

COMPARISON OF SULFUR AMINO ACID UTILIZATION FOR GSH SYNTHESIS BETWEEN HepG2 CELLS AND CULTURED RAT HEPATOCYTES

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Abstract—HepG2 cells are widely used as a model of human hepatocytes for studies of drug metabolism and toxicity. However, GSH metabolism in HepG2 cells is poorly characterized. This report describes the utilization of sulfur amino acids for GSH synthesis in HepG2 cells. In contrast to primary cultures of rat hepatocytes, which rely mostly on methionine for GSH synthesis, HepG2 cells use cystine. Their inability to utilize methionine for GSH synthesis was not due to lack of methionine uptake or low cellular ATP levels, but rather to the lack of *S*-adenosyl-methionine synthetase activity. When HepG2 cells were cultured overnight in medium containing cystine as the only sulfur amino acid, addition of glutamate or acivicin had minimal to no effect on cell GSH; however, addition of threonine significantly depleted cell GSH. When cystine (0.18 mM) uptake was measured, glutamate (2.5 mM), which inhibited cystine uptake in cultured rat hepatocytes, had a minimal effect in HepG2 cells. Instead, threonine (20 mM) strongly inhibited the apparent uptake of cystine by HepG2 cells. Strong inhibition by threonine of apparent cystine uptake was actually due to inhibition of cysteine uptake, which resulted from GSH-cysteine mixed disulfide exchange. Radio-HPLC confirmed this. After incubating cells with [³⁵S]cystine (0.18 mM) for 10 min, the total counts inside the cell matched the counts in the uptake medium in the form of GSH-cysteine mixed disulfide. Finally, HepG2 cells took up cysteine by both Na⁺-dependent and -independent mechanisms. The former exhibited high affinity and low capacity, whereas the latter exhibited the opposite. At a physiologic concentration of cysteine (10 μM), 68% of cysteine uptake occurred via the Na⁺-dependent system and 32% via system L₁.

Key words: *S*-adenosyl-L-methionine synthetase; cysteine transport; GSH-cysteine mixed disulfide exchange

HepG2 cells, a human hepatoblastoma derived cell line, display morphology and function similar to that of liver parenchymal cells, and they are able to proliferate in culture and produce major serum proteins [1, 2]. HepG2 cells also retain drug-metabolizing capabilities, including the cytochrome P450-dependent mixed-function oxidases and glucuronic acid and sulfate-conjugating activities [3, 4]. Due to these characteristics, HepG2 cells are widely used as an *in vitro* model of human hepatocytes for studies of carcinogen activation and drug metabolism [5, 6].

Glutathione (GSH)[†] is a vital cellular defense against toxins and free radicals, and an understanding of its metabolism is essential when studying carcinogen activation and drug metabolism. Most

studies of GSH metabolism have been done in the rat where the liver is central to interorgan homeostasis of GSH and is the major source of plasma GSH [7]. Recently, we characterized the transport of GSH by HepG2 cells and found it to be very similar to that in rat hepatocytes [8].

Another important aspect of GSH metabolism is its synthesis. The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine, and glycine, involves two ATP-requiring enzymatic steps: (1) formation of γ -glutamyl-L-cysteine from L-glutamate and L-cysteine and (2) formation of GSH from γ -glutamyl-L-cysteine and glycine. The first step, catalyzed by γ -glutamylcysteine synthetase, is rate-limiting. Under normal physiologic conditions, the availability of its precursor, L-cysteine, governs the rate of GSH synthesis [9, 10]. In normal rat hepatocytes, major sources of cellular cysteine are uptake of cysteine, uptake and subsequent transsulfuration of methionine, or, in cultured rat hepatocytes, uptake of cystine [11-13]. In contrast, very little is known about the uptake of sulfur amino acids and their utilization for GSH synthesis in HepG2 cells. Since these cells are widely used in studies of drug metabolism and toxicity, it is important to characterize the uptake and utilization of sulfur amino acid precursors of GSH.

MATERIALS AND METHODS

Materials. GSH, bovine serum albumin, NADPH,

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[†] Abbreviations: GSH, reduced glutathione; FBS, fetal bovine serum; PPG, propargylglycine; DTT, dithiothreitol; DEM, diethyl maleate; DME, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium; MeAIB, α -(methylamino)isobutyric acid; BCH, 2-amino-2-norbornane-carboxylic acid; TMA, tetramethylammonium; BSO, DL-buthionine-*S*-*R*-sulfoximine; SAF, sulfur amino acid-free; GGT, γ -glutamyl transpeptidase; SAM, *S*-adenosyl-L-methionine; MET, methionine; CYS, cysteine; and THR, threonine.

5,5'-dithiobis (2-nitrobenzoic acid), sodium EDTA, GSSG reductase, fetal bovine serum (FBS), L-(S-5S)- α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), propargylglycine (PPG), dithiothreitol (DTT), diethyl maleate (DEM), α -(methylamino)-isobutyric acid (MeAIB), threonine, tetramethylammonium (TMA) chloride, 2-amino-2-norbornane-carboxylic acid (BCH), DL-homocysteine, serine, digitonin and HEPES were purchased from the Sigma Chemical Co. (St Louis, MO). DL-Buthionine-S-R-sulfoximine (BSO) was obtained from Schweizerhall (South Plainfield, NJ). Dulbecco's Modified Eagle's medium (DME) was purchased from GIBCO (Grand Island, NY), DME/F12 medium and custom-made sulfur amino acid-free DME/F12 medium (SAF) were purchased from Irvine Scientific (Irvine, CA). L-[³⁵S]Methionine (1120 Ci/mmol), L-[³⁵S]cystine (250 mCi/mmol) and L-[³⁵S]cysteine (> 600 Ci/mmol) were obtained from Amersham (Arlington Heights, IL); L-[methyl-³H]methionine (214 mCi/mmol) was obtained from Du Pont (Wilmington, DE). All other reagents were of analytical grade and were obtained from commercial sources.

Animals. Male Sprague-Dawley rats (Harlan Laboratory Animals, Inc., San Diego, CA), weighing 260–320 g, were maintained on Purina rodent chow (Ralston Purina Co., St Louis, MO) and water *ad lib*.

Cell culture—HepG2 cells. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). HepG2 cells were routinely cultured in DME containing 10% FBS, penicillin (100 μ g/mL) and streptomycin (0.1 mg/mL) in a humidified atmosphere of 5% CO₂, 95% air and were subcultured every 5–7 days at a cell density of 2×10^6 cells/75 cm² flask. Medium was changed every 3–4 days. Cells were discarded after subculturing for 5 months, and the original stock that had been frozen was thawed to continue the culture. To measure sulfur amino acid transport and cell GSH levels, HepG2 cells were detached by trypsin-EDTA (0.05%, 0.02%, respectively), and 2×10^6 cells in 5 mL medium were seeded on 60×15 mm plastic dishes, at 37° in 5% CO₂-95% air atmosphere and studied 24 hr later. The viability of these cells was typically >95% as determined by trypan blue exclusion. To study the effects of different sulfur amino acids on GSH levels, SAF medium supplemented with either methionine, cystine, homocysteine or vehicle was used as plating medium. These media were compared to a DME/F12 control. In addition, 10% FBS was included with all media.

Preparation of collagen and collagen-coated culture plates. Rat tail collagen was prepared according to the method described by Michalopoulos and Pitot [14] with slight modifications. Collagen from rat tails was allowed to crystallize in $10 \times 70\%$ ethanol at room temperature for 3–4 hr. Crystallized collagen was then suspended in 0.1% acetic acid (300 mL/g collagen) and stirred for 48 hr at 4°. The solution was then centrifuged at 500 g for 15–20 min, and the supernatant was sterilized by irradiation (10,000 rad). When coating plates, 0.5 or 1 mL of collagen solution was spread over the surface of a 35 mm or 60 mm

plate, respectively. Coated plates were dried at 25° and then washed with sterile phosphate-buffered saline prior to use.

Preparation of primary cultures of rat hepatocytes. Hepatocytes were isolated aseptically according to the method of Moldeus *et al.* [15]. Initial cell viability was $\geq 90\%$ as determined by 0.2% trypan blue exclusion. The plating medium was DME/F12 containing high glucose (3151 mg/L), methionine (1 mM) and 10% FBS. For experiments designed to examine the effect of glutamate on cystine utilization, SAF medium supplemented with cystine (0.18 mM) was used instead of DME/F12 as plating medium. Cells (2×10^6) in 5 mL plating medium were plated on 60×15 mm dishes precoated with rat tail collagen incubated at 37° in 5% CO₂, 95% air. Two to three hours after plating, medium was changed to remove FBS and dead, unattached cells. Cell attachment averaged $\sim 60\%$.

Measurement of cell GSH, ATP levels, and cell size. Cultured rat hepatocytes and HepG2 cells were detached by trypsin-EDTA (0.05%, 0.02%, respectively) and treated with 10% trichloroacetic acid in order to extract cellular GSH. The mixture was centrifuged at 13,000 g in a microfuge (Beckman Instruments) for 1 min to remove the denatured proteins, and GSH was measured in the supernatant by the recycling method of Tietze [16].

To measure ATP concentrations from cultured rat hepatocytes and HepG2 cells, approximately $1-2 \times 10^6$ cells were collected by detaching cells with trypsin-EDTA, and cellular ATP levels were determined by HPLC according to the method of Jones [17].

Cell counting was done by the Coulter counter and hemocytometer, whereas cell diameter was estimated by the Channelyzer (Coulter counter).

Uptake of methionine, cystine and cysteine by HepG2 cells. HepG2 cells were seeded at $1-2 \times 10^6$ cells in 3–5 mL DME supplemented with 10% FBS on $35 \times 10-60 \times 15$ mm plastic dishes. Uptake studies were done 24 hr after plating. The technique used for measuring sulfur amino acid uptake was adapted from Takada and Bannai [18]. The medium used for washing and stopping uptake was 10 mM phosphate-buffered saline, pH 7.4, with 0.01% CaCl₂, 0.01% MgCl₂·6H₂O, and 0.1% glucose. Uptake medium was the same medium supplemented with non-radioactive methionine (1 mM) and cystine (0.18 mM) in order to simulate concentrations present in the culture medium, or cysteine in various concentrations. In the case of cysteine, uptake medium also contained DTT (2–5 mM) to prevent auto-oxidation of cysteine. HepG2 cells were washed once with 2 mL of prewarmed buffer, and transport was initiated by adding 1 mL of uptake medium containing [³⁵S]methionine (0.5 to 1.0 μ Ci), [³⁵S]cystine (0.5 μ Ci), or [³⁵S]cysteine (0.5 to 1.0 μ Ci). After incubation at 37° for the required time, uptake was terminated by washing five times with 2 mL of ice-cold wash buffer. The number of washes was determined by counting dpm in the supernatant after each wash. After the last wash, no radioactivity was recovered in the supernatant. Cells were then dissolved with 0.5 mL of 0.5 N NaOH; an aliquot was used for determining

the radioactivity and another for protein assay by Bio-Rad. To correct for trapping, cell-associated radioactivity counts at 4° were measured in parallel. Duplicate plates were used for each condition. The difference between counts at 37° and 4° represented true uptake. Uptake was expressed as nanomoles per mg protein per minute.

In the case of cystine uptake, insulin (1 µg/mL) and hydrocortisone (50 nM) were added to the culture medium since cystine uptake in cultured rat hepatocytes is induced by these hormones [18, 19]. Before initiating uptake studies, cells were pretreated with acivicin (0.5 mM for 1 hr) which is an irreversible inhibitor of γ -glutamyl transpeptidase (GGT), and DEM (0.5 mM for 20 min) which depleted the cell GSH by 40% by forming thioether conjugate with GSH. Pretreatment with acivicin was necessary because HepG2 cells express very high GGT activity [20, 21] which can break down GSH released from the cells. Breakdown of GSH will then liberate cysteine, which can undergo thiol-disulfide exchange with [³⁵S]cystine in the uptake medium, and generate [³⁵S]cysteine, which can be taken up. We have shown previously that acivicin (0.5 mM) completely blocked the GGT activity of HepG2 after 1 hr of pretreatment [8], according to the method described [22]. Pretreatment with DEM to lower cell GSH was intended to minimize GSH released from the cells, which can undergo extracellular GSH-cystine mixed disulfide exchange and generate [³⁵S]cysteine that can be taken up. In some experiments, in order to quantitate and verify the molecular forms of the radioisotope in the uptake medium and inside the cell, cells were detached with trypsin-EDTA after washing and, along with the uptake medium at the end of the incubation period, were derivatized for radio-HPLC according to the method of Fariss and Reed [23].

To determine the Na⁺-independent component of cysteine and cystine uptake, Na⁺ was replaced by choline or TMA. These two replacements yielded similar results and were pooled for data analysis. The Na⁺-dependent component was the difference in uptake with and without Na⁺.

In experiments assessing the effects of various amino acids on cysteine uptake, a 60-min depletion period in Na⁺-free (TMA substituted) buffer prior to the uptake assays was included to avoid possible *trans*-effects.

Assay of S-adenosyl-L-methionine (SAM) synthetase activity. SAM-synthetase activity was assayed according to previously published methods [24] with slight modifications. The routine assay medium contained in a final volume of 1 mL was 100 mM Tris-HCl, 200 mM KCl, 10 mM MgCl₂, 1 mM DTT, 5 mM ATP, 5 mM L-methionine, and 1.0 µCi of L-[methyl-³H]methionine at pH 7.8. Cell-free protein extracts from cultured rat hepatocytes and HepG2 cells were obtained as previously described [19]. The reaction was initiated by adding digitonin (50 µM)-treated cell-free protein extract (0.25 to 0.5 mL containing 0.5 to 0.8 mg protein as determined by the Bio-Rad assay) to the above medium and incubating for 30 min at 37°. The reaction was stopped with 50 mL of cold, distilled water. The diluted incubation mixture was immediately applied

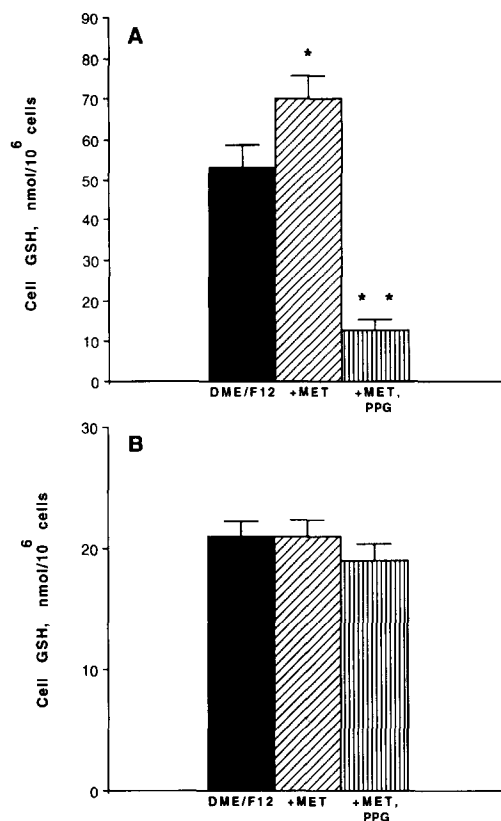


Fig. 1. Role of methionine in GSH synthesis in primary cultures of rat hepatocytes (A) and HepG2 cells (B). Cell GSH levels in rat hepatocytes (panel A) and HepG2 cells (panel B) cultured overnight in DME/F12 medium alone, supplemented with 1 mM methionine (MET), or 1 mM methionine plus 2 mM PPG are shown. Results are means \pm SEM from 7 cell preparations (rat) and 4 experiments (HepG2 cells) done in parallel. Key: (*) $P < 0.05$ vs DME/F12, and (**) $P < 0.05$ vs DME/F12 and DME/F12 plus methionine by ANOVA.

to a 2 mL Dowex 50 W column. After complete passage of the solution, the column was washed with 20 mL of water, and the [³H]SAM formed was then eluted with two 5-mL aliquots of 3 N NH₄OH. Each eluate was added to 10 mL of ScintiVerse E for scintillation counting. The reaction was reported to be linear with time for at least 30 min [24]. An assay blank was obtained by performing the incubation in the absence of any cell-free protein extract. Activity of SAM synthetase was expressed as nmol/mg/30 min after subtracting the blank. All determinations were performed in duplicate.

Statistical analysis. For each experiment, means from duplicate plates were used, and the means of multiple experiments were compared by paired Student's *t*-tests in the case of two comparisons or one-way ANOVAs followed by Fisher's tests for multiple comparisons. Two-tailed tests were used unless otherwise noted. Any *P* less than 0.05 was considered significant.

Kinetic data were fitted with the Michaelis-

Menten equation using the SAAM program [25] to determine V_{\max} and K_m values. The standard errors of the mean values at each concentration of the substrate were submitted as statistical weights (proportional to the inverse of standard errors).

RESULTS

Comparison of the role of methionine in GSH synthesis in cultured rat hepatocytes and HepG2 cells.

For primary cultures of rat hepatocytes, the most important sulfur amino acid GSH precursor is methionine. This is because cysteine readily auto-oxidizes to cystine in culture medium and, although culture conditions induce cystine uptake, its uptake rate is minimal as compared with methionine [11–13]. Figure 1 shows the differences between primary cultures of rat hepatocytes and HepG2 cells in their utilization of methionine for GSH synthesis. In cultured rat hepatocytes, supplementing DME/F12 medium (containing ~0.12 mM methionine) with 1 mM methionine significantly increased cell GSH in rat hepatocytes, whereas blocking methionine utilization with PPG, an irreversible inhibitor of the terminal enzyme of the transsulfuration pathway [26], significantly lowered cell GSH. These same maneuvers had no effect on GSH of HepG2 cells.

Note that HepG2 cell GSH content was much lower than that of cultured rat hepatocytes. However, the actual cellular GSH concentration was probably comparable, since HepG2 cells were smaller than cultured rat hepatocytes. The median cell diameters as estimated by Channelyzer (Coulter counter) for HepG2 and cultured rat hepatocytes were 13 ± 0.2 and $17\text{--}18 \mu\text{m}$, respectively. Thus, the cell volume of HepG2 cells was ~40–45% of cultured rat hepatocytes.

To define the role of methionine and cystine in supporting GSH synthesis in HepG2 cells, GSH of HepG2 cells incubated overnight in SAF medium, or SAF medium supplemented with either methionine (0.12 mM) or cystine (0.18 mM) was compared with that of cells incubated in DME/F12. Figure 2 shows that cell GSH was depleted to near 0 in both SAF and SAF + methionine media, but was the same as control in SAF + cystine medium. Cell viability was still >90% despite cell GSH being near 0. Thus, in contrast to cultured rat hepatocytes, which relied mostly on methionine for GSH synthesis, cystine but not methionine supported GSH synthesis in HepG2 cells.

Potential mechanisms of failure to utilize methionine for GSH synthesis in HepG2 cells. Three potential mechanisms explain the failure to utilize methionine for GSH synthesis in HepG2 cells. The first is lack of methionine transport. The second is lack of the transsulfuration pathway. The third is low cellular ATP levels—important since in rats the K_m of SAM synthetase for ATP is 2 mM whereas the K_m for ATP of the two GSH synthesis enzymes is approximately 0.1 mM [27]. Thus, fluctuations in cellular ATP levels can critically affect the activity of transsulfuration.

We first measured methionine (1 mM) uptake by HepG2 cells and found it to be linear up to 1 min and averaged 23.7 ± 4.4 nmol/mg/min ($N = 3$

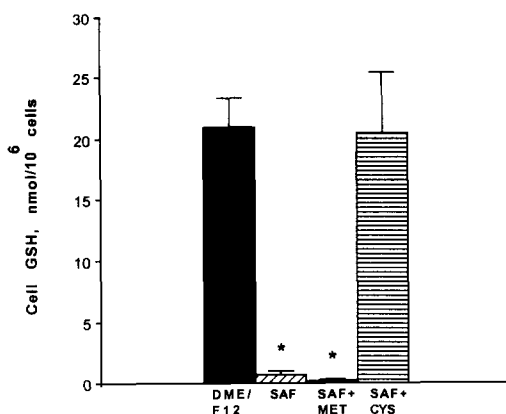


Fig. 2. Utilization of methionine and cystine for GSH synthesis by HepG2 cells. HepG2 cells were cultured overnight in DME/F12, SAF, SAF + methionine (0.12 mM), or SAF + cystine (CYS) (0.18 mM). Results are means \pm SEM from 6 experiments. Key: (*) $P < 0.05$ vs DME/F12 control by ANOVA.

experiments). This differs from the 4 nmol/mg/min that we reported for cultured rat hepatocytes under identical conditions [19]. For comparison, total amounts of protein per 10^6 cells are ~1.5 and ~0.5 mg for cultured rat hepatocytes and HepG2 cells, respectively. The ATP content of HepG2 cells was 10.8 ± 1.9 nmol/ 10^6 cells, and that of cultured rat hepatocytes measured in parallel was 23.3 ± 3.2 nmol/ 10^6 cells ($N = 3$ for both cell preparations for rat hepatocytes and HepG2 cells). Given the differences in cell size, the cellular ATP concentrations were quite comparable.

To assess whether HepG2 cells lack the transsulfuration pathway and where the defect may lie, we compared cell GSH level after overnight incubation of HepG2 cells in SAF medium containing either cystine (0.18 mM) alone, serine plus DTT (1 mM each), methionine, serine plus DTT (1 mM each), homocysteine, serine plus DTT (1 mM each) or vehicle. As Table 1 shows, homocysteine but not methionine was able to maintain cell GSH. Thus, in HepG2 cells, the inability to utilize methionine for GSH synthesis was due to lack of the transsulfuration pathway, with the defect occurring prior to the formation of homocysteine.

To see if the defect lies at the level of SAM synthetase, we compared the activity of SAM synthetase in HepG2 cells with that in cultured rat hepatocytes. The activities of SAM synthetase in cell-free protein extract from cultured rat hepatocytes were 1.61 and 1.95 nmol/mg/30 min, but were undetectable in HepG2 cells (results are expressed as mean of two determinations from two experiments). Equivalent amounts of protein (0.5 to 0.8 mg) were used per assay for cultured rat hepatocytes and HepG2 cells. In terms of number of cells, the amount of protein was equivalent to ~4–6 $\times 10^5$ cultured rat hepatocytes and 2–3 $\times 10^6$ HepG2 cells. Even given the differences in cell volume, more than the equivalent amount of HepG2 cells was used per

Table 1. Effects of sulfur amino acids in maintaining GSH in HepG2 cells

Condition	Cell GSH (nmol/10 ⁶ cells)
SAF control	1.0 ± 0.3
+ Cystine	22.4 ± 1.5*
+ Serine and DTT	1.0 ± 0.3
+ Methionine, serine and DTT	0.8 ± 0.2
+ Homocysteine, serine and DTT	15.2 ± 2.0†

Results are expressed as means ± SEM from 4 experiments. Cell GSH was measured after HepG2 cells were cultured overnight in SAF medium containing either cystine (0.18 mM), serine (1 mM) plus DTT (1 mM), methionine (1 mM), serine (1 mM) plus DTT (1 mM), homocysteine (1 mM), serine (1 mM) plus DTT (1 mM), or vehicle (SAF control).

*† Significant differences: *P < 0.05 vs SAF control, and †P < 0.05 vs SAF control as well as serine plus DTT by ANOVA followed by Fisher's test.

assay. The level of SAM synthetase activity in cultured rat hepatocytes was similar to that reported from normal human liver cytosol (~15 nmol/mg/30 min) [24]. It should be noted that the protein extracts contained cytosol and other solubilized cell constituents. Thus, the specific enzyme activities of SAM synthetase in these extracts are lower than that of cytosol due to the presence of a greater fraction of total cellular protein in the digitonin extracts. For comparison, as we previously reported [19], 35–40 mg of cytosolic protein was obtained from 1 g of rat liver (~10⁸ cells), whereas 1.3 to 1.5 mg of digitonin-treated cell-free protein extract was obtained from 10⁶ cells. Thus, the equivalent amount of SAM synthetase activity per mg cytosolic protein would be ~8 nmol/mg/30 min. In fact, this was confirmed by measuring SAM synthetase activity from rat liver cytosol (mean of two determinations from two samples = 7.6 and 7.1 nmol/mg/30 min). When cell-free protein extract from cultured rat hepatocytes was mixed with that from HepG2 cells in a 1:1 ratio, SAM synthetase activity then measured 2.40 and 2.13 nmol/mg protein of cultured rat hepatocyte cell-free extract/30 min (mean of two determinations from two experiments). Thus, the lack of SAM synthetase activity in HepG2 cells was not due to the presence of an inhibitory substance.

Ways in which cystine can support GSH synthesis in HepG2 cells. Cystine can support GSH synthesis by one or more of the following mechanisms: (1) the very high GGT activity of HepG2 cells [20, 21] can break down any GSH released, liberating enough cysteine for uptake and resynthesis into GSH, (2) the γ -glutamyl moiety from GSH breakdown can be transferred to cystine (the best acceptor) forming γ -glutamylcystine which can be taken up, then reduced intracellularly to γ -glutamylcysteine and synthesized into GSH, bypassing the rate-limiting step, (3) cystine uptake, and (4) GSH-cystine mixed disulfide exchange liberating cysteine for uptake.

Contributions from breakdown of GSH by GGT. Since HepG2 cells express high GGT activity [20, 21],

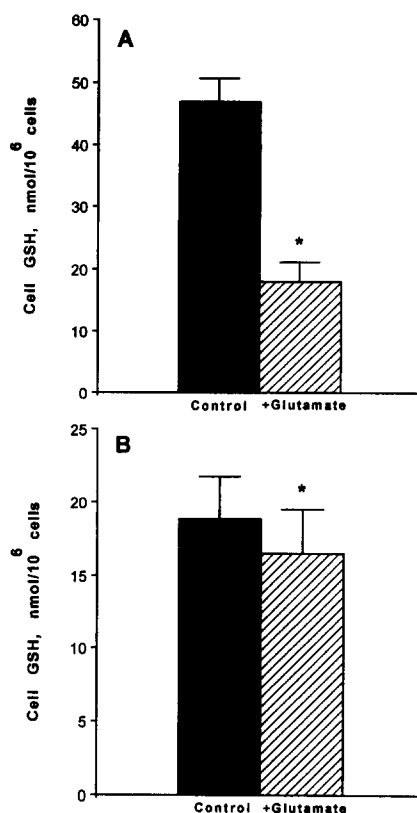


Fig. 3. Effect of glutamate on cell GSH in cultured rat hepatocytes (A) and HepG2 cells (B). Cell GSH levels in rat hepatocytes (panel A) and HepG2 cells (panel B) cultured overnight in SAF + cystine (0.18 mM) medium supplemented with insulin (1 μ g/mL) and hydrocortisone (50 nM) alone (control) versus with the addition of 2.5 mM glutamate are shown. Results are means ± SEM from 4 cell preparations (rat) and 10 experiments (HepG2 cells). Key: (*) P < 0.01 vs SAF + cystine by the paired *t*-test.

one way to maintain cell GSH is via utilization of cysteine that results from the breakdown of released GSH. To exclude GSH breakdown providing enough cysteine to maintain GSH level as the sole explanation, we examined the effect of overnight incubation with acivicin on cell GSH. Acivicin (0.5 mM) had no effect on cell GSH [cell GSH, in nmol/10⁶ cells, of control in SAF + cystine (0.18 mM) medium = 15.1 ± 0.8, and that of SAF + cystine + acivicin = 16.4 ± 0.8; results are expressed as means ± SEM from 5 experiments]. Furthermore, if this mechanism was operational, GSH values should not be near 0 in SAF and SAF + methionine media.

Since γ -glutamylcystine bypasses the rate-limiting step in GSH synthesis, we examined the effect of BSO (10 mM), an irreversible inhibitor of γ -glutamylcystine synthetase [9, 10], on cell GSH. Cell GSH was near 0 after the addition of BSO to culture medium [control cell GSH (nmol/10⁶ cells) = 15.7 ± 0.6, plus BSO overnight = 0.9 ± 0.4, results are expressed as means ± SEM from 4 experiments, P < 0.001 between controls and BSO-treated cells

by paired *t*-test]. Cell viability was still >95% despite such low cell GSH values. Although one cannot exclude a role for γ -glutamylcystine uptake, our data argue against this mechanism being a major source of GSH precursor.

Cystine uptake. Under normal physiologic conditions, cystine uptake is believed to be negligible in both freshly isolated rat hepatocytes and the perfused rat liver but is induced under culture conditions [11, 12, 18, 19]. In cultured rat hepatocytes, cystine is mainly taken up by the X_c^- system and is induced after a 12-hr lag, especially under low density conditions and in the presence of insulin and corticosteroids [18]. We first compared GSH levels of HepG2 cells with those of cultured rat hepatocytes after an overnight incubation in SAF + cystine (0.18 mM) medium with or without the addition of glutamate (2.5 mM), which is known to block cystine uptake by the X_c^- system [18, 19]. Figure 3 shows striking differences in how the two responded to the addition of glutamate. As we previously reported [19], addition of glutamate led to a profound depletion of cell GSH in rat hepatocytes cultured in SAF + cystine medium. However, addition of glutamate had very little effect on GSH of HepG2 cells under identical conditions.

Next we measured uptake of cystine by HepG2 cells. Cystine (0.18 mM) uptake, as estimated by accumulation of temperature-dependent cell-associated radioactivity counts, occurred linearly up to 10 min; thus, all subsequent experiments were done with a 10-min incubation. The effects of various agents were assessed by adding them to the uptake buffer. Any difference in the osmolarity of added agents was compensated for by adding an equivalent molar amount of sucrose to the controls. Table 2 shows the effects of various agents on cystine uptake under Na^+ -containing and Na^+ -free conditions. In contrast to cultured rat hepatocytes, where glutamate inhibited cystine uptake by 85% under identical conditions [19], glutamate had a negligible effect on cystine uptake in HepG2 cells. This finding is consistent with our finding of a negligible effect of glutamate on cell GSH (Fig. 3). Addition of MeAIB (20 mM), an inhibitor of the A system, or lysine (10 mM), a known inhibitor of cystine uptake in renal tubular cells [28], had no significant effect on cystine uptake in HepG2 cells. Removal of Na^+ lowered apparent cystine uptake by 34%. The major inhibitor of cystine uptake examined was threonine (20 mM), which inhibited apparent cystine uptake by ~70% under both Na^+ -containing and Na^+ -free conditions. Since threonine is a specific inhibitor of the ASC system, which reportedly mediates most of cysteine transport by rat hepatocytes [11, 12, 29, 30], we suspected that the apparent cystine uptake was actually cysteine uptake, which formed as a result of extracellular GSH-cystine mixed disulfide exchange in which cystine in the uptake medium reacted with GSH effluxed from the cells. This generated GSH-cysteine mixed disulfide and cysteine in equimolar ratios. In fact, when we examined the molecular forms of the radioisotope in the uptake medium and the cell by radio-HPLC, all of the cell-associated radioactivity could be accounted for by GSH-cysteine mixed disulfide in the uptake medium

Table 2. Characterization of cystine uptake by HepG2 cells

Condition	Cystine uptake (% of control)	N
Na^+ -containing		
+ Glutamate (2.5 mM)	81 \pm 5*	4
+ MeAIB (20 mM)	81 \pm 5	3
+ Threonine (20 mM)	27 \pm 8*	3
+ MeAIB and threonine	37 \pm 4*	5
+ MeAIB, threonine, and glutamate	25 \pm 1*	4
+ Lysine (10 mM)	78 \pm 2	3
Na^+ -free		
Cystine alone	66 \pm 3*	3
+ MeAIB and threonine	30 \pm 9†	3

Results are expressed as means \pm SEM per cent of control (Na^+ -containing) from N, the number of experiments. HepG2 cells were pretreated with acivicin (0.5 mM, 1 hr) and DEM (0.5 mM, 20 min) in order to minimize the breakdown of released GSH and the amount of GSH-cystine mixed disulfide formation, respectively. Cystine (0.18 mM) uptake was determined by measuring the cell associated counts after 10 min of incubation. For the Na^+ -free condition, choline replaced Na^+ . Na^+ -containing control cystine uptake = 0.21 \pm 0.02 nmol/mg/min (mean \pm SEM from 7 experiments). Uptake values refer to net uptake, where 4° blanks have been subtracted.

* \dagger Significant differences: * P < 0.05 vs Na^+ -containing control, and † P < 0.05 vs both Na^+ -containing and Na^+ -free controls by ANOVA followed by Fisher's test.

(in two separate experiments, cell-associated cpm = 1934 and 2282, whereas cpm in the uptake medium in the form of GSH-cysteine mixed disulfide = 2177 and 2347, respectively). Thus, there was very little, if any, cystine uptake by HepG2 cells.

Cysteine uptake by HepG2 cells. To confirm that it was cysteine uptake that was inhibited by threonine, we characterized cysteine uptake in HepG2 cells. Cysteine (0.18 mM) uptake was rapid and linear only to 45 sec, thus all studies were done with a 30-sec incubation. Figure 4 shows the effects of MeAIB and threonine on cysteine (0.18 mM) uptake by HepG2 cells. Similar to what has been reported for rat hepatocytes [29–31], most of cysteine uptake was inhibited by threonine in HepG2 cells. However, cysteine uptake by the threonine-inhibitable ASC system has been reported to be Na^+ dependent [11, 12, 29–31]. This, coupled with our observation that threonine still inhibited apparent cystine uptake under Na^+ -free conditions, led us to a more detailed characterization of cysteine uptake by HepG2 cells.

We examined the kinetics of cysteine uptake under Na^+ -containing and Na^+ -free conditions in HepG2 cells. Figure 5 shows total cell-associated radioactivity counts under Na^+ -containing and Na^+ -free conditions at 37° and 4°. The difference between 37° and 4° was taken as true uptake for all subsequent experiments. Figure 6 shows the kinetics of cysteine uptake under Na^+ -containing and Na^+ -free conditions. The difference between cysteine uptake under Na^+ -containing and Na^+ -free conditions represents the Na^+ -dependent component of cysteine uptake. The

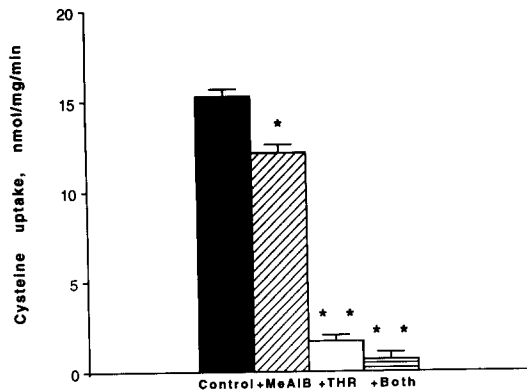


Fig. 4. Effects of MeAIB and threonine (THR) alone or together on cysteine uptake by HepG2 cells. Results are expressed as means \pm SEM from 3 experiments. Cysteine (0.18 mM) uptake of controls versus that of adding MeAIB (20 mM), THR (20 mM), or both to the uptake buffer is shown. To compensate for differences in osmolarity, an equivalent amount of sucrose was added to the controls. The addition of extra sucrose did not change the uptake value in the controls. DTT (2 mM) was present in the uptake buffer to prevent oxidation of cysteine during the uptake experiment. Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs control by ANOVA.

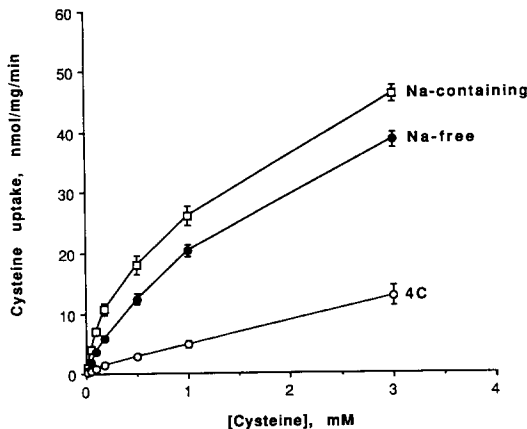


Fig. 5. Cysteine uptake by HepG2 cells. HepG2 cells were incubated with [35 S]cysteine and unlabeled cysteine (0.01 to 3 mM) for 30 sec. Cell-associated radioactivities at 37° under Na⁺-containing, Na⁺-free, and 4° conditions are shown. The 4° values were essentially the same for both Na⁺-containing and Na⁺-free conditions and were pooled. Uptake buffer also contained 5 mM DTT to prevent oxidation of cysteine. Results are expressed as means \pm SEM from 5 experiments. The difference between 37° and 4° represents true uptake.

lines represent computer fits of the Michaelis-Menten equation to the data using the SAAM program [25]. The inset represents Eadie-Hofstee plots of the same data. Note that there appear to be at least two components for total cysteine uptake on the Eadie-Hofstee plot, but only a single component

for both the Na⁺-dependent and Na⁺-independent systems. These were confirmed with the SAAM program. The kinetic parameters (mean \pm SD) as estimated by the SAAM program were: $K_m = 0.11 \pm 0.01$ mM and $V_{max} = 7.06 \pm 0.16$ nmol/mg/min for the Na⁺-dependent uptake component; and $K_m = 1.36 \pm 0.13$ mM and $V_{max} = 38.7 \pm 2.38$ nmol/mg/min for the Na⁺-independent uptake component.

Table 3 shows the effects of various natural and artificial amino acids on Na⁺-dependent and Na⁺-independent cysteine uptake in HepG2 cells. At the physiologic cysteine concentration of 10 μ M, its Na⁺-dependent uptake was strongly inhibited by L-threonine and L-alanine, which are substrates for the ASC system, moderately by L-methionine and L-leucine, and minimally by L-phenylalanine. BCH, L-glutamate, MeAIB, and L-histidine did not affect the Na⁺-dependent cysteine uptake significantly. In contrast, the Na⁺-independent cysteine uptake was strongly inhibited by L-methionine and L-histidine, which are substrates for the L₁ system, as well as L-threonine, L-alanine and L-leucine. BCH and L-phenylalanine also inhibited Na⁺-independent cysteine uptake moderately, whereas glutamate and MeAIB had no effect. These results suggest that the ASC and the L₁ systems mediate the Na⁺-dependent and the Na⁺-independent cysteine uptake in HepG2 cells, respectively.

DISCUSSION

The liver has one of the highest concentrations of GSH and plays the central role in the interorgan homeostasis of GSH. Hepatic GSH detoxifies and eliminates xenobiotics and serves as a reservoir of cysteine [9, 10, 13]. The synthesis and transport of hepatic GSH have been studied intensively. However, these studies have been performed mostly in the rat and very little information is known about human hepatocytes. In the rat, synthesis of hepatic GSH is critically dependent on the availability of its precursor, L-cysteine [9, 10]. Factors important in regulating the availability of cysteine in the rat hepatocyte include cysteine transport, cystine transport which is negligible normally but is induced under culture conditions, and methionine transport with its subsequent conversion to cysteine via the transsulfuration pathway [11–13, 18]. Thus, membrane transport of cysteine, cystine, and methionine along with the activity of the enzymes of the transsulfuration and GSH synthesis pathways are important factors determining the rate of hepatic GSH synthesis.

Membrane transport of cysteine, cystine and methionine have been well characterized in both freshly isolated and cultured rat hepatocytes [11, 12, 32]. Cysteine is transported mostly by the Na⁺-dependent ASC system [11–13, 29, 30, 32]; in cultured rat hepatocytes, cystine is transported by the Na⁺-independent X_c⁻ system [11, 12, 18, 19]; and methionine is transported by the Na⁺-independent L system [11, 12, 32]. In contrast, few studies have characterized the uptake of these sulfur amino acids in liver cells of human origin [33–36]. With regards to the HepG2 cell line specifically, one recent study found that systems A, ASC and L were all present

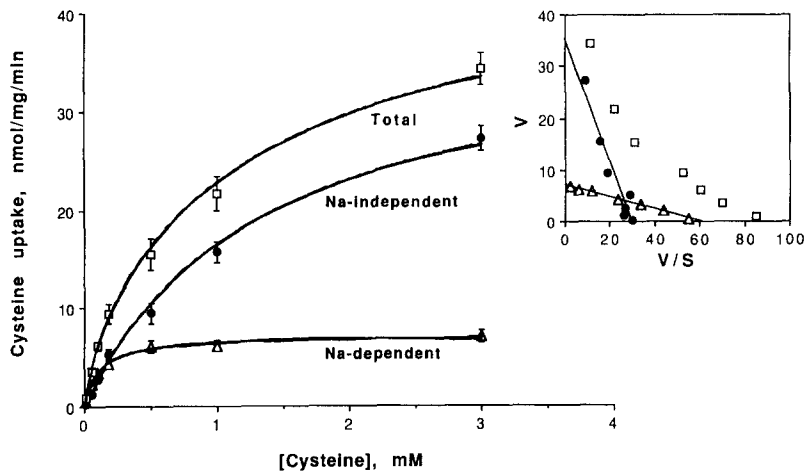


Fig. 6. Kinetics of cysteine uptake in HepG2 cells. Cysteine uptake (net) was determined under Na^+ -containing or Na^+ -free conditions (see Materials and Methods for details). The difference between the two represents Na^+ -dependent cysteine uptake. Results are expressed as means \pm SEM from 5 experiments. The lines represent computer fits of the Michaelis-Menten equation to the data using the SAAM program. K_m and V_{\max} (mean \pm SD) were determined by the program. The K_m values were 1.36 ± 0.13 and 0.11 ± 0.01 mM, and the V_{\max} values were 38.7 ± 2.38 and 7.06 ± 0.16 nmol/mg/min for the Na^+ -independent and Na^+ -dependent cysteine uptake systems, respectively. The inset shows the Eadie-Hofstee plots of mean total cysteine uptake (\square), Na^+ -independent uptake (\bullet), and Na^+ -dependent uptake (\triangle) from the same experiments. Data for Na^+ -independent and -dependent uptake were fitted by linear regression analysis ($R^2 = 0.96$ and 0.995 , respectively). The velocity (V) is given as nmol/mg/min, and the substrate concentration (S) is millimolar. The kinetic parameters as estimated by linear regression of the Eadie-Hofstee plots ($K_m = 1.19$ and 0.12 mM; $V_{\max} = 35.2$ and 7.09 nmol/mg/min for the Na^+ -independent and Na^+ -dependent components, respectively) were very similar to those estimated by the SAAM program.

Table 3. Specificity of L-cysteine uptake in HepG2 cells

Inhibitor (2.5 mM)	L-Cysteine uptake (% of control)	
	Na^+ -dependent	Na^+ -independent
L-Threonine	$8 \pm 3^*$	$25 \pm 5^*$
L-Alanine	$8 \pm 3^*$	$30 \pm 7^*$
L-Phenylalanine	$69 \pm 6^*$	$46 \pm 7^*$
L-Leucine	$41 \pm 2^*$	$24 \pm 3^*$
L-Methionine	$19 \pm 2^*$	$13 \pm 2^*$
BCH	93 ± 3	$43 \pm 2^*$
L-Histidine	75 ± 6	$20 \pm 7^*$
MeIAB	81 ± 3	92 ± 1
L-Glutamate	73 ± 9	105 ± 6

Results are expressed as means \pm SEM per cent of control from 4–5 experiments for each condition. To avoid possible *trans*-effects, a 60-min depletion period in Na^+ -free buffer prior to the uptake assays was included. Cysteine uptake was measured in HepG2 cells after incubation with [^{35}S]cysteine and unlabeled cysteine ($10 \mu\text{M}$) \pm various inhibitors (2.5 mM) for 30 sec (see Materials and Methods for details) in both Na^+ -containing and Na^+ -free buffers. Uptake buffer also contained 5 mM DTT to prevent oxidation of cysteine. For the Na^+ -free condition, TMA replaced Na^+ . Control total cysteine uptake (in the presence of Na^+) = 0.86 ± 0.04 , Na^+ -independent uptake = 0.28 ± 0.03 , and Na^+ -dependent uptake = 0.58 ± 0.04 nmol/mg/min. Uptake values refer to net uptake, where 4° blanks have been subtracted.

* $P < 0.05$ vs its respective control by ANOVA followed by Fisher's test.

[34]. However, no study has characterized the uptake of GSH precursor sulfur amino acids and their utilization for GSH synthesis in human liver cells.

In the transsulfuration pathway, methionine is sequentially converted to cysteine via several enzymatic steps [26]. The first step requires ATP and involves the conversion of methionine to SAM. In contrast to the two GSH synthesizing enzymes that have a low K_m for ATP (~ 0.1 mM), the K_m of SAM synthetase for ATP is high (~ 2 mM). Therefore, hypoxic depletion of ATP is more likely to affect GSH synthesis from methionine than cysteine [27]. Subsequent demethylation of SAM and the removal of the adenosyl moiety yields homocysteine. Homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine synthase. Cleavage of cystathionine, catalyzed by cystathionase, then releases free cysteine.

Primary cultures of rat hepatocytes are known to rely mostly on methionine present in culture medium to support GSH synthesis because cysteine present in the medium readily auto-oxidizes to cystine [13], and although cystine uptake is induced under culture, its rate of uptake is only 20% that of methionine [11]. Our results are confirmatory in stressing the importance of methionine and the transsulfuration pathway in maintaining GSH levels of rat hepatocytes in culture. In contrast, methionine cannot maintain the GSH of HepG2 cells. This observation was also made by Duthie *et al.* [37] when they noted that HepG2 cells could not synthesize GSH when

incubated with methionine alone for 2 hr, but could with cysteine. They concluded that HepG2 cells lack the transsulfuration pathway. However, they did not examine methionine uptake, cellular ATP levels, or define where the defect may lie. Our study excluded both lack of methionine uptake and low cellular ATP levels as explanations. Furthermore, it showed that homocysteine was able to maintain cell GSH, which means the defect occurs prior to the utilization of homocysteine.

Low activity of the transsulfuration pathway has been reported in the rat fetal liver [38], in cirrhotic human liver [24, 39], and during prolonged culture of rat hepatocytes [40]. In the first and the last case, the defect occurs after the formation of homocysteine. Specifically, the fetal liver has a low activity of cystathionase [38], and during prolonged culture, rat hepatocytes have low activities of both cystathionine synthase and cystathionase [13, 40]. In the case of the cirrhotic human liver, the defect lies in loss of the high-molecular-weight form of SAM synthetase [24, 39]. Reduced activity of SAM synthetase has also been reported in rat Novikof ascites hepatoma cells [41]. Thus, abnormal activity of SAM synthetase may be associated with hepatic dysfunction and malignant degeneration. In fact, when we measured the activity of SAM synthetase in HepG2 cells, it was undetectable but was restored to the same level as cultured rat hepatocytes when cell-free protein extracts from the two types of cells were mixed, excluding the presence of an inhibitory substance against SAM synthetase in HepG2 cells. Thus, the inability of HepG2 cells to utilize methionine for GSH synthesis is due to absence of SAM synthetase activity. More work will be necessary to define precisely the molecular mechanism of the defect.

Of the different ways cystine can support GSH synthesis, the uptake of cystine via the glutamate-inhibitable X_c^- system is the most important for rat hepatocytes cultured in the presence of cystine as the sole sulfur amino acid. This was evident by a 60% fall in cell GSH when its uptake was blocked by glutamate. However, glutamate had a minimal effect on the GSH of HepG2 cells under identical conditions. This was apparently due to lack of cystine transport in HepG2 cells. In fact, our study points to the importance of the role of GSH-cystine mixed disulfide exchange when examining cystine uptake. Most studies that measured cystine uptake were actually measuring cell-associated radioactivity counts after incubation with cystine, as was our study. It only became apparent that the cell-associated radioactivity counts after incubation with cystine were due to cysteine uptake, liberated as a result of GSH-cystine mixed disulfide exchange, when we noted threonine but not glutamate was potentially inhibitory. Radio-HPLC also confirmed this as the total counts inside the cell matched those in the uptake medium in the form of GSH-cysteine mixed disulfide. Thus, in HepG2 cells the most important mechanism by which cystine maintained cell GSH was by forming mixed disulfides with GSH released from the cells, thereby liberating cysteine for uptake. This is a well known phenomenon in freshly isolated rat hepatocytes [42].

Previous work on cysteine transport in rat

hepatocytes suggests that cysteine is predominantly transported by system ASC [29, 30], with perhaps a small contribution from system A [31]. The K_m for Na^+ -dependent cysteine transport (presumably system ASC) was found to be ~ 2 mM, and V_{max} was 800 nmol/g of intracellular water/min [30]. Even though Kilberg *et al.* [30] observed a significant Na^+ -independent component in cysteine uptake, they dismissed it since choline was used as a substitute for Na^+ , which they felt might have partially fulfilled the role ordinarily played by Na^+ . More recently, Mailliard and Kilberg [35] studied cysteine transport by human liver plasma membrane vesicles and found that virtually all of cysteine uptake (50 μ M) occurred via Na^+ -dependent routes—25% by system A, 35% by system ASC, and another 40% by a yet to be characterized system(s). In our studies, we used choline as well as TMA as Na^+ replacements, and there was clearly a Na^+ -independent cysteine transport system. In HepG2 cells, the Na^+ -dependent cysteine transport system exhibits high affinity (K_m of 0.1 mM, which is much lower than reported for the rat hepatocytes) and low capacity (V_{max} of 7 nmol/mg/min), whereas the Na^+ -independent cysteine transport system exhibits low affinity (K_m of 1.36 mM) and high capacity (V_{max} of 39 nmol/mg/min). Thus, HepG2 cells have much higher affinity for cysteine uptake than cultured rat hepatocytes. Interestingly, Saiki *et al.* [43] suggested that a high-affinity low-capacity cysteine transport system exists in zone III of rat liver.

In HepG2 cells, at a low cysteine concentration (10 μ M) the Na^+ -dependent cysteine transport system resembles the ASC system. At a higher cysteine concentration (0.18 mM), system A may contribute to a small degree as well, since MeAIB significantly inhibited cysteine uptake (Fig. 4). Similar to what Kilberg and colleagues described [30], the Na^+ -dependent cysteine uptake was strongly inhibited by threonine and alanine and to a mild-moderate degree by phenylalanine and leucine. We found that methionine also inhibited Na^+ -dependent cysteine uptake. This finding is consistent with previous reports that methionine uptake can also occur via systems ASC and A [29, 30]. The Na^+ -independent cysteine transport system in HepG2 cells is most likely the L_1 system. Of note, cultured human fibroblasts also transport cysteine via the L system [44], and recently Simmons and colleagues [45] described Na^+ -independent cysteine uptake by rat liver canalicular membrane vesicles which also appears to be system L. Our assignment of Na^+ -independent cysteine transport in HepG2 cells to system L_1 is based on a previous report that both the L_1 and L_2 systems are known to be inhibited by BCH, leucine and phenylalanine equally, whereas histidine and methionine inhibit L_1 more than L_2 , and threonine inhibits only L_1 [46]. Interestingly, system L_2 is predominant in freshly isolated adult rat hepatocytes and declines over the initial 24–48 hr in cultures, whereas system L_1 increases during culture [46] and is predominant in fetal hepatocytes. Thus, its presence in HepG2 cells may be a feature of being in culture and/or transformed cells.

In summary, we have characterized the uptake and utilization of methionine, cystine and cysteine

for GSH synthesis in HepG2 cells. They differed from cultured rat hepatocytes in lack of SAM synthetase activity and cystine transport, and in the transport of cysteine. HepG2 cells are being increasingly used as a human liver cell model. Therefore, our findings must be considered when using these cells to perform studies involving GSH metabolism.

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