Amino Acids (2000) 19: 705–728

Amino
Acids

Cysteine dioxygenase and γ-glutamylcysteine synthetase activities in primary cultured hepatocytes respond to sulfur amino acid supplementation in a reciprocal manner*

J. Ohta[1,2], **Y. H. Kwon**[2], and **M. H. Stipanuk**[2]

[1] Department of Biochemistry, Okayama University Medical School, Okayama, Japan
[2] Division of Nutritional Sciences, Cornell University, Ithaca, New York, U.S.A.

Accepted September 16, 1999

Summary. Hepatocytes were cultured for 3 days as spheroids (aggregates) or as monolayers in basal medium and in sulfur amino acid-supplemented media. Cultured hepatocytes had low levels of cysteine dioxygenase (CDO) activity and normal levels of γ-glutamylcysteine synthetase (GCS) and cysteine-sulfinate decarboxylase (CSDC) activities compared to freshly isolated cells. CDO activity increased and GCS activity decreased in a dose-response manner in cells cultured in either methionine- or cysteine-supplemented media. CSDC activity was not significantly affected by methionine supplementation. Changes in CDO and GCS were associated with changes in cysteine catabolism to taurine plus sulfate and in synthesis of glutathione, respectively. These responses are similar to those observed in liver of intact rats fed diets supplemented with sulfur amino acids. A near-maximal response of CDO or GCS activity was observed when the medium contained 1.0 mmol/L of methionine plus cyst(e)ine. Changes in CDO and GCS activities did not appear to be mediated by changes in the intracellular glutathione concentration. Cultured hepatocytes offer a useful model for further studies of cysteine metabolism and its regulation in response to sulfur amino acid availability.

Keywords: Amino acids – Cysteine – Cysteine dioxygenase – Glutathione – γ-Glutamylcysteine synthetase – Hepatocytes – Taurine

Introduction

Cysteine is metabolized in mammalian liver mainly to taurine, sulfate, glutathione and protein as summarized in Fig. 1. We have studied the effects

* A preliminary report of a portion of this work was presented at the International Taurine Symposium '95, June 27–July 1, 1995, Osaka, Japan.

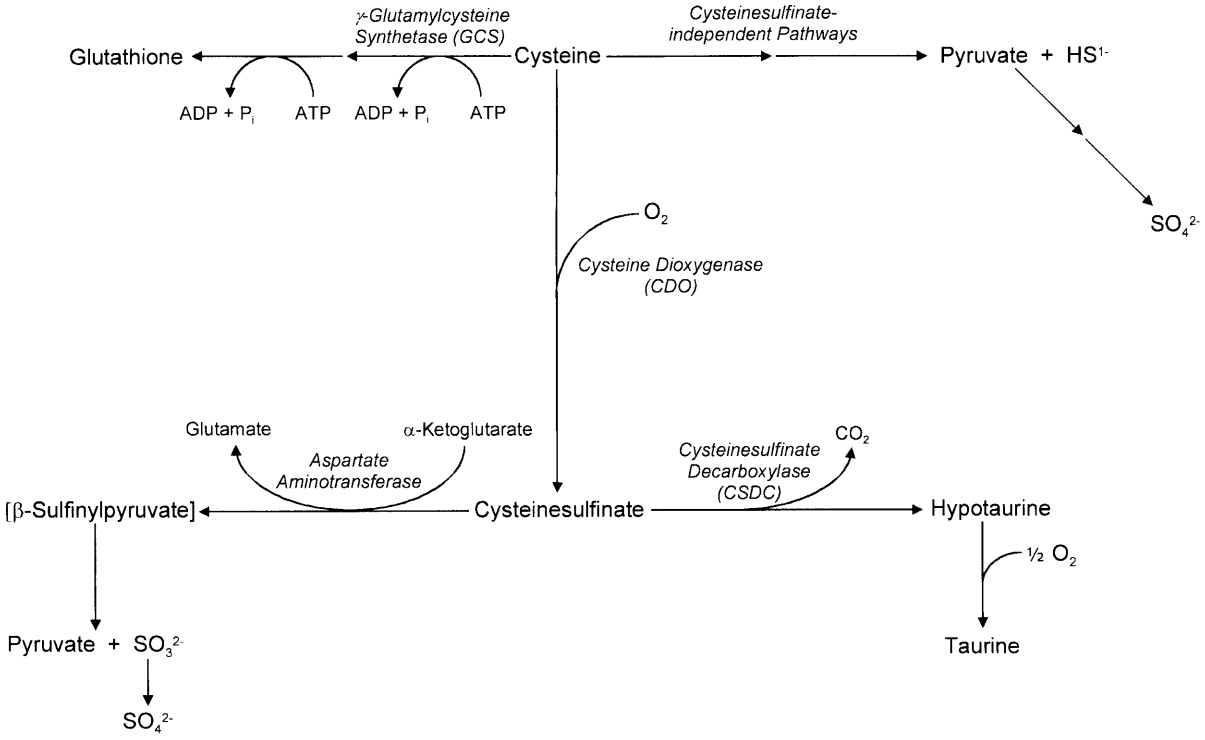

Fig. 1. Major metabolic pathways using cysteine as substrate

of dietary protein and sulfur amino acid level on cysteine metabolism using freshly isolated hepatocytes obtained from rats fed various diets (Bagley and Stipanuk, 1994, 1995; Bella and Stipanuk, 1995, 1996; Bella et al., 1996; Coloso et al., 1990). These studies have shown that cysteine catabolism and glutathione synthesis in liver are regulated reciprocally in response to dietary levels of protein, cystine or methionine. In addition, these studies have shown that the activities of cysteine dioxygenase (CDO) and γ-glutamylcysteine synthetase (γ-GCS) are regulated reciprocally in response to dietary protein or sulfur amino acid level. On the other hand, cysteinesulfinate decarboxylase (CSDC), which is responsible for taurine formation, responds more strongly to changes in dietary protein than to changes in dietary sulfur amino acid content. The exact mechanism and the direct effectors of the regulation of cysteine metabolism are of interest because taurine, sulfate and glutathione are essential for important physiological processes including taurine conjugation of bile acids, synthesis of sulfated proteoglycans, detoxification of xenobiotics via conjugation with glutathione or sulfate, and removal of peroxides.

Primary cultures of rat hepatocytes would provide some advantages over freshly isolated cells for studies of the regulation of cysteine metabolism. Isolated cells are viable for only a few hours after isolation compared to several days for cultured cells. Furthermore, treatment of cultured cells, as compared with treatment of the intact animal followed by cell isolation, allows

assessment of treatment effects specifically at the cellular level. Hence, the purpose of these studies was to develop a hepatocyte culture system for studies of cysteine metabolism and to determine if sulfur amino acid supplementation of cultured hepatocytes had similar effects on hepatic metabolism as did feeding diets supplemented with sulfur amino acids.

Hepatocyte-derived cell lines have lost much of their capacity for sulfur amino acid oxidation. For example, in the HepG2 cell line, both cysteine dioxygenase (McCann et al., 1994) and the high-Km, liver-specific, isozyme of *S*-adenosylmethionine synthetase are not expressed (Lu and Huang, 1994; Cai et al., 1996). Furthermore, activities of essential enzymes of sulfur amino acid metabolism have not been detected in several studies of hepatoma cell lines; cysteine dioxygenase activity was not found in AH 2440 and AH109A cells, and cystathionine synthase activity was not found in HTC cells (Sakakibara et al., 1976; Goss, 1986). Monolayer primary cultures of hepatocytes are also known to lose many of the differentiated functions of liver cells and specifically to undergo changes in activities of cysteine-metabolizing enzymes and of cyst(e)ine transport (Meredith, 1987; Takada and Bannai, 1984). Hutson et al. (1987) reported that insulin, glucocorticoid, and supplemental amino acids were necessary for maintenance of a maximal rate for secretion of albumin (the major secretory protein synthesized by liver) as well as maintenance of hepatocyte monolayer cultures beyond 2 to 3 days. Recent work with hepatocytes cultured as spheroids (multicellular aggregates with well-defined cellular morphology) have suggested that aggregate cultures maintain certain highly differentiated functions of liver parenchymal cells such as secretion of albumin and transferrin and high glucokinase activity (Koide et al., 1990; Landry et al., 1985; Tong et al., 1992; Yuasa et al., 1993). On the other hand, addition of corticosteroids (dexamethasone) to culture systems has been reported to not favor spheroid formation (Yuasa et al., 1993), and corticosteroids are not normally added to medium when hepatocytes are cultured as spheroids. Because corticosteroids may play a role in maintenance of liver-specific amino acid metabolism, it is not clear whether spheroid cultures would be a good model for the study of cysteine metabolism.

Therefore, we compared certain aspects of cysteine metabolism in freshly isolated hepatocytes, hepatocytes cultured as conventional monolayers on collagen-coated plates, and hepatocytes cultured as spheroids on poly(2-hydroxyethyl methacrylate)-coated plates. Based on these initial studies, the monolayer system was further developed for studies of cysteine metabolism, and the time course and dose-response for changes in CDO and GCS activities in response to supplemental sulfur amino acids were determined.

Materials and methods

Materials

Collagenase was purchased from Boehringer-Mannheim (Indianapolis, IN). Williams' medium E (WE medium), murine natural epidermal growth factor (EGF), bovine insulin,

and an antibiotic-antimycotic mixture that contained the sodium salt of penicillin G, streptomycin sulfate and amphotericin B were purchased from Gibco BRL (Grand Island, NY). Sulfur amino acid-free Williams' medium E (SAF-WE medium) was prepared without cysteine, cystine, methionine, and glutathione by Gibco BRL. Calf skin collagen type I, poly(2-hydroxyethyl methacrylate) (poly-HEMA), L-methionine, L-cysteine, bathocuproine disulfonate (BCS), Na_2SeO_3, and dexamethasone were obtained from Sigma (St. Louis, MO). Tissue culture dishes and plates [35 × 10 mm (Primaria) or 60 × 15 mm dishes and Multiwell 6-well plates and 24-well plates] were purchased from Becton Dickinson (Franklin Lakes, NJ).

L-[^{35}S]Cysteine was purchased from Amersham (Product # SJ141, Arlington Heights, IL) as the hydrochloride. L-[^{35}S]Cysteine was purified prior to use by applying it to a 0.6 × 4 cm Dowex 1-X8 column (200–400 mesh, acetate form) and eluting it with approximately 20 mL of 0.01 M hydrochloric acid. Aliquots of the eluate (50 or 100 μCi/tube) were lyophilized and stored at −70°C until used.

Preparation of culture dishes

Culture dishes or wells were coated with collagen or poly-HEMA before use. To coat vessels with collagen, sterile calf skin collagen I solution, 0.5 mg/mL in 50 mmol/L acetic acid, was spread over the surface of vessels (20 μl per cm^2 of surface area), and the solvent was evaporated by placing the plates under UV-light for 16 h. To coat vessels with poly-HEMA, 2.5% (w/v) poly-HEMA solution in 95% (w/v) ethanol was applied to tissue culture plates or wells (0.2 mL per cm^2); the solvent was evaporated, and the coated surfaces were then sterilized by placing the plates under UV-light for 16 h.

Culture medium

For experiments 1 and 2, cells were cultured in WE medium supplemented with 10 μg/mL insulin, 50 ng/mL EGF, 3 nM Na_2SeO_3, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, and either no additional sulfur amino acids or 2 mmol/L or 10 mmol/L L-methionine (Enat et al., 1984). In experiment 3 with monolayer cultures only, the insulin concentration was reduced to 1 μg/mL in the basal medium, and the basal medium was supplemented with amino acids to increase amino acid concentrations to those used by Hutson et al. (1987), with 50 nmol/L dexamethasone, or with 2 mmol/L L-methionine as indicated. For experiments 4 and 5, the basal WE medium was modified to contain 1 μg/mL insulin and 50 nmol/L dexamethasone, and all cells were initially plated in this basal medium. At 4 h, the medium was changed and replaced with fresh basal medium, or with medium containing the indicated concentrations of L-methionine or L-cysteine plus BCS (0.05 mmol/L). BCS was included with cysteine to minimize oxidation of cysteine to cystine (Coloso et al., 1990). The osmolarity of the unsupplemented and supplemented media was adjusted to 350 mOsmol/L by adjusting the concentration of sodium chloride.

Animals and hepatocytes

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed in stainless steel wire mesh cages in a room maintained at 20°C and 60–70% humidity with light from 2000 to 0800. Rats were given ad libitum access to water and a nonpurified diet (Prolab RMH 1000, Agway, Syracuse, NY). Rats weighed approximately 250 g when they were used for isolation of hepatocytes. The care and use of animals was approved by the Cornell University Institutional Animal Care and Use Committee.

Hepatocytes were isolated aseptically by collagenase perfusion as described by Berry et al. (1991). Initial cell viability was more than 85% as determined by 0.2% (w/v) Trypan blue exclusion. The freshly isolated hepatocytes were resuspended in WE medium to give a concentration of 1×10^7 to 1.5×10^7 cells per mL. The hepatocyte suspension in WE

medium was diluted with the designated culture medium to yield a final cell concentration of 5×10^5 to 7.5×10^5 cells per mL and used for cell culture.

For culture of hepatocytes as spheroids, 2 mL of the diluted cell suspension was plated on a 35 mm diameter dish precoated with poly-HEMA. For monolayer culture, the diluted cell suspension was plated on collagen-coated vessels (0.2 mL per cm^2). The plated cells were cultured at 37°C in 5% CO_2/95% air. In experiments 1 and 2, half of the treatment culture medium was changed 4 h after plating and then every 24 h. In experiment 3, the treatment culture medium was completely changed at 4 h after plating, 20 h later (i.e., 24 h), and again at 48 h. In experiments 4 and 5, the basal culture medium was replaced with treatment culture medium at 4 h and then every 24 h (i.e., 28 h, 52 h). Thus, cells were allowed to attach in basal medium over a 4-h period and were then cultured in the designated treatment medium for the indicated times.

Measurement of enzyme activities

To measure enzyme activities, cells were harvested and treated as follows. Freshly isolated hepatocytes (1.2×10^7 cells) suspended in SAF-WE medium were collected by centrifugation at 1,000 × g for 30-s and washed twice with 1 mL of phosphate-buffered saline by resuspension and centrifugation. Monolayer cultures of hepatocytes were each washed twice with 1 mL of phosphate-buffered saline per well or dish, and cells were collected by scraping. For hepatocytes cultured on the poly-HEMA dishes, spheroids (aggregates of hepatocytes) that were floating in the culture medium were collected by transferring the culture medium and spheroids to centrifuge tubes and by centrifugation at 100 × g for 30 s; the pelleted cells were washed twice with 1 mL of phosphate-buffered saline by resuspension and centrifugation. Harvested cells were resuspended in 50 nmol/L Mes [2-(N-morphilino)ethanesulfonic acid], pH 6.0, to give a final cell concentration of approximately 6.5×10^6 cells per mL, and the suspension was sonicated for three 15-s periods using a High Intensity Ultrasonic Processor (Sonics and Materials Inc., Dansbury, CT) to disrupt the cells.

CDO activity was measured according to the method of Bagley et al. (1995) except that, in this study, the volume of the reaction mixture was reduced to 0.2 mL and the amount of [^{35}S]cysteine was increased to 10–15 μCi per 0.2 mL of reaction mixture. The activity was calculated as the rate of production of [^{35}S]cysteinesulfinate from [^{35}S]cysteine. CSDC activity was measured as described by Daniels and Stipanuk (1982) as modified by Bella and Stipanuk (1995) except that the volume of the reaction mixture was reduced to 0.2 mL. The rate of production of hypotaurine plus taurine from cysteinesulfinate was measured. GCS activity was assayed by measuring the production of γ-glutamylcysteine plus glutathione in incubation mixtures that contained 1.0 mmol/L cysteine, 20 mmol/L glutamate and 10 mmol/L glycine. Assay was done as described by Bella et al. (1999).

Measurement of protein, taurine and glutathione content of hepatocytes

Protein in the homogenates was measured by the method of Smith et al. (1985) using bicinchoninic acid; bovine serum albumin was used as the standard. Enzyme activities were expressed as nmol product formed·min^{-1}·mg $protein^{-1}$. The taurine (taurine plus hypotaurine) and glutathione concentrations in a sample of the harvested hepatocytes were determined using the same HPLC procedures that were used to measure products of the CSDC and GCS activity assays, respectively (Bagley et al., 1995; Fariss and Reed, 1987; Stipanuk et al., 1992).

[^{35}S]Cysteine metabolism

Studies of cysteine metabolism were carried out over 6 h in SAF-WE medium with 0.2 mmol/L L-[^{35}S]cysteine added as substrate. Three days after plating, hepatocytes that had been cultured on either collagen- or poly-HEMA-coated plates and in either basal WE medium or WE medium supplemented with 2 mmol/L L-methionine were similarly

used for studies of [^{35}S]cysteine metabolism. Monolayers (3.1–4.7 μg DNA/well) were each washed twice with 0.3 mL of SAF-WE medium, and then new SAF-WE medium was added to the well. Spheroids from six 35 × 10 mm dishes coated with poly-HEMA were collected by centrifugation at 100 × g for 30 s and were washed twice with 1 mL of SAF-WE medium by resuspension and centrifugation. About one-eighth of the resulting pellet (1.4–1.9 μg DNA/well) was added back with fresh SAF-WE medium into poly-HEMA-coated dishes. Immediately after preparation, freshly isolated hepatocytes (2×10^5 cells/well; 7.7–9.3 μg DNA/well) were suspended in SAF-WE medium in both collagen-coated wells and poly-HEMA-coated plates; this was done to provide the same incubation conditions for freshly isolated cells as for cultured cells.

The harvested cultured and the freshly-isolated cells, in culture dishes as indicated, were preincubated for 20 min in 0.3 mL of SAF-WE medium. After the preincubation period, 0.1 mL of 0.8 mmol/L L-[^{35}S]cysteine (0.15 Ci/mmol) plus 2.0 mmol/L BCS in SAF-WE medium was added to each incubation mixture to give a final concentration of 0.2 mmol/L L-cysteine and 0.5 mmol/L BCS. Incubations with [^{35}S]cysteine were carried out at 37°C for 6 h.

Reactions were stopped, and the acid supernatants and the protein precipitate obtained were analysed for formation of labeled products. ^{35}S-Labeled sulfate, taurine, and glutathione were measured as described previously (Bella and Stipanuk, 1995; Fariss and Reed, 1987; Stipanuk et al., 1992; Coloso and Stipanuk, 1989). To measure [^{35}S]cysteine incorporation into protein, the precipitated protein pellets from incubation mixtures stopped with perchloric acid were each washed twice with 0.5 mL of 0.5 mol/L perchloric acid, then with 0.5 mL of 0.17 M sulfosalicylic acid, and finally with 0.5 mL of water. The pellet was resuspended with 0.5 mL of 10% (w/v) hydrogen peroxide, and the resulting suspension was incubated for 15 min at room temperature to oxidize cysteine to cysteic acid, to break all disulfide bonds, and to remove cysteine not attached by peptide bonds. The pellet was washed with 1 mL of water and was then resuspended in 1 mL of water. The resuspended pellet was mixed with 1.5 mL of Solvable (NEN Research Products, Boston, MA) in a scintillation vial and placed in a shaking water bath at 50°C for 60 min. Then Ecoscint liquid scintillation cocktail (National Diagnostics, Atlanta, GA) was added, and radioactivity was measured by liquid scintillation spectrometry.

Production of ^{35}S-metabolites was expressed as nmol product formed·6 h^{-1}·μg DNA^{-1}. DNA was measured by the Hoechst 33258 method using calf thymus DNA as the standard (Labarca and Paigen, 1980). The viability of the cells was evaluated for each experiment by measuring lactate dehydrogenase leakage; leakage of total hepatocyte lactate dehydrogenase activity into the incubation medium was less than 10% during the 6-h incubation period for all culture conditions and hepatocyte preparations and was considered acceptable.

Statistical analyses

Data were analyzed with one-way or two-way ANOVA followed by comparison of means with Tukey's ω-procedure, linear contrasts, or paired t-tests (Minitab for Windows Release 10.2, Minitab Inc., State College, PA and Microsoft Excel Version 5.0, Microsoft Corp, Cambridge, MA). When variances were unequal, values were transformed ($\log_{10}$) prior to statistical analyses as indicated in the table footnotes. Differences were accepted as significant at $p \leq 0.05$.

Results

Experiment 1: Effects of cell culture, type of culture, and L-methionine supplementation on enzyme activities

Hepatocytes were cultured both as spheroids and as monolayers, and measurements in culture cells were compared to values for the freshly isolated

hepatocytes. Three days after plating, many spheroids with well-defined cellular morphology were observed on the dishes precoated with poly-HEMA. Spheroids corresponding to 5–23% of the initially plated hepatocytes were recovered. The diameter of the recovered spheroids was roughly estimated to be 100 to 300 μm for all of the preparations. On the dishes precoated with collagen, ~50% of the plated cells were recovered as a monolayer of cells at three days after the start of culture. In either spheroid or monolayer cultures, morphological differences among cells cultured with the different methionine levels were not detected by light-microscopic observation. Hepatocytes cultured as spheroids showed lower hexokinase and higher glucokinase activities than did hepatocytes cultured as monolayers (data not reported).

Activities of key enzymes of cysteine metabolism are reported in Table 1 for freshly isolated hepatocytes and for hepatocytes cultured on either collagen- or poly-HEMA-coated plates for three days. The activity of CDO and the activity of CSDC were significantly lower in cultured hepatocytes than in freshly isolated cells, whereas γ-GCS activity in cultured hepatocytes was similar to or greater than that in freshly isolated hepatocytes. The type of culture (or surface coating of the culture plates) did not have a clear effect on CDO or GCS activities, but CSDC activity was significantly higher (10.4 nmol·min^{-1}·mg protein^{-1}) in hepatocytes cultured as monolayers on collagen-coated dishes than in hepatocytes cultured as spheroids on poly-HEMA-coated dishes (6.9 nmol·min^{-1}·mg protein^{-1}).

Methionine supplementation of the culture medium clearly affected the activities of CDO and GCS but not that of CSDC (Table 1). CDO activity in hepatocytes cultured in WE medium supplemented with methionine (either 2 mmol/L or 10 mmol/L) tended to be higher than that in hepatocytes cultured in unsupplemented basal WE, whereas GCS activity in hepatocytes cultured in methionine-supplemented medium tended to be lower than that for cells cultured in basal WE medium. However, when individual means were compared, methionine supplementation significantly decreased GCS activity in spheroidal cultures but not in monolayer cultures and significantly increased CDO activity in monolayer cultures but not in spheroidal cultures. Hence, although the type of culture or coating material did not significantly affect GCS or CDO activity, the magnitude of the decrease in GCS activity in response to methionine supplementation was significantly greater in hepatocytes cultured as spheroids, whereas the magnitude of increase in CDO activity was greater in hepatocytes cultured as monolayers (linear contrasts, $p < 0.05$). CSDC activity was not affected by methionine supplementation of the culture medium in either the monolayer or spheroidal cultures. For all three enzyme activities, similar results were obtained with supplemention of the culture medium with either 10 mmol/L methionine or 2 mmol/L methionine, which suggests that 2 mmol/L of supplemental methionine was sufficient to yield a maximal effect.

The cellular taurine and glutathione contents of freshly isolated cells and of cultured hepatocytes are shown in Table 1. Freshly isolated hepatocytes had relatively low concentrations of both taurine and glutathione, which may

Table 1. Cysteine dioxygenase, cysteinesulfinate decarboxylase, and γ-glutamylcysteine synthetase activities and taurine and glutathione concentrations in freshly isolated hepatocytes and in hepatocytes cultured as monolayers or as spheroids in medium with different L-methionine concentrations

Hepatocytes	Culture medium	Cysteine dioxygenase	Cysteinesulfinate decarboxylase	γ-Glutamylcysteine synthetase	Taurine content	Glutathione content
		nmol·min^{-1}·mg protein^{-1}			nmol·mg protein^{-1}	
Freshly isolated cells	–	0.45 ± 0.14^{c}	22.2 ± 0.6^{c}	0.37 ± 0.05^{a}	2.2 ± 1.1^{a}	27.4 ± 2.0^{a}
Monolayer cultures	Basal	0.07 ± 0.01^{a}	10.4 ± 0.4^{b}	0.97 ± 0.25^{bc}	5.7 ± 0.6^{a}	77.2 ± 8.0^{cd}
	Basal + 2 mmol/L L-Met	0.17 ± 0.01^{b}	11.7 ± 0.2^{b}	0.70 ± 0.14^{ab}	61.6 ± 4.3^{b}	90.9 ± 2.7^{d}
	Basal + 10 mmol/L L-Met	0.19 ± 0.03^{b}	10.6 ± 0.5^{b}	0.80 ± 0.08^{abc}	68.4 ± 6.2^{b}	96.7 ± 8.1^{d}
Spheroidal cultures	Basal	0.12 ± 0.03^{ab}	6.9 ± 0.5^{a}	1.20 ± 0.11^{c}	Trace	54.9 ± 3.4^{b}
	Basal + 2 mmol/L L-Met	0.18 ± 0.04^{b}	7.4 ± 0.9^{a}	0.44 ± 0.07^{a}	48.1 ± 2.8^{b}	78.5 ± 7.1^{cd}
	Basal + 10 mmol/L L-Met	0.16 ± 0.02^{b}	6.5 ± 0.6^{a}	0.51 ± 0.08^{ab}	48.8 ± 4.3^{b}	68.4 ± 3.3^{bc}

Values are means ± SEM for n = 3 rats. Values within a column and not followed by the same superscript letter are significantly different ($p \leq 0.05$) by Tukey's ω-procedure following two way analysis of variance (factor 1, rat; factor 2, treatment of hepatocytes). The data for cysteine dioxygenase and γ-glutamylcysteine synthetase were transformed to $\log_{10}$ prior to this statistical analysis. For each preparation of hepatocytes (with each preparation representing cells from one rat liver), measurements were made on the freshly isolated cells at the time of isolation and measurements were made 3 days (~70 h) later on cultured cells from the same hepatocyte preparation, with culture being carried out on two types of plating surfaces and in medium with three different methionine concentrations. The basal WE medium itself contained 0.1 mmol/L methionine and 0.49 mmol/L cyst(e)ine; hence, the total methionine in the supplemented media was 2.1 mmol/L methionine for medium supplemented with 2 mmol/L L-methionine and 10.1 mmol/L methionine for medium supplemented with 10 mmol/L L-methionine.

be due to loss of these compounds during the isolation process. The taurine content of hepatocytes cultured in basal WE medium did not significantly increase, but the glutathione content of hepatocytes cultured in basal medium was significantly ($p > 0.05$) increased compared with that in freshly isolated cells.

Both taurine and glutathione content increased in response to methionine supplementation of the medium. The taurine content of cultured cells was markedly higher (11 times or more) in hepatocytes cultured in medium with supplemental L-methionine than in those cultured in basal medium. The glutathione content was 18 to 43% higher in hepatocytes cultured in methionine-supplemented medium than in those cultured in basal medium. The observation that the basal medium contained a sufficient level of sulfur amino acids to support substantial accumulation of glutathione, but not of taurine, is consistent with our observations in intact rats and in freshly isolated cells: when sulfur amino acid availability is limited, glutathione synthesis has a higher priority for cysteine utilization than does cysteine catabolism and taurine synthesis (Bella and Stipanuk, 1995, 1996; Bella et al., 1996). For both taurine and glutathione contents, there was no difference in concentrations between cells cultured with 2 mmol/L and 10 mmol/L supplemental methionine; this indicates that 2 mmol/L of supplemental methionine or a total sulfur amino acid [methionine + cyst(e)ine] concentration of 2.6 mmol/L in the culture medium was sufficient to support maximal accumulation of both glutathione and taurine.

Experiment 2. Effect of cell culture and L-methionine supplementation on cysteine metabolism by hepatocytes

Production of labeled metabolites from L-[^{35}S]cysteine (added to SAF-WE medium) was measured for freshly isolated hepatocytes (with incubations being carried out in the same culture dishes used for cultured cells) and for both monolayer and spheroidal cultures of hepatocytes derived from the same hepatocyte preparations. The accumulations of labeled metabolites over the 6-h period of incubation with [^{35}S]cysteine are reported in Table 2. The amount of [^{35}S]taurine formed by cultured hepatocytes was not significantly different from that accumulated by freshly isolated hepatocytes. [^{35}S]Taurine accumulation tended to be higher for hepatocytes that had been cultured in WE medium supplemented with 2 mmol/L L-methionine as compared to that for hepatocytes that had been cultured in basal WE medium, but means (basal vs methionine-supplemented) were significantly different only for hepatocytes cultured as spheroids.

Production of [^{35}S]sulfate by cultured hepatocytes tended to be higher than that by freshly isolated hepatocytes, although individual means for freshly isolated hepatocytes and for cells that had been cultured in basal WE medium were not significantly different (Table 2). Analysis by linear contrasts clearly demonstrated a highly significant effect of culture ($p < 0.002$), with all cultured hepatocytes displaying greater [^{35}S]sulfate production than freshly

Table 2. Comparison of cysteine metabolism in freshly isolated hepatocytes and hepatocytes cultured as monolayers or as spheroids and with different L-methionine levels

Hepatocytes	Culture medium	[^{35}S]Taurine (taurine)	[^{35}S]Sulfate	[^{35}S]Glutathione (glutathione)	[^{35}S]Cysteine incorporation into protein
		pmol·(6 h)$^{-1}$·μg DNA^{-1}			
Freshly isolated hepatocytes (in collagen dish)[1]	–	276 ± 78^{ab} (754 ± 215^{a})	261 ± 37^{ab}	411 ± 79^{a} (1,144 ± 106^{a})	121 ± 12^{a}
(in poly-HEMA dish)[2]	–	243 ± 60^{ab} (568 ± 208^{a})	223 ± 23^{a}	479 ± 67^{ab} (1,375 ± 86^{ab})	110 ± 7^{a}
Monolayer cultures					
	Basal	115 ± 11^{a} (189 ± 68^{a})	434 ± 13^{ab}	759 ± 49^{ab} (2,992 ± 133^{ab})	84 ± 11^{a}
	Basal + 2 mmol/L L-Met	343 ± 36^{ab} (691 ± 117^{a})	459 ± 30^{b}	615 ± 30^{ab} (2,056 ± 241^{ab})	85 ± 4^{a}
Spheroidal cultures					
	Basal	194 ± 94^{a} (537 ± 466^{a})	401 ± 108^{ab}	1,234 ± 326^{b} (4,414 ± 761^{b})	161 ± 66^{a}
	Basal + 2 mmol/L L-Met	634 ± 190^{b} (1,970 ± 912^{a})	464 ± 95^{b}	427 ± 70^{a} (1,555 ± 276^{a})	146 ± 24^{a}

Values are means ± SEM for conversion of exogenous radiolabeled substrate to radiolabeled products; n = 3 rats. Values in parentheses in the columns for [^{35}S]taurine and [^{35}S]GSH show accumulation of molar amounts of taurine or glutathione. Values within a column and not followed by the same superscript letter are significantly different ($p \leq 0.05$) by Tukey's ω-procedure following two-way analysis of variance (factor 1, rat; factor 2, treatment of hepatocytes). The data for taurine, glutathione, protein, and taurine plus sulfate were transformed to $\log_{10}$ prior to statistical analysis. [1]Freshly isolated hepatocytes were incubated with L-[^{35}S]cysteine in collagen-coated wells. [2]Freshly isolated hepatocytes were incubated with L-[^{35}S]cysteine in poly(2-hydroxyethyl methacrylate)-coated wells.

isolated hepatocytes. However, neither the type of culture nor methionine supplementation had a significant effect on [^{35}S]sulfate accumulation from [^{35}S]cysteine by cultured hepatocytes. These results suggest that much of the sulfate production in cultured hepatocytes may occur predominantly by cysteinesulfinate-independent pathways that do not require cysteine dioxygenase, because cysteine dioxygenase activity was lower in cultured hepatocytes than in freshly isolated cells and was not induced in excess of the basal levels in freshly isolated cells by culture in the presence of excess sulfur amino acids.

The extent of [^{35}S]glutathione accumulation in hepatocytes cultured in basal WE medium was greater than that in freshly isolated hepatocytes (Table 2). Glutathione accumulation tended to be higher in cells cultured in basal medium than in hepatocytes cultured in methionine-supplemented medium; the mean for [^{35}S]glutathione accumulation in spheroids cultured with supplemental methionine was only 35% of that for spheroids that had been cultured in basal medium.

Overall, the rate of [^{35}S]cysteine incorporation into protein in cultured hepatocytes was comparable to that in freshly isolated hepatocytes (Table 2). However, analysis by linear contrast indicated that monolayer cultures incorporated less [^{35}S]cysteine into protein than did spheroidal cultures ($p < 0.01$).

Although less precise because of the presence of large amounts of unlabeled glutathione and taurine in cells prior to incubation with [^{35}S]cysteine, the measured molar accumulations of glutathione and taurine (shown in parentheses in Table 2) were approximately 1.9-fold greater than those calculated based on the specific radioactivity of exogenous radiolabeled substrate alone. This is because the specific radioactivity of the exogenous [^{35}S]cysteine was diluted by unlabeled cysteine released by protein or glutathione turnover or formed from methionine, such that the specific radioactivity of the intracellular pool was lower than that of the exogenous substrate. Although it is likely that the different intracellular sulfur amino acid or glutathione contents of cells from various culture treatments affected the extent of dilution of cysteine specific radioactivity, the direct measurements of glutathione and taurine production indicate that the values based on radioactivity are qualitatively correct.

Experiment 3. Effect of dexamethasone and additional amino acids on CDO and GCS activities in cultured hepatocytes

Because hepatocytes cultured as spheroids did not show any obvious major advantages over monolayer cultures for the study of cysteine metabolism but are more difficult to manipulate experimentally, we decided to further explore the monolayer culture system as a possible model for studies of cysteine metabolism.

CDO and GCS values in cells cultured with 50 nmol/L dexamethasone were not significantly different from those in cells cultured without

dexamethasone, but the magnitude of the effect of methionine supplementation on CDO and GCS activities was greater when dexamethasone was included in the medium (Fig. 2). This observation suggested that glucocorticoids may play a permissive role in the adaptive changes in enzyme activities that occur in response to changes in sulfur amino acid availability.

Addition of a higher concentration of all twenty amino acids to the culture medium [based on the amounts used by Hutson et al. (1987)] had no statistically significant effect on either CDO or GCS activity as compared with that in cells cultured in basal WE medium (Fig. 2). However, the CDO activity tended to be higher and the GCS activity tended to be lower, which could be explained by the higher methionine level in the amino acid-supplemented medium. The WE + amino acids medium contained 0.41 mmol/L L-methionine as compared with 0.1 mmol/L L-methionine in the basal WE medium, and both media contained the same amount of cyst(e)ine, 0.49 mmol/L. Cells cultured in the amino acid-supplemented medium did not respond significantly ($p > 0.05$) to addition of 2 mmol/L L-methionine, regardless of whether dexamethasone was present. Both of these observations suggest that as little as 0.41 mmol/L L-methionine or 0.90 mmol/L total sulfur amino acids in the medium may be sufficient to obtain a maximal sulfur amino acid effect on activities of cysteine-metabolizing enzymes.

Experiment 4. Time course of response of cultured cells to supplemental methionine or cysteine

The time course of CDO and GCS activities in hepatocytes cultured for 3 days in medium that contained no supplemental amino acids or 2 mmol/L supplemental L-methionine or 2 mmol/L supplemental L-cysteine (plus BCS) is shown in Fig. 3. CDO activity tended to decrease during the first 24 h of culture, particularly in basal medium. A higher level of CDO activity was retained in cells supplemented with L-cysteine or L-methionine. By 48-h, CDO activity was significantly greater ($p \leq 0.05$; paired t-test) in hepatocytes cultured in medium supplemented with L-cysteine than in cells cultured in basal medium. By 72 h CDO activity was significantly greater in hepatocytes cultured with either supplemental methionine or cysteine. Hence, cultured hepatocytes tended to lose CDO activity in response to culture, but supplemental sulfur amino acids minimized or prevented this decline in CDO activity. Cysteine was as effective as methionine in this experiment in which equivalent molar concentrations were added to the culture medium.

GCS activity remained at initial levels in cells cultured in basal medium, but decreased in cells cultured in methionine or cysteine supplemented medium. Decreases in GCS activity in cysteine- or methionine-supplemented cells were apparent by 12 h and essentially maximal by 48 h. Decreases in response to cysteine supplementation were statistically significant at both 48 and 72 h, and those in response to methionine supplementation were statistically significant at 72 h. As for effects on CDO activity, cysteine was as effective as an equi-sulfur amount of methionine.

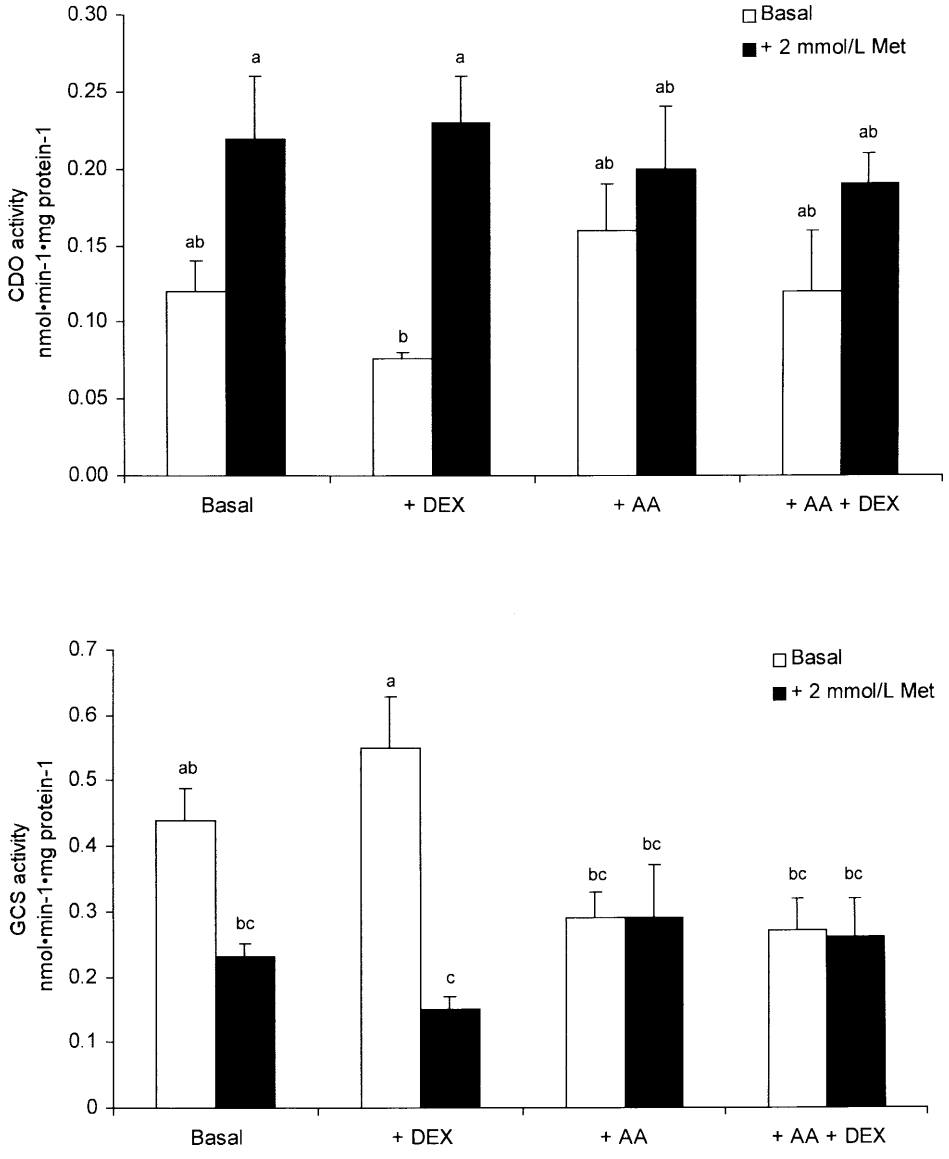

Fig. 2. The effect of addition of supplemental amino acids and dexamethasone to the culture medium on the activities of CDO and GCS. Monolayer cultures of hepatocytes were cultured in basal WE medium with or without supplemental amino acids (*+AA*) (Hutson et al., 1987) and with or without 50 nmol/L dexamethasone (*+DEX*) as indicated. Cells were cultured under each of these conditions with (shaded bars) or without (open bars) a supplement of 2 mmol/L L-methionine. Total L-methionine concentration was 0.1, 0.41, 2.1, and 2.41 mmol/L in the basal medium, the +AA medium, the basal medium plus 2 mmol/L L-methionine, and the +AA medium plus 2 mmol/L L-methionine, respectively. Both the basal WE medium and the +AA medium contained 0.49 mmol/L L-cyst(e)ine. Results are expressed as the means ± SEM for three experiments. Bars with different superscripts indicate that the means are significantly different ($p \leq 0.05$) by ANOVA and Tukey's ω-procedure

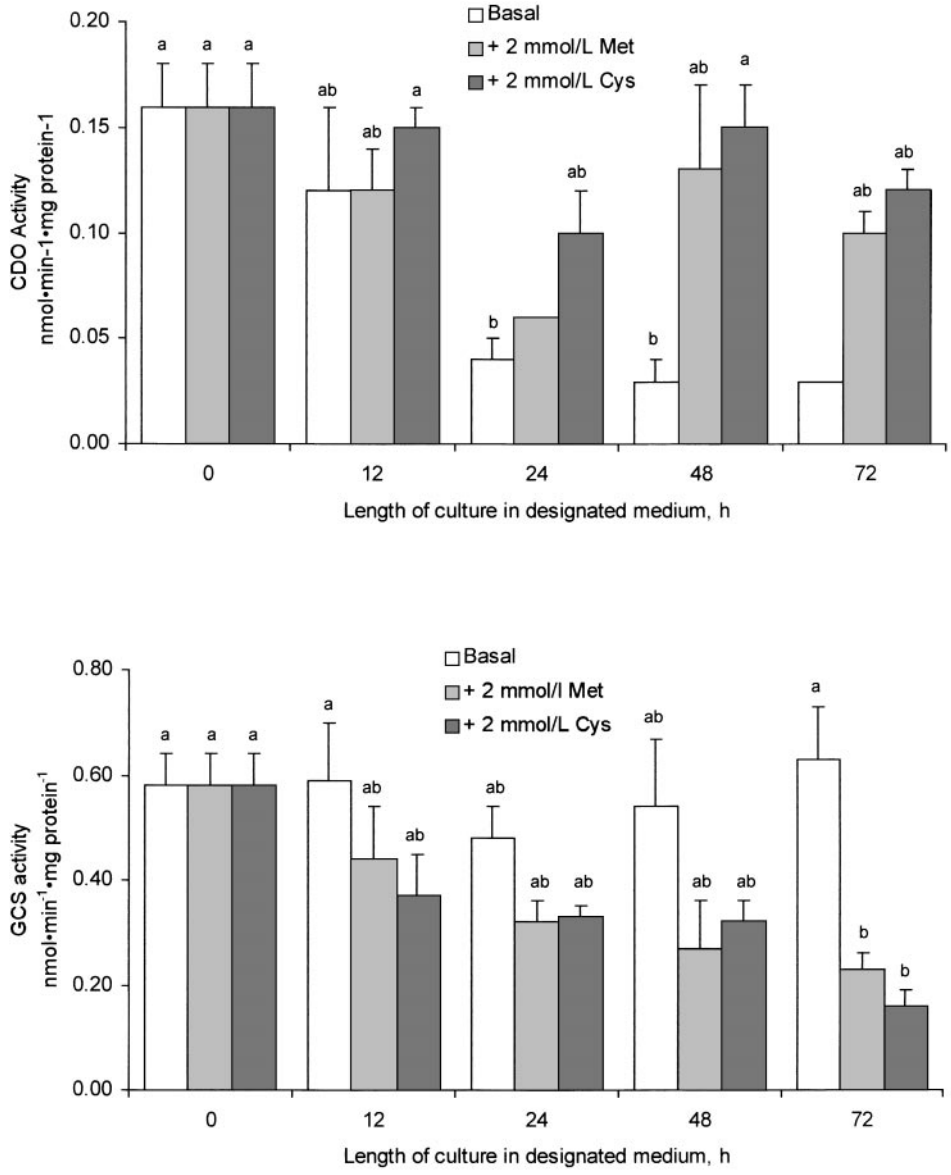

Fig. 3. Time course of CDO and GCS activities in hepatocytes cultured as monolayers in basal WE medium or in WE medium supplemented with methionine (*Met, 2 mmol/L*) or cysteine (*Cys, 2 mmol/L*). Results are expressed as the means ± SEM for three experiments. Bars with different superscript letters indicate that the means are significantly different ($p \leq 0.05$) by ANOVA and Tukey's ω-procedure. The basal WE medium contained 0.10 mmol/L L-methionine and 0.49 mmol/L L-cyst(e)ine

Based on this time course study, use of cells for studies of cysteine metabolism after 3 days of culture in the designated medium, as was done in the other experiments, seems appropriate. Also, this study indicated that cells responded to cysteine, suggesting that excess methionine per se was not essential for the sulfur amino acid effect. The possibility that the intracellular

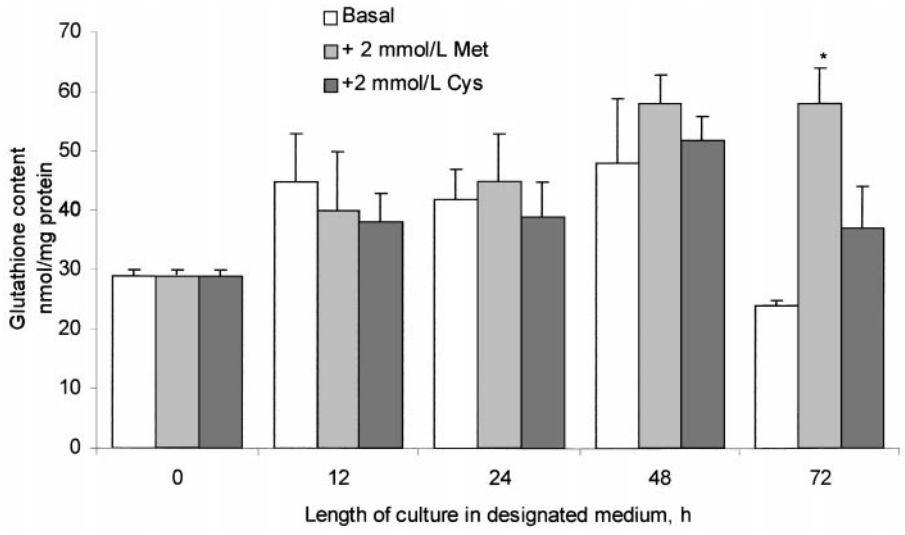

Fig. 4. Changes in glutathione content of hepatocytes during culture in basal or sulfur amino acid supplemented media. Hepatocytes were allowed to attach to collagen-coated plates (4 h) and were then incubated in basal WE medium or basal medium supplemented with 2 mmol/L methionine or 2 mmol/L cysteine for the indicated length of time before measurement of total glutathione content of the cells. Results are means + SEM for three experiments. Values marked by * are significantly different ($p \leq 0.05$) from the value for cells cultured in basal medium for the same length of time by the paired t-test

glutathione content acts as a cellular indicator of sulfur amino acid availability and signals the need for changes in CDO and GCS activities seems unlikely. The glutathione content of cultured cells increased during the first 12 h of culture that followed the 4-h period to allow attachment of cells to collagen, but the glutathione content of cells cultured in sulfur amino acid-supplemented media was generally similar to that of cells cultured in basal medium (Fig. 4).

Experiment 5. Effect of cysteine or methionine concentration in the culture medium on CDO and GCS activities in cultured hepatocytes

To determine the dose-response curves for the effect of cysteine and methionine supplementation of CDO and GCS activities, hepatocytes were cultured for 72 h in WE medium supplemented with various concentrations of cysteine or methionine. Because we did not observe differences in activities of CDO and GCS in cells cultured in media with 2 mmol/L as compared with 10 mmol/L of supplemental methionine and because 0.41 mmol/L methionine (0.90 mmol/L total sulfur amino acids) seemed to yield a near-maximal response, the dose-response of CDO and GCS activities to methionine and cysteine supplementation was tested in medium to which 0.1 to 5 mmol/L supplemental amino acid was added.

Although the concentrations of either methionine and cysteine in the medium refer to those concentrations in the fresh cell culture medium, which was replaced at 24-h intervals, and not to the overall or average concentrations in the medium or concentrations in the cell, we can make rough calculations of the concentrations of total or supplemental sulfur amino acids in the medium required for maximal responsiveness. The effect of methionine on CDO activity was near-maximal at 0.5 mmol/L of supplemental methionine, and the effect of methionine on GCS activity was near-maximal at 0.3 mmol/L of supplemental methionine. The effect of cysteine on CDO activity was near-maximal at 1 to 2 mmol/L of supplemental cysteine, and that of cysteine on GCS activity was near-maximal at 0.5 to 1 mmol/L of supplemental cysteine. The concentration of supplemental methionine that gave a half-maximal increase in CDO activity was 0.27 mmol/L, and the concentration of supplemental methionine that gave a half-maximal decrease in GCS activity was 0.09 mmol/L. The concentration of supplemental cysteine that gave a half-maximal increase in CDO activity was 0.70 mmol/L, and the concentration of supplemental cysteine that gave a half-maximal decrease in GCS activity was 0.32 mmol/L. These observations indicate that lower concentrations of sulfur amino acids were required, in the medium, to exert maximal adaptive downregulation of GCS activity than were required for maximal upregulation of CDO activity. The data also indicate that CDO and GCS respond to either supplemental methionine or cysteine, but lower concentrations of methionine than of cysteine were required in the medium for equivalent effects in the dose-response range of concentrations (0.1 to ~1 mmol/L of supplemental sulfur amino acid). If, however, the maximal response (the plateau of the curves in Fig. 5) to cysteine and methionine are compared, supplemental cysteine brought about a similar increase in CDO activity and a significantly greater decrease ($p \leq 0.05$) in GCS activity compared to the responses to equimolar methionine. The trend observed in experiment 4 (Fig. 3) was similar but not statistically significant.

Discussion

Effect of monolayer vs. spheroidal culture

Spheroidal cultures maintain more-differentiated liver functions such as albumin synthesis, transferrin synthesis, expression of glucokinase, and response of iodothyronine 5′-deiodinase to thyroid hormone (Koide et al., 1990; Menjo et al., 1993; Tong et al., 1992; Yuasa et al., 1993). Because expression of CDO and the biosynthesis of taurine occur predominantly in the liver, we hypothesized that these functions might also be retained better in spheroidal cultures than in monolayer cultures and, hence, that spheroidal cultures might be more suitable as a model for studies of hepatic cysteine metabolism. This was not observed to be the case. No statistical difference in CDO or GCS activity was observed between cells cultured as monolayers on collagen-coated plates and those cultured as spheroids on poly-HEMA-coated plates. Although CSDC activity was significantly lower in hepatocytes cultured as spheroids than in those cultured as monolayers, CSDC activity in

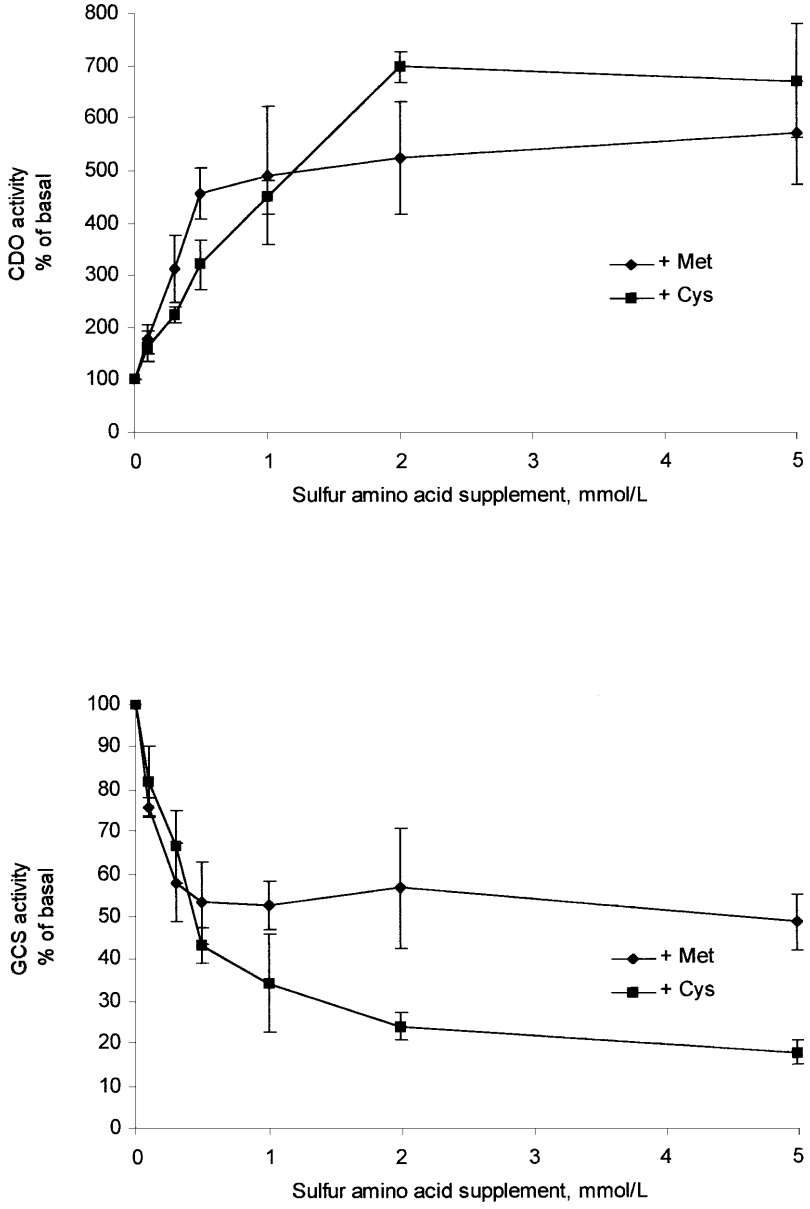

Fig. 5. Effect of sulfur amino acid concentration in the culture medium on the activities of CDO and GCS. Cells were allowed to attach to collagen-coated plates over 4 h, and then cells were cultured in basal WE medium supplemented with various concentrations of L-methionine or L-cysteine for 72 h. Results are expressed as % of control (basal; no sulfur amino acid supplementation) for 3 to 5 experiments. The basal mean activity of CDO was 0.02 nmol·min^{-1}·mg protein^{-1}, and the basal mean activity of GCS was 0.64 nmol·min^{-1}·mg protein^{-1}

cells cultured as monolayers was closer to that observed in freshly isolated cells.

The observation that the magnitude of the effect of methionine on GCS activity and GSH accumulation was greater for cells cultured as spheroids than for those cultured as monolayers suggests that spheroids might behave

more like hepatocytes in situ in the response of GSH synthesis to methionine. On the other hand, the magnitude of the effect of methionine on CDO activity was smaller for cells cultured as spheroids than for those cultured as monolayers, and the magnitude of the effect of methionine on accumulation of catabolites of cysteine (taurine or taurine plus sulfate) was similar for both monolayer and spheroidal cultures. The overall finding (Table 2) that incorporation of [^{35}S]cysteine into protein was greater in spheroids than in monolayers is consistent with observations that spheroids synthesize and secrete various proteins including albumin (Tong et al., 1992).

Whether or not these differences in magnitude of response of spheroidal vs. monolayer cultures to methionine is meaningful needs to be further examined. During the 3-day culture period in experiment 1, CDO activity decreased markedly in both monolayers and spheroids from the levels originally present in the freshly isolated cells. CDO activity in hepatocytes cultured in basal medium was 0.07 nmol·min^{-1}·mg protein^{-1} (monolayers) or 0.12 nmol·min^{-1}·mg protein^{-1} (spheroids); this activity inceased by 1.4-fold (monolayers) or 0.5-fold (spheroids) in response to supplementation of the medium with 2 mmol/L methionine. Both the observed activities and the magnitude of change in response to methionine supplementation were lower than the values observed in previous studies with hepatocytes isolated from rats fed various levels of methionine; CDO activity ranged from 0.1 nmol·min^{-1}·mg protein^{-1} for hepatocytes from rats fed basal low protein diets to 5 nmol·min^{-1}·mg protein^{-1} for hepatocytes from rats fed high levels of methionine (Bagley and Stipanuk, 1994; Bella and Stipanuk, 1995, 1996). Whether this difference in response is due to dedifferentiation of the hepatocytes or to differences in exposure of hepatocytes to other signals and/or effectors needs to be further studied. It should be noted, however, that in the slightly modified culture systems used in experiments 4 and 5 in this series of studies, larger fold differences (~3-fold) in CDO activity in response to sulfur amino acid supplementation were observed. Nevertheless, the absolute values for CDO activity remained low compared to those observed in liver of intact rats.

GCS activity in cultured cells was similar to that observed in liver of intact rats, and the effect of methionine or cysteine supplementation of the culture medium was similar to that observed in liver of intact rats fed diets with various levels of protein or sulfur amino acids. GCS activity decreased to 20% to 70% of basal levels with sulfur amino acid supplementation.

Effect of sulfur amino acid supplementation of culture medium

Methionine or cysteine supplementation of the culture medium consistently increased the activity of CDO and decreased the activity of GCS. These changes occurred gradually over the three days of culture, becoming significant by 48 or 72 h (Fig. 3).

In experiment 1 (Tables 1 and 2), similar changes in CDO and GCS activities, as well as in cell taurine and glutathione contents, were observed for

cells cultured with 2 mmol/L supplemental methionine as were observed for those cultured with 10 mmol/L supplemental methionine. In experiment 3 (Fig. 2), no effect of addition of 2 mmol/L methionine to the amino acid-supplemented basal medium was observed; the amino acid-supplemented basal medium contained 0.41 mmol/L total methionine (0.90 mmol/L total sulfur amino acids) compared to 0.10 mmol/L methionine (0.59 mmol/L total sulfur amino acids) in the basal WE medium. These experiments together suggested that a near-maximal response to methionine supplementation could be obtained with as little as 0.4 mmol/L methionine or 0.9 mmol/L total sulfur amino acids in the culture medium. A clear dose-response relationship between methionine concentration in the culture medium and CDO and GCS activities was observed in experiment 5 (Fig. 5) in which the response to supplemental methionine in amounts that ranged from 0.1 to 5.0 mmol/L of medium; the basal medium contained 0.10 mmol/L of methionine and 0.41 mmol/L of cyst(e)ine. Concentrations in the culture medium ranging from 1.1 to 6.1 mmol/L were measured. The concentration of supplemental methionine (or total sulfur amino acids, equivalent to supplemental plus basal) in fresh medium that gave a half-maximal increase in CDO activity was 0.27 mmol/L of supplemental methionine (total of 0.78 mmol/L of total sulfur amino acids), and the concentration of methionine that gave a half-maximal decrease in GCS activity was 0.09 mmol/L of supplemental methionine (total of 0.60 mmol/L of total sulfur amino acids). Both effects were essentially maximal when the level of supplemental methionine was 0.5 mmol/L and the total sulfur amino acid concentration was 1.0 mmol/L.

Comparison of methionine and cysteine for their effectiveness in increasing CDO activity or decreasing GCS activity indicate that the two sulfur-containing amino acids were similarly effective when added to culture medium in equimolar amounts, 2 mmol/L (Fig. 3). The dose-response study (Fig. 5) suggested, however, that higher amounts of cysteine were required in the culture media to bring about half-maximal or near-maximal changes in activity of either CDO or GCS. Although lower concentrations of supplemental methionine were required for a near-maximal response, the response of both CDO and GCS at the plateau was of greater magnitude when cysteine rather than methionine was used for supplementation of the culture medium. The fact that cysteine was effective suggests that methionine per se is not required for these changes in CDO and GCS activities. One might expect that these adaptive effects could be mediated by changes in intracellular glutathione content, but the total cellular glutathione concentration was similar for hepatocytes cultured with and without supplemental sulfur amino acids. One might expect that a lower concentration of cysteine would be required in the medium for maximal changes in CDO or GCS activities if the adaptive changes are mediated by cysteine at the cellular level. However, it is difficult to extrapolate from concentrations present in fresh medium added at 24-h intervals or concentrations present in animal diets to concentrations of amino acids in the hepatocytes. Differences in transport, oxidative degradation in the medium, differences in rate of utilization, and other factors could account for the apparently greater responsiveness to

methionine than cysteine. It is interesting that methionine was also more effective than cyst(e)ine in bringing about increases in CDO activity and cysteine catabolism and decreases in the capacity for glutathione synthesis when equimolar amounts were added to rat diets (Bagley and Stipanuk, 1995).

As observed in studies in intact rats, CDO and GCS respond in opposite directions to changes in sulfur amino acid availability. Increases in CDO activity are generally accompanied by decreases in GCS activity. The lower concentration of either supplemental methionine or cysteine required to bring about half-maximal or near-maximal changes in GCS activity, as compared with those required for changes in CDO activity, suggest that glutathione synthesis would be partially suppressed before substantial enhancement of CDO activity and cysteine catabolism would occur.

These observations support results of dietary studies conducted in our laboratory (Bagley and Stipanuk, 1994, 1995; Bella and Stipanuk, 1995, 1996; Bella et al., 1996). In dietary studies, hepatocytes from rats fed higher levels of protein or supplemental methionine or cystine had higher levels of CDO and lower levels of GCS. A dose-response relationship was observed for both protein and sulfur amino acids. Hence, the similar effects of supplemental methionine or cysteine at the whole animal and the cellular levels imply that methionine has a central role in the regulation of CDO and GCS activities in the liver and offer further support to our hypothesis that CDO and GCS share a coordinated, but reciprocal, mode of regulation.

CSDC activity in cultured hepatocytes was not affected by the addition of methionine to the culture medium. This is also consistent with our observations in intact animals. Although CSDC was consistently decreased by increases in the dietary protein level, it was not affected by supplemental sulfur amino acids except at extremely high levels (Bagley and Stipanuk, 1994, 1995; Bella and Stipanuk, 1995, 1996; Bella et al., 1996). These studies with cultured cells offer further evidence that the major mode of regulation of CSDC activity is distinct from that for regulation of CDO and GCS activities.

Relationship beween enzyme activities and metabolism of cysteine to particular metabolites

In previous studies with freshly isolated hepatocytes, we have observed a parallel increase in total cysteine catabolism (taurine + sulfate production) with increases in CDO activity (Bagley and Stipanuk, 1994, 1995). This relationship was not apparent in this study if freshly isolated cells are compared to cultured cells, but did seem to hold for cultured cells with respect to the observed increase in both CDO activity and accumulation of taurine + sulfate from [^{35}S]cysteine in response to methionine supplementation of the culture medium. It seems likely that other factors besides CDO activity must be considered when freshly isolated cells are compared to cultured cells. For example, cysteine availability and possibly its intracellular form (cysteine vs. cystine) may be different for cultured cells than for freshly isolated

hepatocytes. It is known that system x_c^-, which transports the anionic form of cystine, is induced in cultured hepatocytes (Takada and Bannai, 1984). Increased transport of cystine could increase cysteine catabolism as a result of increasing the intracellular cysteine availability and by providing cystine as substrate for desulfhydration by cystathionine γ-lyase. A greater distribution of cysteine to taurine vs. sulfate was consistently associated with higher CDO activity, supporting the position that CDO plays a major role, often more important than CSDC, in determining the extent of taurine production by liver (Bagley and Stipanuk, 1994, 1995; Bella and Stipanuk, 1995, 1996; Bella et al., 1996).

Accumulation of glutathione during the metabolism experiments corresponded reasonably well with the GCS activity observed in freshly isolated cells and in hepatocytes cultured on different surfaces and with different levels of methionine. This suggests that GCS activity plays an important role in determining the extent of glutathione synthesis in hepatocytes.

Primary cultured hepatocytes as a model for studies of the adaptive regulation of cysteine metabolism

Expression of CDO is clearly a differentiated function of liver parenchymal cells that is readily loss when cells are transformed (McCann et al., 1994; Sakakibara et al., 1976). Our studies demonstate that CDO activity decreases in primary hepatocytes cultured as either monolayers or spheroids compared to the levels of activity present in the freshly isolated cells. Although CDO activity in hepatocytes maintained in primary culture continued to decrease throughout the first 24 h of culture when basal medium was used, CDO activity in primary cultures of hepatocytes responded to sulfur amino acid supplementation of the medium and levels of activity comparable to those in freshly attached cells on substrata or to those observed in liver of rats fed diets with low or moderate levels of protein were maintained (Bella and Stipanuk, 1995, 1996; Bella et al., 1996). Either cysteine or methionine supplementation of the medium to provide approximately 1 mmol/L total methionine plus cyst(e)ine was sufficient to maximally enhance CDO activity in cultured hepatocytes. The induced levels of CDO in cultured cells never reached the levels (2 nmol·min^{-1}·mg protein^{-1} or more) observed in intact rats fed diets high in protein or sulfur amino acids, however.

Absolute levels of GCS activity and the response of GCS activity to changes in sulfur amino acid availability were similar to those observed in intact rats (Bella et al., 1996). GCS activity was maintained relatively close to levels in freshly isolated cells or rat liver throughout three days of culture, and GCS activity responded to methionine or cysteine supplementation with 30 to 80% decreases in activity, which is close to the magnitude of responses (40% to 75%) observed in intact rats fed diets with high levels of protein or sulfur amino acids (Bella et al., 1996, 1999).

Monolayer culture of primary hepatocytes is promising as a system for further studies of the mechanism by which sulfur amino acids alter the

activities of these two enzymes. Our limited studies suggest that spheroidal cultures hold no advantages over monolayers cultures for these studies, and experimentally monolayer cultures are easier to manipulate and yield higher numbers of viable cells. The monolayer cultures of hepatocytes yielded cells with substantial CDO, CSDC and GCS activities. The responsiveness of these key enzymes to sulfur amino acid availability was similar to that observed in liver of intact rats: CDO increased with sulfur amino acid supplementation, GCS decreased with sulfur amino acid supplementation, and CSDC was relatively unresponsive to sulfur amino acid supplementation. Furthermore, metabolism of [^{35}S]cysteine gave patterns of metabolism consistent with observed enzyme activities as observed with freshly isolated hepatocytes. We plan to use primary hepatocytes to further explore the mechanisms by which sulfur amino acids bring about adaptive changes in the activities of CDO and GCS.

Acknowledgements

The authors wish to thank Lawrence Hirschberger for his technical assistance with these studies.

This work was supported by National Research Initiative Competitive Grants Program/United States Department of Agriculture Competitive Research Grant #92-37200-7583 and by United States Department of Agriculture/Cooperative State Research Education and Extension Service Grant #94-34324-0987.

References

Bagley PJ, Stipanuk MH (1994) The activities of rat hepatic cysteine dioxygenase and cysteinesulfinate decarboxylase are regulated in a reciprocal manner in response to dietary casein level. J Nutr 124: 2410–2421

Bagley PJ, Stipanuk MH (1995) Rats fed a low protein diet supplemented with sulfur amino acids have increased cysteine dioxygenase activity and increased taurine production in hepatocytes. J Nutr 125: 933–940

Bagley PJ, Hirschberger LL, Stipanuk MH (1995) Evaluation and modification of an assay procedure for cysteine dioxygenase activity: high-performance liquid chromatography method for measurement of cysteine sulfinate and demonstration of physiological relevance of cysteine dioxygenase activity in cysteine catabolism. Anal Biochem 227: 40–48

Bella DL, Stipanuk MH (1995) Effects of protein, methionine, or chloride on acid-base balance and on cysteine catabolism. Am J Physiol 269: E910–E917

Bella DL, Stipanuk MH (1996) High levels of dietary protein or methionine have different effects on cysteine metabolism in rat hepatocytes. In: Huxtable R, Azuma J, Nakagawa M, Kuriyama K (eds) Taurine: basic and clinical aspects. Plenum Press, New York, pp 73–84

Bella DL, Kwon YH, Stipanuk MH (1996) Variations in dietary protein but not in dietary fat plus cellulose or carbohydrate levels affect cysteine metabolism in rat isolated hepatocytes. J Nutr 126: 2179–2187

Bella DL, Hirschberger LL, Hosokawa Y, Stipanuk MH (1999) The mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver in vivo. Am J Physiol 276 (in press)

Berry MN, Edwards AM, Borritt GJ (1991) Isolated hepatocytes: preparation, properties and applications. Elsevier, New York, pp 16–32

Cai J, Sun WM, Hwang JJ, Stain SC, Lu SC (1996) Changes in *S*-adenosylmethionine synthetase in human liver cancer: Molecular characterization and significance. Hepatology 24: 1090–1097

Coloso RM, Stipanuk MH (1989) Metabolism of cyst(e)ine in rat enterocytes. J Nutr 119: 1914–1924

Coloso RM, Drake MR, Stipanuk MH (1990) Effect of bathocuproine disulfonate, a copper chelator, on cyst(e)ine metabolism by freshly isolated rat hepatocytes. Am J Physiol 259: E443–E450

Daniels KM, Stipanuk MH (1982) The effect of dietary cysteine level on cysteine metabolism in rats. J Nutr 112: 2130–2141

Enat R, Jefferson DM, Ruiz-Opazo N, Gatmaitan Z, Leinwand LA, Reid LM (1984) Hepatocyte proliferation in vitro: its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. Proc Natl Acad Sci USA 81: 1411–1415

Fariss MW, Reed DJ (1987) High performance liquid chromatography of thiols and disulfides: dinitrophenyl derivatives. Methods Enzymol 143: 101–109

Goss SJ (1986) Characterization of cystathionine synthase as a selectable, liver-specific trait in rat hepatoma. J Cell Sci 82: 309–320

Hutson SM, Stinson-Fisher C, Shiman R, Jefferson LS (1987) Regulation of albumin synthesis by hormones and amino acids in primary cultures of rat hepatocytes. Am J Physiol 252: E291–E298

Koide N, Sakaguchi K, Koide Y, Asano K, Kawaguchi M, Matsushima H, Takenami T, Shinji T, Mori M, Tsuji T (1990) Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. Exp Cell Res 186: 227–235

Labarca C, Paigen K (1980) A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102: 344–352

Landry J, Bernier D, Quellet C, Goyette R, Marceau N (1985) Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. J Cell Biol 101: 914–923

Lu SC, Huang H-Y (1994) Comparison of sulfur amino acid utilization for GSH synthesis between HepG2 cells and cultured rat hepatocytes. Biochem Pharmacol 47: 859–869

McCann KP, Akbari MT, Williams AC, Ramsden DB (1994) Human cysteine dioxygenase type I: primary structure derived from base sequencing of cDNA. Biochim Biophys Acta 1209: 107–110

Menjo M, Murata Y, Fujii T, Nimura Y, Seo H (1993) Effects of thyroid and glucocorticoid hormones on the level of messenger ribonucleic acid for iodothyronine type I 5′-deiodinase in rat primary hepatocyte cultures grown as spheroids. Endocrinology 133: 2984–2990

Meredith MJ (1987) Cystathionase activity and glutathione metabolism in redifferentiating rat hepatocyte primary cultures. Cell Biol Toxicol 3: 361–377

Sakakibara S, Yamaguchi KY, Hosokawa Y, Kohashi N, Ueda I, Sakamoto Y (1976) Purification and some properties of rat liver cysteine oxidase. Biochim Biophys Acta 422: 273–279

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85

Stipanuk MH, Coloso RM, Garcia RAG, Banks M (1992) Cysteine concentration regulates cysteine metabolism to glutathione, sulfate and taurine in rat hepatocytes. J Nutr 122: 420–427

Takada A, Bannai S (1984) Transport of cystine in isolated rat hepatocytes in primary culture. J Biol Chem 259: 2441–2445

Tong JZ, De Lagausie P, Furlan V, Cresteil T, Bernard O, Alvarez F (1992) Long-term culture of adult rat hepatocyte spheroids. Exp Cell Res 200: 326–332

Yuasa C, Tomita Y, Shono M, Ishimura K, Ichihara A (1993) Importance of cell aggregation for expression of liver functions and regeneration demonstrated with primary cultured hepatocytes. J Cell Physiol 156: 522–530

Authors' address: Martha H. Stipanuk, Ph.D., 225 Savage Hall, Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853-6301, U.S.A.,
Fax 607 255-1033, E-mail: mhs6@cornell.edu

Received June 2, 1999