al. 1990) and *in vivo* (Mudd et al. 1980). In comparison with other organs, liver has the most complex methionine metabolism (Martinov et al. 2010). Deficiencies of liver-specific enzymes responsible for methionine consumption lead to dramatic elevation of blood methionine levels (Chamberlin et al. 2000; Lu et al. 2001; Mudd et al. 2001). Also, an interruption of methionine resynthesis from its metabolite homocysteine in liver causes pronounced decrease in blood methionine levels (Shinohara et al. 2006).

Thus, liver is the key organ that maintains normal methionine levels in blood. A number of theoretical and experimental studies have shown that kinetic properties of liver enzymes involved in methionine metabolism provide possibilities for very complicated metabolic behavior, including metabolic switch from methionine conservation at low methionine concentrations to methionine disposal at high methionine concentrations and stabilization of methionine levels (Korendyaseva et al. 2008; Martinov et al. 2000; Nijhout et al. 2006; Prudova et al. 2005; Reed et al. 2004). However, complete understanding of methionine metabolism regulation at the level of cells needs understanding of methionine distribution between intracellular and extracellular compartments. Transport limitations and uneven distribution between cells and extracellular compartments can significantly modify intracellular methionine metabolism. There are many papers related to investigation of methionine metabolism. On the other hand, only a few papers deal with methionine transport in hepatocytes. Thus, transmembrane transport of methionine and its distribution between hepatocytes and media remain poorly studied. While methionine can be transported across hepatocyte membrane by  $\text{Na}^+$ -dependent amino acid transport system, the rate of passive ( $\text{Na}^+$ -independent) transport is at least six times higher (Kilberg et al. 1981; Schreiber and Schreiber 1972). At physiological methionine concentrations the rate of methionine transport in hepatocytes is approximately 100  $\mu\text{mol}/\text{min}$  L cells (Aw et al. 1986; Kilberg et al. 1981) that is fast enough to provide equilibration of methionine between cells and incubation medium in approximately 1 min. However, experiments with radioactive methionine give contradictory results regarding the equilibrium distribution of methionine between cells and medium, the time it takes to reach equilibrium and the reversibility of methionine transport in hepatocytes. In the paper of Schreiber and Schreiber (1972) it was found that in methionine concentration ranges from 18  $\mu\text{mol}/\text{L}$  to 10  $\text{mmol}/\text{L}$ , its transport in hepatocytes is reversible, the equilibrium between intracellular and extracellular methionine is reached within about 1 min and the equilibrium

intracellular and extracellular methionine concentrations are equal. On the other hand, later publication of Aw et al. (1986) has shown that when methionine concentration increases from 100  $\mu\text{mol}/\text{L}$  to 1  $\text{mmol}/\text{L}$ , the time of methionine equilibration in hepatocytes increases from 1 to almost 30 min, and the ratio between equilibrium intracellular and extracellular methionine concentrations decreases twofold, from 0.6 to 0.3. Also, the data of this work does not confirm the reversibility of methionine transport in hepatocytes. These contradictions could be the result of essential methodological defects. Indeed, during investigations of methionine transport the metabolic consumption of methionine in cells was not taken into account. Our theoretical and experimental results have shown that regulation of methionine metabolism in hepatocytes is associated with the fast accumulation of a large amount of metabolic intermediates, mainly *S*-adenosylmethionine (Korendyaseva et al. 2008). Thus, if radioactive methionine is used, the buildup of radioactive products of methionine metabolism in hepatocytes can provide a significant contribution to the distribution of radioactivity between cells and incubation medium. Further, in the above-mentioned papers, before radioactivity analysis cells were washed out of the medium that, in case of reversible methionine transport, could lead to uncontrolled efflux of labeled methionine from the cells.

In our work we tried to fill the gap in understanding of methionine transport in hepatocytes. For the first time the rate and reversibility of methionine transport in hepatocytes, and distribution of methionine between hepatocytes and incubation medium have been evaluated using direct measurements of methionine concentration.

## Materials and methods

### Preparation of hepatocytes

The experimental protocol was approved by the Scientific Council of the National Research Center for Hematology. Hepatocytes were isolated from liver of Wistar female rats ( $250 \pm 30$  g) under sodium thiopental narcosis (60 mg/kg *i.p.*). The liver was perfused at a pressure of 120 mm  $\text{H}_2\text{O}$  with the media maintained at 37°C and saturated with carbogen gas mixture (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ). The following solution was used as a basic perfusion medium: 115 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L  $\text{KH}_2\text{PO}_4$ , 10 mmol/L glucose, and 25 mmol/L HEPES buffer, pH 7.5. Immediately after portal vein cannulation, the liver was perfused with 500 ml of basic medium supplemented with 0.5 mmol/L EGTA, followed by 13 min recirculation of 150 ml basic medium containing 2 mmol/L  $\text{CaCl}_2$  and 0.013% collagenase (type IV, Sigma). Finally, the liver

was dispersed in a plastic dish in 40–50 ml of basic medium at room temperature. The resulting cell suspension was passed through a 50- $\mu\text{m}$  nylon mesh. After sedimentation at 1 g, hepatocytes were washed three times by resuspension in 45 ml of basic medium and centrifugation for 1 min at 20 g and room temperature. Following the third wash, the cell pellet was resuspended in eight volumes of basic medium supplemented with 1 mmol/L  $\text{CaCl}_2$ , 1 mmol/L  $\text{MgCl}_2$ , 40  $\mu\text{mol/L}$  methionine and 2% BSA at room temperature. Cell count and viability were determined using a hemocytometer after staining with 0.4% trypan blue. Cells were used in experiments if viability was at least 92%.

#### Distribution of methionine between hepatocytes and incubation medium

Distribution of methionine between cells and incubation medium was determined using a suspension of freshly isolated hepatocytes containing  $\sim 10^7$  viable cells/mL. Aliquots of suspension were placed in glass conical flasks and incubated under air atmosphere at 37°C in orbital shaker at 100 RPM. Typically, a 50-ml flask contained 5 ml of cell suspension. Methionine was added to the cell suspension to a final concentration of 60–1,500  $\mu\text{mol/L}$ . Samples of the suspension were collected from flasks at the specified time intervals after methionine addition. Rapid separation of hepatocytes and medium was achieved by centrifugation through dibutylphthalate (Fariss et al. 1985). To this end 0.2 ml of 10% perchloric acid was placed into a 1.85-ml centrifuge tube and 0.45 ml of dibutylphthalate was placed above the acid. An aliquot of hepatocyte suspension (1.2 ml) was carefully placed above dibutylphthalate layer, the centrifuge tube was closed with stopper and centrifuged for 1 min at 14,000g at room temperature. A sample of the incubation medium was taken from the upper layer, fixed with a half volume of 10% perchloric acid, centrifuged for 3 min at 1,500g, and the supernatant was neutralized with saturated solution of  $\text{K}_2\text{CO}_3$  to pH  $\sim 8$ . Then, the liquid above dibutylphthalate layer was removed completely, the denatured cell pellet under dibutylphthalate layer was resuspended with narrow stainless steel wire, and the tube was centrifuged again for 3 min at 1,500g. Following centrifugation, perchloric cell extract was aspirated from beneath the dibutylphthalate layer, and neutralized to pH  $\sim 8$  with saturated solution of  $\text{K}_2\text{CO}_3$ . Neutralized samples were stored at  $-20^\circ\text{C}$  until they were used for methionine analysis.

#### Reversibility of methionine transport

To analyze the reversibility of methionine transport in hepatocytes, cells were loaded with methionine during

15 min incubation at 37°C in suspension containing  $10^7$  cells/mL and 1,500  $\mu\text{mol/L}$  methionine. The suspension was then centrifuged for 30 s at 250g at room temperature. The supernatant was completely removed, cells were resuspended in methionine-free medium to the concentration of  $\sim 10^7$  cells/mL and incubated under air atmosphere at 37°C in orbital shaker at 100 RPM. Samples for methionine analysis in incubation medium and in cells were collected and treated as described above.

#### Methionine analysis

To measure methionine concentration, samples were derivatized with 2,4-dinitrofluorobenzene and analyzed by HPLC as described elsewhere (Aw et al. 1986; Korendyaseva et al. 2008). Intracellular methionine concentration was calculated by taking into account volume occupied by hepatocytes in suspension. To measure this volume, cell suspension was centrifuged in a narrow capillary for 30 s at 170g and the ratio of cell column to total capillary content was determined. The ratio of total cell volume to cell count in suspension gives us the average hepatocyte volume of  $(10 \pm 2) \times 10^{-6}$   $\mu\text{l}$  (mean  $\pm$  SD,  $n = 10$ ). This value is in good agreement with literature data (Katayama et al. 2001) and with our own light microscopy data regarding size of rat hepatocytes. Thus, centrifugation of hepatocyte suspension in a capillary provides the correct method for the measurement of cell volume in suspension.

#### Statistical analysis

Statistical analysis of the experimental data was done using Origin 7.0 software (OriginLab Corporation, USA).

#### Mathematical model

In order to explore the possible impact of hepatocyte methionine metabolism on the distribution of methionine between cells and medium, we constructed a simple, qualitative mathematical model. The model describes the kinetics of intracellular methionine concentration as a difference between the rates of methionine transport and metabolic consumption:

$$\frac{dM_{\text{in}}}{dt} = V_t - V_c$$

where  $M_{\text{in}}$  denotes intracellular methionine concentration,  $V_t$  denotes the net methionine transport rate across the cell membrane and  $V_c$  denotes the net rate of metabolic methionine consumption inside the cell. There is no net methionine production in the model. While all mammalian cells express methionine synthase, this enzyme usually only provides recycling of intracellular methionine from its

metabolite homocysteine. The net methionine production can be obtained in case of some external source of homocysteine that does not correspond to our experimental conditions. Methionine transport rate in the model is described as a linear function of the difference between methionine concentrations in the medium and inside the cell:

$$V_t = K_t(M_{\text{ex}} - M_{\text{in}})$$

where  $K_t$  is a constant representing the diffusion coefficient of methionine through the cell membrane and  $M_{\text{ex}}$  denotes the extracellular methionine concentration. In accordance with available experimental data (Korendyaseva et al. 2008), the rate of metabolic consumption of methionine is described by sigmoidal dependence on methionine concentration:

$$V_c = \frac{K_c}{1 + \left(\frac{K_m}{M_{\text{in}}}\right)^4}$$

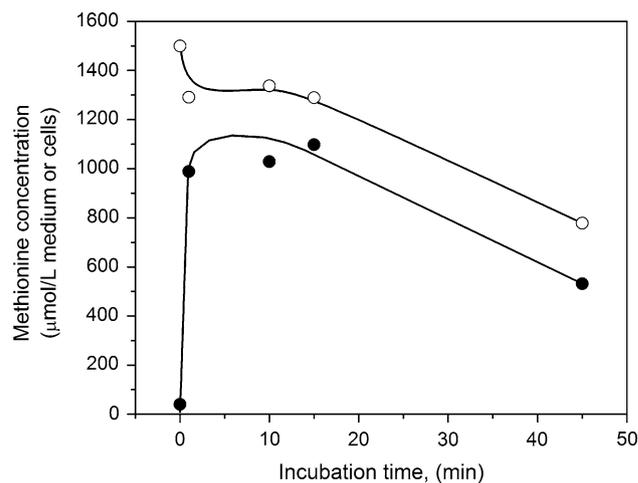
where constant  $K_c$  denotes the maximal rate of metabolic methionine consumption in hepatocytes and the constant  $K_m$  represents methionine concentration at which the rate of metabolic methionine consumption reaches half of its maximal value. Steady-state ratio between intracellular and extracellular methionine concentrations can be obtained from the following equation:

$$K_t(M_{\text{ex}} - M_{\text{in}}) = \frac{K_c}{1 + \left(\frac{K_m}{M_{\text{in}}}\right)^4}$$

Our data as well as literature data regarding the accumulation of added-to-incubation-medium methionine inside hepatocytes (Aw et al. 1986; Kilberg et al. 1981; Schreiber and Schreiber 1972) provides an estimation for  $K_t$  of approximately 1 per minute. Initial values for constants  $K_c$  and  $K_m$  were assumed to be 150  $\mu\text{mol}/\text{min L cells}$  and 100  $\mu\text{mol}/\text{L}$ , respectively, based on currently available experimental data (Korendyaseva et al. 2008).

## Results

When added to hepatocyte suspension, methionine rapidly enters the cells. Within 1 min after addition of methionine to a 10% hepatocyte suspension, cellular methionine levels increase to 50–100% of medium methionine concentration. Methionine concentration in the cells can increase slightly during the next few minutes and then both intracellular and extracellular methionine concentrations decrease due to metabolic consumption (Fig. 1). The decrease in methionine concentration does not cause a significant change in the ratio between intracellular and extracellular methionine concentrations. The ratios obtained at different methionine



**Fig. 1** Kinetics of methionine concentration in the medium (*open circles*) and in hepatocytes (*black circles*) after addition of methionine to the medium to a final concentration of 1,500  $\mu\text{mol}/\text{L}$

concentration ranges are presented in Table 1. It can be seen that the ratio between intracellular and extracellular methionine concentrations has a maximal value at low (physiological) methionine concentrations and a pronounced (statistically significant) minimum at methionine concentrations close to 400  $\mu\text{mol}/\text{L}$ .

Our mathematical model shows that the distribution of methionine between hepatocytes and incubation medium can be explained by the interaction between methionine transport and metabolic consumption. Theoretical curves have pronounced minima which depend on model parameters. As one can see from Fig. 2a, b, a decrease in methionine transport activity ( $K_t$ ) influences the dependence of methionine distribution between cells and medium on its concentration by the same way as an increase in activity of methionine metabolic consumption ( $K_c$ ). The minimum on the corresponding curve becomes more pronounced and shifts towards higher methionine concentrations. Parameter  $K_m$  represents methionine concentration providing half-maximal activity of metabolic methionine consumption. Increase in  $K_m$  value leads to a less pronounced minimum on the curve which shifts towards higher methionine concentrations (Fig. 2c). Variations in the  $K_m$  value do not change theoretical curves at high methionine concentrations. Despite its qualitative nature the model provides a good quantitative description for the experimental dependence of methionine distribution between hepatocytes and incubation medium on the methionine concentration even at initial values of  $K_t$  and  $K_c$  and  $K_m = 150 \mu\text{mol}/\text{L}$  (Fig. 2c). In this case the theoretical curve lies within the deviation of the experimental results. Model parameters can be easily adjusted to fit the mean experimental data. By decreasing the  $K_t$  value or increasing the  $K_c$  value the theoretical curve can be moved closer to

**Table 1** The dependence of steady-state distribution of methionine between hepatocytes and incubation medium on methionine concentration in the medium

$M_{\text{ex}}$	$M_{\text{in}}/M_{\text{ex}}^{\text{a}}$	Number of experiments	Statistical significance of the difference between current $M_{\text{in}}/M_{\text{ex}}$ value and the maximal $M_{\text{in}}/M_{\text{ex}}$ value ( $1.06 \pm 0.38$ )
$64 \pm 19$	$1.06 \pm 0.38$	10	–
$152 \pm 39$	$0.89 \pm 0.26$	5	NS, $p > 0.2$
$413 \pm 55$	$0.67 \pm 0.16$	13	S, $p < 0.003$
$1,204 \pm 104$	$0.82 \pm 0.06$	3	NS, $p > 0.1$

<sup>a</sup> The ratios values between intracellular and extracellular methionine concentrations were obtained as follows. Methionine concentrations in cells ( $M_{\text{in}}$ ) and in the medium ( $M_{\text{ex}}$ ) were measured in micromoles per liter of cells and in micromoles per liter of medium, respectively. In each particular experiment we chose the first time point in which intracellular methionine concentration had a maximal value or did not further increase within the frames of experimental error (not more than 10%). Usually it was the first or the second none zero time point. The ratio of  $M_{\text{in}}/M_{\text{ex}}$  was calculated for this point and for the following points (if exist) in which methionine concentration varied within  $\pm 10\%$ . In Fig. 1, for example, the ratio was calculated for the second, third and fourth points. Using these data we calculated the average  $M_{\text{in}}/M_{\text{ex}}$  ratio and corresponding average  $M_{\text{ex}}$  for each experiment. The average data of all experiments were combined in four concentration intervals (30–80, 100–200, 300–400, and 1,000–1,300  $\mu\text{mol/L}$ ). Finally, the average values of  $M_{\text{in}}/M_{\text{ex}}$  ratio and  $M_{\text{ex}}$  were calculated for each concentration interval and presented as mean  $\pm$  SD in the Table 1

NS not significant difference, S significant difference

the mean experimental point obtained at high methionine concentrations. Then, by increasing the  $K_m$  value the theoretical curve can be adjusted to fit the mean experimental points obtained at intermediate and low methionine concentrations. The result of such fitting procedure is shown in Fig. 2 by a dashed line obtained at the following parameter values:  $K_t = 0.8/\text{min}$ ,  $K_c = 150 \mu\text{mol/min L cells}$ , and  $K_m = 220 \mu\text{mol/L}$ . These parameter values are close to experimental estimations of corresponding parameters in rodent hepatocytes (Table 2).

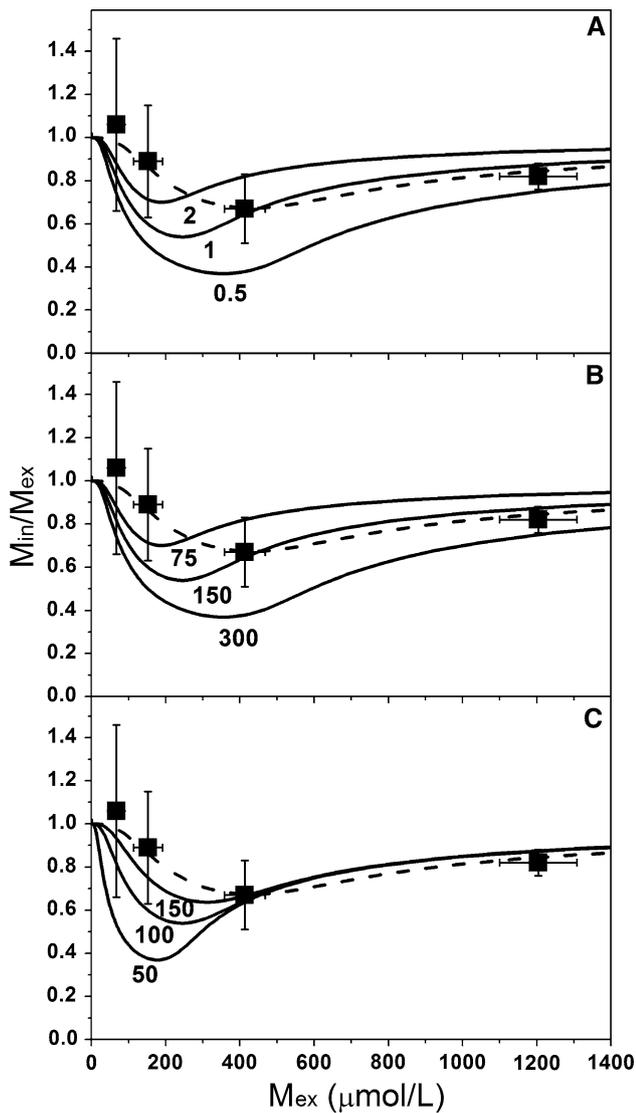
The pronounced dependence of methionine distribution between hepatocytes and medium on methionine metabolism, revealed by the model, suggests that this distribution is actually at a steady state rather than at equilibrium.

Methionine transport in hepatocytes is reversible. If loaded with methionine hepatocytes are resuspended in excess of methionine-free medium, then methionine concentration in the cells rapidly decreases and this is accompanied by a sharp accumulation of methionine in the medium (Fig. 3). During the first minute cells lose 60–80% of the methionine that has accumulated in the medium in equivalent quantity. As time proceeds, the methionine concentration in the cells continues to decrease approaching the concentration in the medium.

## Discussion

Our results reveal fast and reversible transport of methionine in hepatocytes. In concentration range from 60  $\mu\text{mol/L}$  to 1.5  $\text{mmol/L}$ , methionine rapidly passes through the hepatocyte membrane and reaches stable distribution between cells and incubation medium within a few

minutes. Normal physiological concentration of methionine in human and rodent plasma is in the range of tens of micromolars (Gupta et al. 2009; Guttormsen et al. 2004; Jacobs et al. 2001). In case of deficiency of liver methionine adenosyltransferase (the first enzyme in methionine metabolism system) methionine concentration in plasma can increase to millimolar levels (Chamberlin et al. 2000; Lu et al. 2001). Thus, our experimental conditions cover the physiologically relevant range of methionine concentrations. Contrary to data obtained with radioactive labels (Schreiber and Schreiber 1972), direct measurements of methionine show that its distribution between cells and incubation medium significantly depends on its concentration (Table 1; Fig. 2). According to our model, the dependence of the ratio between intracellular and extracellular methionine on methionine concentration is a consequence of different dependencies of methionine transport and metabolic consumption on methionine concentration. If methionine concentration increases from 0 to 200–400  $\mu\text{mol/L}$ , the rate of its metabolic consumption increases faster than the rate of transmembrane transport. As a result, methionine concentration in the cells increases less than in the medium and the ratio between intracellular and extracellular methionine concentrations decreases. For increases in the medium methionine concentration above 400  $\mu\text{mol/L}$ , the rate of methionine transport increases to a greater extent than the rate of its metabolic consumption and the ratio between intracellular and extracellular methionine concentrations begins to increase. Our model provides a good approximation for the dependence of methionine distribution between cells and incubation medium on its concentration (Fig. 2). This approximation can be obtained at physiologically relevant parameter



**Fig. 2** The dependence of steady-state distribution of methionine between hepatocytes and incubation medium on methionine concentration in the medium. *Solid lines* represent results of model simulation obtained at the following parameter values: **a**  $K_c = 150 \mu\text{mol}/\text{min L cells}$ ,  $K_m = 100 \mu\text{mol}/\text{L}$ , at different  $K_t$  values; **b**  $K_t = 1/\text{min}$ ,  $K_m = 100 \mu\text{mol}/\text{L}$ , at different  $K_c$  values; **c**  $K_t = 1/\text{min}$ ,  $K_c = 150 \mu\text{mol}/\text{min L cells}$ , at different  $K_m$  values. Values of variable parameters are shown under corresponding curves. Symbols show experimental data presented in Table 1. *Dashed line* represents result of model simulation at  $K_t = 0.8/\text{min}$ ,  $K_c = 150 \mu\text{mol}/\text{min L cells}$ , and  $K_m = 220 \mu\text{mol}/\text{L}$  providing the best fit to the experimental data

values and proves that the distribution of methionine between hepatocytes and incubation medium is at steady state rather than at equilibrium. Moreover, good description of experimental results in the model proves that the assumption regarding linear dependence of methionine transport rate on methionine concentration is correct and within tested methionine concentration interval this

dependence actually provides a good approximation for net methionine transport in hepatocytes.

In our experiments hepatocytes were separated from incubation medium by centrifugation through dibutylphthalate. It was shown earlier that after centrifugation through dibutylphthalate, packed hepatocytes may contain about 20% of incubation medium (Fariss et al. 1985). This contamination can affect the ratios between intracellular and extracellular methionine concentrations presented in Table 1 and Fig. 2. Simple calculations show, however, that the ratio values, obtained in our experiments may be overestimated by less than 10% that is not crucial for presented results.

Our previous experimental data regarding methionine metabolism regulation in hepatocytes demonstrate that an increase in methionine concentration from 0 to 100–200  $\mu\text{mol}/\text{L}$  causes sharp accumulation of a large amount of *S*-adenosylmethionine in the cells (Korendyaseva et al. 2008). In the case of radioactive methionine usage, this can lead to an overestimation of methionine level in the cells, and thus to an overestimation of the ratio between intracellular and extracellular methionine concentrations, particularly in the methionine concentration range from 100 to 1,000  $\mu\text{mol}/\text{L}$ . This can explain the independence of methionine distribution between cells and incubation medium, obtained earlier with radioactive methionine (Schreiber and Schreiber 1972). Also, accumulation of radioactive *S*-adenosylmethionine in hepatocytes can distort data regarding the reversibility of methionine transport. The cell membrane is impermeable to *S*-adenosylmethionine. Therefore in methionine-free medium, *S*-adenosylmethionine will remain inside the cells increasing intracellular radioactivity and producing the illusion of incomplete efflux of methionine from hepatocytes. Direct measurements of methionine demonstrate that in methionine-free medium it rapidly exits hepatocytes and distributes almost uniformly between cells and medium.

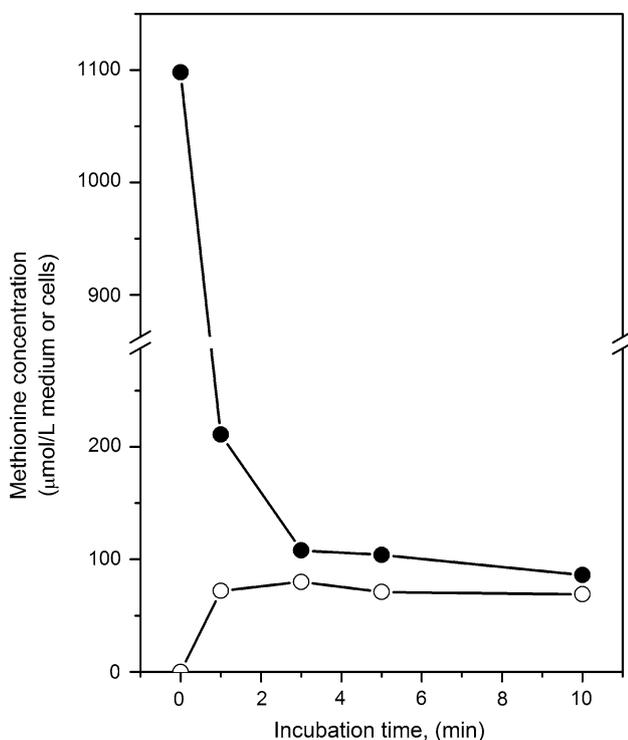
According to literature data, packed hepatocytes may contain about 25% of incubation medium (Krebs et al. 1974). This medium can be a source of additional extracellular methionine after resuspension of packed hepatocytes in methionine-free medium. However, our estimations show that the contamination of hepatocytes with incubation medium can provide less than 50% of the initial increase in medium methionine levels observed after resuspension of hepatocytes in methionine-free medium. Thus, at least more than 50% of methionine appears in the medium due to its efflux from the cells. In this way, even in the case of significant contamination of packed hepatocytes with methionine containing medium, our results confirm the reversibility of methionine transport in these cells.

Methionine can be transported across hepatocyte cell membrane by a number of  $\text{Na}^+$ -dependent and

**Table 2** Model parameter values providing the best fit for the experimental data and experimental estimations for these parameter values

Parameter	Units	Model value	Experimental estimation	Reference
$K_r$	$\text{min}^{-1}$	0.8	$>0.2^a$	Schreiber and Schreiber (1972)
			$0.32^b$	Kilberg et al. (1981)
			$1.0^c$	Aw et al. (1986)
$K_c$	$\mu\text{mol}/\text{min L cells}$	150	$100 \pm 40$	Korendyaseva et al. (2008)
$K_m$	$\mu\text{mol}/\text{L}$	220	50–100	Korendyaseva et al. (2008)

The experimental estimations for  $K_r$  were obtained at methionine concentrations of <sup>a</sup> 500  $\mu\text{mol}/\text{L}$  to 10  $\text{mmol}/\text{L}$ , <sup>b</sup> 100  $\mu\text{mol}/\text{L}$ , <sup>c</sup> 100  $\mu\text{mol}/\text{L}$



**Fig. 3** Kinetics of methionine concentration in the medium (*open circles*) and in hepatocytes (*black circles*) after resuspension of preloaded with methionine hepatocytes in methionine-free medium. Cells were loaded with methionine to a final concentration of 1,098  $\mu\text{mol}/\text{L}$  cells during 15 min incubation in the medium with initial methionine concentration of 1,500  $\mu\text{mol}/\text{L}$ . Then suspension was centrifuged, supernatant was completely removed and cells were resuspended in 10 volumes of methionine-free medium

$\text{Na}^+$ -independent transporters. Two  $\text{Na}^+$ -dependent amino acid transport systems (A and ASC) are able to transport methionine in hepatocytes (Kilberg et al. 1981). However, between two identified transporters of ASC system one (ASCT1) probably does not transport methionine (Arriza et al. 1993; Shafiqat et al. 1993) while the other one (ASCT2) is not expressed in liver (Utsunomiya-Tate et al. 1996). Between three identified transporters of system A (ATA1, ATA2, and ATA3) two, ATA2 (SNAT2) and ATA3 (SNAT4) are expressed in liver and can transport methionine (Hatanaka et al. 2001; Sugawara et al. 2000a, b; Tanaka et al.

2005). However, the kinetics of methionine transport by these transporters was not studied even in model systems. As was mentioned above, at methionine concentration of 100  $\mu\text{mol}/\text{L}$  a contribution of  $\text{Na}^+$ -dependent transporters to total methionine transport in hepatocytes is less than 20% (Kilberg et al. 1981). Between  $\text{Na}^+$ -independent transport systems (L,  $y^+\text{L}$ , asc,  $x_c^-$ ,  $b^{0,+}$ ), probably only system L can transport methionine in hepatocytes (Palacin et al. 1998; Wagner et al. 2001; Weissbach et al. 1982). So far four separate transporters were identified within system L: LAT1, LAT2 (del Amo et al. 2008), LAT3 (Babu et al. 2003), LAT4 (Bodoy et al. 2005). Transporter LAT4 is not expressed in liver (Bodoy et al. 2005). Transporters LAT1 and LAT2 work as amino acid exchangers (del Amo et al. 2008; Rossier et al. 1999), and low level expression of these transporters was found in mouse, rabbit and human liver (del Amo et al. 2008; Wagner et al. 2001). However, at least presence of functional LAT1 transporter in liver is questionable (Fukuhara et al. 2007). Transporter LAT3 is expressed in liver, and can provide methionine transport via facilitated diffusion (Babu et al. 2003; Fukuhara et al. 2007). The highest activity of LAT3 transporter expressed in *Xenopus* oocytes is associated with low (millimolar range) substrate affinity (Babu et al. 2003). Unfortunately, kinetics of methionine transport by potential  $\text{Na}^+$ -independent transporters actually has not been studied.

$\text{Na}$ -dependent transporters and amino acid exchangers cannot provide rapid methionine efflux from hepatocytes against  $\text{Na}^+$  gradient and into the amino-acid-free medium that was observed in our experiments. Properties of methionine transport in hepatocytes observed in our experiments and reported in other papers (Kilberg et al. 1981; Schreiber and Schreiber 1972) remind properties of LAT3 transporter (Babu et al. 2003). Thus, it looks plausible that methionine transport in hepatocytes is provided mainly by LAT3 transporter.

While the steady-state distribution of methionine between hepatocytes and incubation medium *in vitro* depends on the methionine concentration, it remains close to uniform distribution for all studied concentration ranges. The distribution of methionine between hepatocytes and blood *in vivo* can be affected by amino acid availability,

hormonal status and other factors (Christie et al. 2001; Jacobs et al. 2001). However, under normal physiological conditions in vivo, the liver and blood methionine concentrations are close to each other (Gupta et al. 2009; Jacobs et al. 2001) in accordance with our in vitro data demonstrating almost uniform distribution of methionine between hepatocytes and extracellular compartments. The fast and uniform distribution of methionine between hepatocytes and extracellular compartments provides a possibility for effective regulation of blood methionine levels due to methionine metabolism in hepatocytes.

## References

- Arriza JL, Kavanaugh MP, Fairman WA, Wu YN, Murdoch GH, North RA, Amara SG (1993) Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. *J Biol Chem* 268:15329–15332
- Aw TY, Ookhtens M, Kaplowitz N (1986) Mechanism of inhibition of glutathione efflux by methionine from isolated rat hepatocytes. *Am J Physiol* 251:G354–G361
- Babu E, Kanai Y, Chairoungdua A, Kim DK, Iribe Y, Tangtrongsup S, Jutabha P, Li Y, Ahmed N, Sakamoto S, Anzai N, Nagamori S, Endou H (2003) Identification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters. *J Biol Chem* 278:43838–43845
- Beaudin AE, Stover PJ (2009) Insights into metabolic mechanisms underlying folate-responsive neural tube defects: a minireview. *Birth Defects Res A Clin Mol Teratol* 85:274–284
- Bodoy S, Martin L, Zorzano A, Palacin M, Estevez R, Bertran J (2005) Identification of LAT4, a novel amino acid transporter with system L activity. *J Biol Chem* 280:12002–12011
- Chamberlin ME, Ubagai T, Mudd SH, Thomas J, Pao VY, Nguyen TK, Levy HL, Greene C, Freehauf C, Chou JY (2000) Methionine adenosyltransferase I/III deficiency: novel mutations and clinical variations. *Am J Hum Genet* 66:347–355
- Christie GR, Hyde R, Hundal HS (2001) Regulation of amino acid transporters by amino acid availability. *Curr Opin Clin Nutr Metab Care* 4:425–431
- del Amo EM, Urtti A, Yliperttula M (2008) Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2. *Eur J Pharm Sci* 35:161–174
- Fariss MW, Brown MK, Schmitz JA, Reed DJ (1985) Mechanism of chemical-induced toxicity I. Use of a rapid centrifugation technique for the separation of viable and nonviable hepatocytes. *Toxicol Appl Pharmacol* 79:283–295
- Finkelstein JD, Martin JJ (1986) Methionine metabolism in mammals. Adaptation to methionine excess. *J Biol Chem* 261:1582–1587
- Finkelstein JD, Kyle WE, Harris BJ, Martin JJ (1982) Methionine metabolism in mammals: concentration of metabolites in rat tissues. *J Nutr* 112:1011–1018
- Forslund AH, Hambræus L, van BH, Holmback U, El-Khoury AE, Hjorth G, Olsson R, Stridsberg M, Wide L, Akerfeldt T, Regan M, Young VR (2000) Inverse relationship between protein intake and plasma free amino acids in healthy men at physical exercise. *Am J Physiol Endocrinol Metab* 278:E857–E867
- Fukuhara D, Kanai Y, Chairoungdua A, Babu E, Bessho F, Kawano T, Akimoto Y, Endou H, Yan K (2007) Protein characterization of NA<sup>+</sup>-independent system L amino acid transporter 3 in mice: a potential role in supply of branched-chain amino acids under nutrient starvation. *Am J Pathol* 170:888–898
- Gupta S, Kuhnisch J, Mustafa A, Lhotak S, Schlachterman A, Sliker MJ, Klein-Szanto A, High KA, Austin RC, Kruger WD (2009) Mouse models of cystathionine beta-synthase deficiency reveal significant threshold effects of hyperhomocysteinemia. *FASEB J* 23:883–893
- Guttormsen AB, Solheim E, Refsum H (2004) Variation in plasma cystathionine and its relation to changes in plasma concentrations of homocysteine and methionine in healthy subjects during a 24-h observation period. *Am J Clin Nutr* 79:76–79
- Hatanaka T, Huang W, Ling R, Prasad PD, Sugawara M, Leibach FH, Ganapathy V (2001) Evidence for the transport of neutral as well as cationic amino acids by ATA3, a novel and liver-specific subtype of amino acid transport system A. *Biochim Biophys Acta* 1510:10–17
- Jacobs RL, Stead LM, Brosnan ME, Brosnan JT (2001) Hyperglucagonemia in rats results in decreased plasma homocysteine and increased flux through the transsulfuration pathway in liver. *J Biol Chem* 276:43740–43747
- Katayama S, Tatenos C, Asahara T, Yoshizato K (2001) Size-dependent in vivo growth potential of adult rat hepatocytes. *Am J Pathol* 158:97–105
- Kilberg MS, Handlogten ME, Christensen HN (1981) Characteristics of system ASC for transport of neutral amino acids in the isolated rat hepatocyte. *J Biol Chem* 256:3304–3312
- Korendyaseva TK, Kuvatov DN, Volkov VA, Martinov MV, Vitvitsky VM, Banerjee R, Ataulakhanov FI (2008) An allosteric mechanism for switching between parallel tracks in mammalian sulfur metabolism. *PLoS Comput Biol* 4:e1000076
- Krebs HA, Cornell N, Lund P, Hems R (1974) Isolated liver cells as experimental material. In: Lundquist F, Tygstrup N (eds) Regulation of hepatic metabolism. Academic Press Inc, New York, pp 726–750
- Lu SC, Alvarez L, Huang Z-Z, Chen L, An W, Corrales FJ, Avila MA, Kanel G, Mato JM (2001) Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proc Natl Acad Sci USA* 98:5560–5565
- Martignoni E, Tassorelli C, Nappi G, Zangaglia R, Pacchetti C, Blandini F (2007) Homocysteine and Parkinson's disease: a dangerous liaison? *J Neurol Sci* 257:31–37
- Martinov MV, Vitvitsky VM, Mosharov EV, Banerjee R, Ataulakhanov FI (2000) A substrate switch: a new mode of regulation in the methionine metabolic pathway. *J Theor Biol* 204:521–532
- Martinov MV, Vitvitsky VM, Banerjee R, Ataulakhanov FI (2010) The logic of the hepatic methionine metabolic cycle. *Biochim Biophys Acta* 1804:89–96
- Mato JM, Martinez-Chantar ML, Lu SC (2008) Methionine metabolism and liver disease. *Annu Rev Nutr* 28:273–293
- Mudd SH, Ebert MH, Scriver CR (1980) Labile methyl group balances in the human: the role of sarcosine. *Metabolism* 29:707–720
- Mudd SH, Cerone R, Schiaffino MC, Fantasia AR, Minniti G, Caruso U, Lorini R, Watkins D, Matiaszuk N, Rosenblatt DS, Schwahn B, Rozen R, LeGros L, Kotb M, Capdevila A, Luka Z, Finkelstein JD, Tangerman A, Stabler SP, Allen RH, Wagner C (2001) Glycine N-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninaemia. *J Inher Metab Dis* 24:448–464
- Nijhout HF, Reed MC, Anderson DF, Mattingly JC, James SJ, Ulrich CM (2006) Long-range allosteric interactions between the folate and methionine cycles stabilize DNA methylation reaction rate. *Epigenetics* 1:81–87

- Palacin M, Estevez R, Bertran J, Zorzano A (1998) Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* 78:969–1054
- Prudova A, Martinov MV, Vitvitsky VM, Ataullakhanov FI, Banerjee R (2005) Analysis of pathological defects in methionine metabolism using a simple mathematical model. *Biochim Biophys Acta* 1741:331–338
- Rao AM, Drake MR, Stipanuk MH (1990) Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes. *J Nutr* 120:837–845
- Reed MC, Nijhout HF, Sparks R, Ulrich CM (2004) A mathematical model of the methionine cycle. *J Theor Biol* 226:33–43
- Rossier G, Meier C, Bauch C, Summa V, Sordat B, Verrey F, Kuhn LC (1999) LAT2, a new basolateral 4F2hc/CD98-associated amino acid transporter of kidney and intestine. *J Biol Chem* 274:34948–34954
- Schreiber G, Schreiber M (1972) Protein synthesis in single cell suspensions from rat liver. I. General properties of the system and permeability of the cells for leucine and methionine. *J Biol Chem* 247:6340–6346
- Shafiqat S, Tamarappoo BK, Kilberg MS, Puranam RS, McNamara JO, Guadano-Ferraz A, Freneau RT Jr (1993) Cloning and expression of a novel Na(+)-dependent neutral amino acid transporter structurally related to mammalian Na+/glutamate cotransporters. *J Biol Chem* 268:15351–15355
- Shinohara Y, Hasegawa H, Ogawa K, Tagoku K, Hashimoto T (2006) Distinct effects of folate and choline deficiency on plasma kinetics of methionine and homocysteine in rats. *Metabolism* 55:899–906
- Sugawara M, Nakanishi T, Fei Y-J, Huang W, Ganapathy ME, Leibach FH, Ganapathy V (2000a) Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. *J Biol Chem* 275:16473–16477
- Sugawara M, Nakanishi T, Fei YJ, Martindale RG, Ganapathy ME, Leibach FH, Ganapathy V (2000b) Structure and function of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. *Biochim Biophys Acta* 1509:7–13
- Tanaka K, Yamamoto A, Fujita T (2005) Functional expression and adaptive regulation of Na+-dependent neutral amino acid transporter SNAT2/ATA2 in normal human astrocytes under amino acid starved condition. *Neurosci Lett* 378:70–75
- Tchantchou F, Shea TB (2008) Folate deprivation, the methionine cycle, and Alzheimer's disease. *Vitam Horm* 79:83–97
- Utsunomiya-Tate N, Endou H, Kanai Y (1996) Cloning and functional characterization of a system ASC-like Na+-dependent neutral amino acid transporter. *J Biol Chem* 271:14883–14890
- Wagner CA, Lang F, Broer S (2001) Function and structure of heterodimeric amino acid transporters. *Am J Physiol Cell Physiol* 281:C1077–C1093
- Weissbach L, Handlogten ME, Christensen HN, Kilberg MS (1982) Evidence for two Na+-independent neutral amino acid transport systems in primary cultures of rat hepatocytes Time-dependent changes in activity. *J Biol Chem* 257:12006–12011
- Yamada Y, Ichihara S, Nishida T (2008) Molecular genetics of myocardial infarction. *Genomic Med* 2:7–22