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Glutathione Biosynthesis in the Postimplantation Rat Conceptus *in Vitro*

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Glutathione (GSH) biosynthesis in Day 10 rat conceptuses was characterized in whole embryo culture by evaluation of net rates of GSH replenishment in whole conceptuses, embryos, and visceral yolk sacs (VYS); uptake and distribution of [^{35}S]cysteine and [^{35}S]methionine from the culture medium; incorporation of ^{35}S-labeled amino acids into GSH; and efflux of [^{35}S]GSH into the culture medium. Diethyl maleate (DEM, 500 μM) depleted intracellular GSH pools in embryo and VYS to 30% of control values within 45 min. Restoration of GSH pools in VYS began immediately and continued at a rate of 295 pmol/conceptus/hr until GSH concentrations exceeded initial control levels at 4 hr. GSH pools in the embryo remained depleted for over 2 hr, followed by resynthesis at initial rates of 118 pmol/conceptus/hr. [^{35}S]Cysteine (0.2 mM) uptake from the culture medium resulted in whole conceptus accumulations that reached 1.6 nmol/conceptus. The portion of intracellular free cysteine obtained through uptake of extraconceptal amino acid was 40–90 pmol/conceptus and represented less than 20% of the total free intracellular cysteine. [^{35}S]Methionine (0.2 mM) accumulation surpassed that of cysteine at all time points by two- to threefold. [^{35}S]Cysteine, added 2 hr after DEM, was incorporated into GSH at rates of 126 pmol/conceptus/hr during the first hour. By 4 hr, rates of incorporation had declined to 22 pmol/conceptus/hr. L-Buthionine-[*S,R*]-sulfoximine (1 mM) completely eliminated incorporation of [^{35}S]cysteine into GSH. Net efflux of [^{35}S]GSH into the culture medium accounted for less than 40 pmol of total GSH when measured 5 hr after DEM addition. Although effectively transported into the conceptus and readily utilized in protein synthesis, ^{35}S from methionine was not incorporated into GSH under any conditions tested. After chemical depletion, *de novo* GSH synthesis occurs exclusively in the VYS. Embryonic recovery begins only after GSH pools in the VYS are replete. Prolonged embryonic GSH depletion and slower recovery rates indicate that the embryo may be selectively susceptible to chemical insult following depletion of GSH.

Glutathione (L-γ-glutamyl-Lcysteinylglycine, GSH)[1] status has been shown to be an important factor in the ability of therapeutic and chemical agents to elicit embryotoxicity, dysmorphogenesis, and embryolethality in the organogenesis-stage rat embryo (Harris *et al.*, 1987; Slott and Hales, 1987; Wong *et al.*, 1989; Harris *et al.*, 1988; Naya *et al.*, 1990). This tripeptide is found in most animal, plant, and bacterial cells in relatively high concentrations, indicative of the broad spectrum of GSH involvement in detoxication reactions, cellular metabolic regulation, and general maintenance of intracellular redox status (Ziegler, 1985; Gilbert, 1990; Ammon *et al.*, 1989). Selective alterations in GSH status have been reported to influence a number of endogenous reactions including the synthesis of proteins and deoxyribonucleotide precursors of DNA, metabolic processing of estrogens, prostaglandins and leukotrienes, binding and secretion of hormones, and maintenance of membrane integrity (Kosower and Kosower, 1978; Ammon *et al.*, 1989). Recent studies have also begun to focus on the potential role of GSH and related intracellular thiols in the control of development through specific effects on differentiation, growth regulation, and cell proliferation (Noelle and Lawrence, 1981; Allen and Balin, 1989; Allen and Venkatraj, 1992).

A major function of GSH, however, is in the protection of cells from free radicals, reactive oxygen species, and the chemical electrophiles produced from bioactivation of therapeutic and environmental chemicals (Ketterer *et al.*, 1983; Reed, 1985). Several important enzymes utilize GSH as a cofactor or cosubstrate for the purposes of cellular protection and for normal processing of endogenous substrates. Of these, selenium-dependant glutathione peroxidase (GSH-Px,) serves an important function in the protection against damaging oxygen radicals and peroxides through reduction of hydrogen and organic peroxides and in the termination of lipid peroxidation (Flohe *et al.*, 1976). Glutathione disulfide (GSSG), generated through the activity of

[1] Abbreviations used: GSH, L-γ-glutamyl-L-cysteinylglycine (glutathione); GSSG, glutathione disulfide; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; mBBr, monobromobimane; MSA, methanesulfonic acid; DEM, diethyl maleate; VYS, visceral yolk sac; GSHT, glutathione *S*-transferase; GSH-Px, glutathione peroxidase; GSSG-Rd, glutathione disulfide reductase; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; LSC, liquid scintillation counting; GCS, γ-glutamylcysteine synthetase; GC, glutathione synthetase.

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GSH-Px and direct interaction of GSH with oxygen and organic free radicals can be reduced intracellularly to GSH by the cytosolic NADPH-dependent GSSG reductase (GSSG-Rd) (Babson *et al.*, 1981). Inhibition of this pathway has been shown to enhance the embryotoxicity elicited by chemical agents both *in vivo* and *in vitro* (Wong and Wells, 1989; Hiranruengchok and Harris, 1993). Chemical electrophiles react with GSH nonenzymatically or with the aid of glutathione *S*-transferases (GSHT) to form adducts as an important detoxication pathway in a number of organisms (Ketterer, 1982; Ketterer *et al.*, 1983). Evidence supporting the presence of active GSHT in the organogenesis-stage rat conceptus has been equivocal and the potential importance of these enzymes during development has not yet been clearly established (Faustman *et al.*, 1988; Hales and Huang, 1992). Direct evidence that specific GSH adducts are formed with reactive chemicals in the developing conceptus as a means of cellular protection is not yet available. Relatively high concentrations of intracellular GSH found in the conceptus may provide significant cellular protection through direct chemical conjugation and various other reactions involving GSH (Harris *et al.*, 1987; Harris *et al.*, 1986). The protective role of GSH is of increased importance since it has been demonstrated that the postimplantation conceptus possesses significant biotransformation capacity, particularly in the form of active constitutive and inducible cytochrome P-450 monooxygenases (Yang *et al.*, 1988; Juchau *et al.*, 1991; Harris *et al.*, 1989). These enzymes have been shown to produce reactive metabolites in rat conceptuses in sufficient quantities to elicit malformations in the embryo (Juchau *et al.*, 1991; Harris *et al.*, 1989). Numerous therapeutic and environmental agents are known to reach the conceptus and accumulate, especially during early development and prior to placentation (Waddell and Marlowe, 1981). Accumulation of chemicals and their bioactivation to reactive intermediates and subsequent detoxication, especially under conditions of oxidative stress, could easily result in depletion of the GSH pool. Further protection against the deleterious consequences of altered intracellular redox status and compromised detoxication capacity may ultimately depend on the ability of the conceptus to rapidly replenish depleted GSH stores.

Because most cells are unable to transport GSH directly, restoration and maintenance of intracellular GSH pools is facilitated through *de novo* GSH synthesis. This process requires an adequate supply of amino acid precursor, including the normally rate-limiting component, cysteine. Amino acid precursors for GSH synthesis are provided, in part, through cleavage of extracellular GSH to constitutive amino acids by plasma membrane γ-glutamy transpeptidases (EC 2.3.2.2, GGT), followed by intracellular transport using the γ-glutamyl moiety as an amino acid carrier (Meister, 1984). A series of enzymatic reactions referred to as the γ-glutamyl cycle are then required to facilitate the intracellular resynthesis of GSH (Kaplowitz *et al.*, 1985; Meister, 1974). The first reaction involves the combination of glutamate and cysteine via the γ-amine of glutamate to form γ-glutamylcysteine (GC). This reaction is catalyzed by the cytosolic enzyme, γ-glutamylcysteine synthetase (EC 6.3.2.2, GCS) and requires ATP to complete the catalysis. The second reaction also requires ATP and involves the addition of glycine to GC via a peptide bond to form the product GSH. This reaction is catalyzed by cytosolic glutathione synthetase (EC 6.3.2.3). Intracellular GSH levels are controlled by the availability of precursor amino acids (principally cysteine) and by nonallosteric feedback inhibition of GSH on the activity of GCS (Richman and Meister, 1975). In some tissues such as the adult liver, the critical sulfur amino acid precursor, cysteine, can also be provided through transsulfuration of methionine via enzymes of the cystathionine pathway (Reed and Orrenius, 1977). Virtually nothing is known about the overall regulation of GSH synthesis and turnover in the developing conceptus, including the relative capacities of tissues to restore GSH lost as a result of chemical detoxication activity and in response to environmental extremes such as hyperthermia, hyperoxia, and hypoxia (Harris *et al.*, 1991; Fantel *et al.*, 1991; Jenkinson *et al.*, 1986).

The purpose of the current study is to characterize the ability of the postimplantation rat conceptus to synthesize new GSH during the teratogen-sensitive period of early organogenesis. Basal and optimal rates of GSH synthesis have been evaluated by measuring the rate of recovery of GSH pools in the whole conceptus and in individual embryos and visceral yolk sacs (VYS) by high-performance liquid chromatography (HPLC) analysis and also by determining the incorporation of [^{35}S]cysteine or [^{35}S]methionine into new GSH in the whole rat conceptus *in vitro*. The ability of the conceptus to utilize either of the sulfur amino acid precursors indicates the extent of cystathionine pathway involvement in providing the precursor for the synthetic process. Differential restoration of GSH in the embryo and VYS suggest a regulatory role for the VYS in providing appropriate precursors to the embryo for use in GSH synthesis.

MATERIALS AND METHODS

Animals. Primagravida Sprague–Dawley rats were obtained on Days 6–9 of gestation from the Reproductive Sciences Program Small Animal Core, University of Michigan. Detection of a sperm-positive vaginal smear on the morning following copulation confirmed pregnancy and was designated Day 0 of gestation. Pregnant rats were maintained on a 14 hr light/10 hr dark cycle until explanted on Day 10 of gestation. Access to food and water was *ad libitum*.

Embryo culture and glutathione depletion. On the morning of Gestational Day 10, conceptuses (8–10 somites) were removed from the uterus and prepared for culture as has been described in detail elsewhere (Fantel *et*

al., 1979; Harris *et al.*, 1987). Culture media consisted of 33% homologous heat-inactivated rat serum and 66% Hanks' balanced salt solution (HBSS, pH 7.4). Intracellular GSH was depleted in conceptuses by direct addition of diethyl maleate (DEM, 500 μM) to the culture media, followed by incubation in a roller-incubator (37°C). Two variations of this depletion protocol have been used. In the first, the time course of GSH depletion and recovery (shown in Fig. 1) was determined by removal of 2 conceptuses from the culture media at each specified time point (treatment and control). Under these conditions DEM remained in the culture medium for the entire 5-hr period. Visceral yolk sacs were quickly dissected free of the embryo and amnion and the tissues were placed in microcentrifuge tubes containing 100 μl methanesulfonic acid (MSA, 200 mM) and immediately frozen in liquid nitrogen. Subsequent preparation for analysis of GSH and cysteine levels by HPLC is discussed below. In the second DEM protocol, intracellular GSH was depleted prior to the addition of labeled cysteine or methionine in GSH synthesis experiments in order to relieve feedback inhibition and allow for optimal rates of new GSH synthesis. Conceptuses (20–30) were divided into two sealed media bottles containing 15 ml of media which had been warmed (37°C) and saturated with 20% O_2, 5% CO_2, 75% N_2. DEM (500 μM) was added directly to the media and conceptuses were placed in the roller-incubator at 37°C for 2 hr. At the end of this time the conceptuses were removed, rinsed 3× in warm HBSS and transferred to smaller media bottles containing 5–10 conceptuses and 5 ml of fresh media without DEM (prepared for GSH synthesis experiments).

HPLC analysis of glutathione and cysteine. GSH and cysteine were resolved and quantified as their monobromobimane (mBBr, Calbiochem) derivatives using the HPLC method as described and modified from Fahey and Newton (1987) and Harris *et al.* (1991). Whole conceptuses or pooled embryos and visceral yolk sacs were rinsed free of culture media in cold physiological buffered saline (PBS) or HBSS and placed in microcentrifuge tubes containing 100 μl of MSA (200 mM). Samples were frozen immediately in liquid nitrogen and stored at −75°C until prepared for HPLC.

Thawed samples were homogenized by ultrasonic disruption and 100 μl of 4 M sodium methanesulfonate added. Samples were centrifuged at 14,000*g* to pellet precipitated protein. The supernatant was transferred into a clean microcentrifuge tube and the following components were added in succession: 400 μl of 200 mM HEPPS-methanesulfonate (pH 8.0), 20 μl of 5 mM diethylenetriaminepentaacetic acid (DTPA), and 20 μl of 3 mM monobromobimane (mBBr, Thiolyte from Calbiochem). Samples were mixed and allowed to react in the dark for 20 min (25°C) followed by addition of 400 μl of 200 mM MSA to reacidify samples prior to storage at −75°C. Samples remained stable under these conditions for up to 3 months.

HPLC analyses were carried out using a Waters NovaPak C_{18} 4-μm Radial-Pak cartridge preceded by a NovaPak Guard-Pak guard column. The column was equilibrated and the samples were resolved by elution with an isocratic mobile phase consisting of 14.2% methanol (v/v) and 2.5% glacial acetic acid (adjusted to pH 3.4) at a flow rate of 1.0 ml/min. After each run the column was washed and reequilibrated for 15 min with a mobile phase consisting of 90% methanol and 2.5% glacial acetic acid. Detection and quantitation of bimane–GSH or bimane–cysteine products was accomplished using a Waters Model 470 scanning fluorescence detector (λ excitation 360 nm; λ emission 455 nm) followed by peak recording and analysis using a Waters Model 746 data module. Identification and quantitation of thiol peaks was accomplished using concentration curves prepared for each thiol using authentic standards. Quantities of GSH as low as 10 pmol can be accurately detected using this method.

Uptake of ^{35}S-labeled amino acids and their incorporation into GSH. Day 10 or 11 rat conceptuses were grown *in vitro* with the addition of DEM for 2 hr after explant as described above. Conceptuses were then removed, washed 3× in 37°C HBSS and placed in small (60 ml) roller bottles containing 5 ml of warmed, gassed culture media. In uptake experiments, total ^{35}S-labeled-amino acid concentrations in the culture media were varied from 0.1 to 1.0 mM. When [^{35}S]cysteine was added at the concentrations indicated in Fig. 2, unlabeled methionine was also added at a final concentration of 0.2 mM. Likewise, when the various concentrations of labeled methionine were evaluated for uptake, unlabeled cysteine was also added at a final concentration of 0.2 mM. ^{35}S-labeled amino acids were purchased from New England Nuclear–Dupont ([^{35}S]cysteine, 1139.9 Ci/mmol; [^{35}S]methionine, 1139.9 Ci/mmol) and diluted in deionized water with the appropriate amounts of cold cysteine or methionine as needed to obtain specific radioactivities of 25,000–53,000 dpm/nmol. After addition of the labeled amino acid, uptake was determined by removal of single whole conceptuses at the appropriate time points, rinsed 5× in cold PBS, transferred into microcentrifuge tubes with watchmaker's forceps, and homogenized by ultrasonic disruption. The homogenized sample was qualitatively transferred to a scintillation minivial containing 6 ml of LSC cocktail (Biosafe II, RPI) and counted for total radioactivity in a Beckman LS6000IC liquid scintillation counter.

Incorporation of labeled amino acid into newly synthesized GSH was determined under the same conditions as described above for amino acid uptake except two whole conceptuses were removed from the media at each time point, rinsed 5× in PBS, placed in microcentrifuge tubes containing 100 μl MSA (200 mM), frozen immediately in liquid nitrogen, and stored at −75°C. Thawed samples were homogenized by ultrasonic disruption and centrifuged for 5 min at 14,000*g* to pellet precipitated protein. The supernatant was derivatized for HPLC analysis as described above. Fractions (0.5 min) were collected from the HPLC effluent and counted for radioactivity, also as described above. GSH synthesis is expressed as the rate of incorporation of labeled amino acids into new GSH of the whole conceptus as determined by calculation of specific radioactivity in the GSH peak.

^{35}S-labeled amino acid incorporation into protein. The pellets remaining from the initial MSA precipitation of conceptuses evaluated for GSH synthesis were washed 3× by resuspension in ice-cold MSA (200 mM) followed by sonication, removal of the supernatant, and resuspension in MSA. After removal of the last supernatant wash, the pellet was resuspended by sonication in 0.25 N NaOH and the sample was allowed to sit for 1 hr at room temperature to solubilize protein. Identical 15-μl aliquots were removed for protein assay using a microplate reader (Bradford, 1976; Harris *et al.*, 1989), and the remaining sample was added in equal aliquots to LSC vials, acidified with 500 μl MSA, and counted for protein-associated [^{35}S]methionine or [^{35}S]cysteine.

Efflux of [^{35}S]GSH into culture media. The rate of GSH efflux from conceptuses into the culture media was determined under the same conditions used to measure optimal rates of GSH synthesis. Culture bottles containing 8–10 Gestational Day 10 conceptuses were sampled at 0,1,2,3, and 5 hr by the removal of 100-μl aliquots of culture medium for analysis of the [^{35}S]GSH released. Accurate measurement of the total efflux of [^{35}S]GSH required that all oxidized GSH in the form of GSSG or mixed disulfides be reduced prior to bimane derivitization for HPLC. Complete reduction of all oxidized GSH forms and maintenance of the pH optima (pH 8.0) for mBBr derivitization was achieved by incubating the media sample in a circulating water bath (37°C) with buffer, dithiothreitol (DTT, a thiol reductant), GSSG-Rd, and an NADPH-generating system (Lou *et al.*, 1987). This enzyme buffer mixture consisted of 25 mM Na phosphate buffer (pH 8.4), 5 mM EDTA, 1 mM DTT, 0.2 mM $NADP^+$, 2.5 mM glucose 6-phosphate, 2 μg/ml glucose-6-phosphate dehydrogenase, and 4 μg/ml GSSG-Rd (Sigma Chemical Co., St. Louis, MO), in a total volume of 200 μl. After incubation, samples were transferred immediately into micro-filtration tubes (Lida, 30,000 MW cutoff) and centrifuged for 25 min at 6000 rpm. The reduced filtrate was immediately derivatized with mBBr as described previously and HPLC was used to determine the specific [^{35}S]GSH content.

RESULTS

Repletion of GSH Pools Following DEM Exposure

Addition of the α,β-unsaturated carbonyl compound, diethyl maleate (DEM, 500 μM) directly to the culture me-

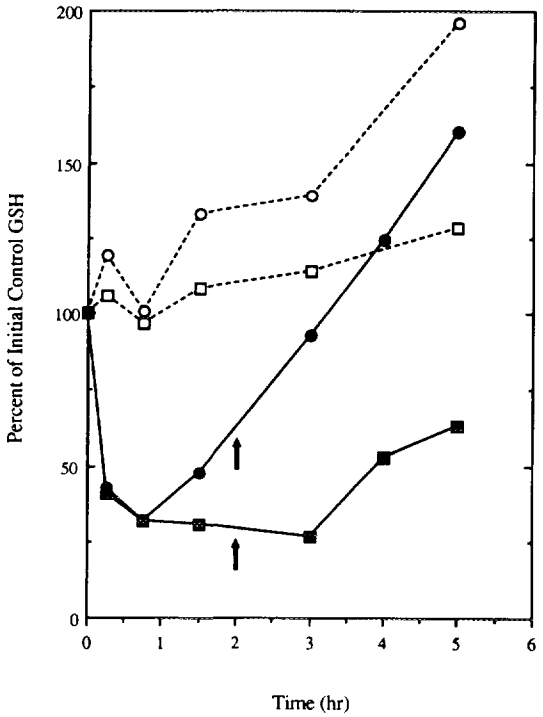

FIG. 1. Glutathione (GSH) depletion by diethyl maleate (DEM) in the embryo and visceral yolk sac (VYS) of the Day 10 rat conceptus grown in whole embryo culture. GSH levels were determined by HPLC analysis as described under Materials and Methods. Initial intracellular GSH concentrations (Time 0) were determined to be 595 ± 73 and 567 ± 45 pmol/embryo and VYS, respectively (mean ± SEM). The arrow at 2 hr indicates the time of sulfur amino acid addition in subsequent experiments. Data is expressed as the percentage of initial control GSH and each data point represents the mean of three or four separate determinations. Points are identified as: ○, control VYS; □, control embryo; ●, DEM (500 μM) VYS; and ■, DEM (500 μM) embryo.

dium resulted in an equally rapid GSH depletion in both embryo and VYS (Fig. 1). This dose of DEM (500 μM) is the highest that could be tolerated without decreasing viability or resulting in visible embryotoxicity. GSH pools in embryo and VYS were reduced to 40% of initial control values by 15 min. Maximum depletion to 28% of initial controls was reached after 45 min of exposure. GSH levels in the VYS began to recover immediately after reaching maximum depletion and continued to increase in a near linear fashion until original control values were exceeded at 4 hr. In the embryo, GSH pools remained depleted to values near 30% of control until 3 hr after DEM exposure and had not returned to original control values by 5 hr. The potential for altering GSH pool replenishment patterns by adding 0.2 mM cysteine and 0.2 mM methionine (2 hr after DEM exposure, as used for the assessment of ^{35}S-labeled amino acid incorporation) was confirmed in a similar manner by HPLC analysis. Patterns of GSH pool replenishment under these conditions were first determined in whole conceptuses in order to assess total GSH synthesis and allow direct comparisons with the ^{35}S-labeled amino acid incorporation studies described below. Net GSH pools in the whole conceptus decreased to 42% of control by 45 min after DEM addition and were replenished at a rate of 273 pmol/conceptus/hr over the next 5 hr. As shown in Table 1, removal of DEM and supplementation of culture medium with cysteine and methionine (2 hr after DEM addition) resulted in a more rapid recovery of embryonic GSH pools than that seen in conceptuses not supplemented with cysteine and methionine and where DEM remained in the medium for the entire time (compare results with Fig. 1). The onset of embryonic recovery was still delayed, however, as shown in Fig. 1. GSH concentrations in DEM-treated embryos (supplemented with cysteine and methionine) were found to slightly exceed original control levels at 4 hr but the recovery was attenuated compared to the additional increases expected in the normal, untreated embryo (Fig. 1). Net intracellular GSH concentrations of 886 ± 65 (mean ± SEM) and 865 ± 45 pmol per embryo and VYS, respectively, were reduced by DEM to 253 ± 49 and 235 ± 38 pmol/embryo or VYS, respectively, within 45 min. Beginning from the point of recovery, GSH pools in the VYS were restored at a rate of 295 pmol/VYS/hr over the subsequent 5 hr. Restoration of embryonic GSH levels was first apparent approximately 2 hr after reaching optimal depletion and increased thereafter at a rate of 118 pmol/embryo/hr over the next 5 hr. In comparison, control conceptuses not treated with DEM synthesized GSH in the VYS at an average rate of 104 pmol/VYS/hr over a 5-hr period while GSH pools in the embryo increased at a slower rate of 49 pmol/embryo/hr (data not shown).

Intracellular cysteine levels, also determined from HPLC data, were found to follow the same general patterns of depletion and repletion as seen for GSH in embryos and VYSs from control and DEM-treated conceptuses (Fig. 1). GSH/cysteine ratios remained relatively constant throughout the period of GSH replenishment and were found to range from 10 to 16 in both embryo and VYS.

^{35}S-Labeled Amino Acid Uptake

A time of 2 hr after DEM exposure was chosen for addition of ^{35}S-labeled cysteine and methionine in subsequent experiments to assess GSH synthesis because GSH was being rapidly replenished in the VYS and overall GSH synthesis in the conceptus was believed to be optimal. When labeled cysteine was added at the various concentrations (0.1–1.0 mM), unlabeled methionine was also added at a final concentration of 0.2 mM. In like manner, when various doses of labeled methionine were added, unlabeled cysteine was also added at a concentration of 0.2 mM. The rate of uptake of each amino acid initially increased in proportion with dose (0.1–1.0 mM) as shown in Fig. 2. Rates of cysteine uptake were highest during the first hour at each

TABLE 1

Net Cysteine and Glutathione (GSH) Contents in Whole Gestational Day 10 Rat Conceptuses, Embryos, and Visceral Yolk Sacs Exposed to Diethyl Maleate (DEM, 500 μM) *in Vitro*

	Glutathione and cysteine content (pmol)					
	Whole conceptus		Embryo		Visceral yolk sac	
Time-treatment	Cysteine	GSH	Cysteine	GSH	Cysteine	GSH
DEM (500 μM) 45 min[b]	564 ± 42[a] 306 ± 69	2868 ± 376 1192 ± 149	44 ± 18 34 ± 10	886 ± 65 253 ± 49	42 ± 29 117 ± 25	865 ± 115 235 ± 38
0 hr[c]	355 ± 66	1552 ± 201	19 ± 5	247 ± 55	48 ± 16	564 ± 51
1 hr	527 ± 68	1476 ± 198	34 ± 6	370 ± 35	54 ± 19	838 ± 76
2 hr	409 ± 63	1943 ± 117	42 ± 4	501 ± 32	99 ± 17	1282 ± 55
3 hr	393 ± 36	2386 ± 151	48 ± 7	756 ± 72	96 ± 30	1490 ± 109
4 hr	405 ± 33	2626 ± 117	86 ± 32	986 ± 200	125 ± 36	1700 ± 199
5 hr	462 ± 63	2812 ± 203	87 ± 32	824 ± 183	115 ± 24	1593 ± 167

[a] Values represent the mean ± SE of at least three separate determinations (HPLC analysis of monobromobimane derivatives).

[b] Point of optimal GSH depletion occurring at ~45 min after DEM addition.

[c] Exposure protocols were the same for all data presented above. The 0-hr time represents the point when conceptuses were removed from the DEM–medium, rinsed, and added to fresh medium containing cysteine (0.2 mM) and methionine (0.2 mM). The removal of DEM and supplementation of amino acids occurred 2 hr after DEM addition.

dose, but rates slowed considerably over subsequent time points. Methionine uptake was similar to that of cysteine at the earliest time point but continued to increase over the entire 5-hr period. Total methionine uptake at the end of 5 hr for the highest dose (1.0 mM) resulted in accumulation in the conceptus at concentrations 2.9 times that of cysteine. Uptake of [^{35}S]cysteine at 0.2 mM under conditions where no prior DEM depletion had occurred resulted in cysteine uptake at rates approximately one-half of the DEM-depleted values (data not shown). Accumulation of free cysteine in tissues of the intact Day 10 conceptus was concurrently determined by HPLC analysis (Fig. 3). Intracellular free cysteine concentrations in the whole conceptus were found to increase rapidly in tissues during the first hour after cysteine addition (0.2 mM; compare Fig. 2). The initial rapid rise in intracellular cysteine reached 82 pmol/conceptus but rapidly decreased to less than 40 pmol/conceptus by 5 hr.

^{35}S-Labeled Amino Acid Incorporation Into Protein

In addition to incorporation into GSH, the fate of net sulfur amino acids taken up from the culture medium was also found to include direct incorporation into protein. Cysteine derived from extracellular sources (i.e., amino acids added to the culture medium) was incorporated into protein and totaled 318 ± 37 pmol for the whole conceptus after 5 hr (Fig. 4). Methionine was incorporated into new proteins at a rate nearly four times that of cysteine, reaching net accumulations in proteins of the conceptus of 1192 ± 202 pmol by 5 hr. The pattern of labeled cysteine incorporation into new protein was similar whether expressed as picomoles per conceptus or picomoles incorporated per milligram conceptal protein.

^{35}S-Labeled Amino Acid Incorporation Into GSH

Rates of new GSH synthesis from precursor amino acids taken up from the culture medium were determined by following the incorporation of labeled sulfur amino acid into GSH. Thiols were resolved by HPLC analysis, fractions collected and the radioactivity specifically incorporated into the GSH peak was used to calculate rates of GSH synthesis from sulfur amino acid added to the medium. Patterns of [^{35}S]cysteine incorporation into GSH of the intact Day 10 conceptus are shown in Fig. 5. In conceptuses previously treated with DEM, cysteine incorporation took place at an optimal rate of 126 pmol/hr/conceptus over the first hour following addition of [^{35}S]cysteine. Net cysteine incorporation into new GSH decreased rapidly after the first hour to rates less than 22.0 pmol/conceptus/hr during the final 2 hr monitored. In conceptuses not treated with DEM, optimal initial rates of incorporation were much lower, on the order of 38 pmol/hr/conceptus and were reduced to 20 pmol/conceptus/hr after 2 hr. Confirmation that an active γ-glutamyl cycle was responsible for the incorporation of [^{35}S]cysteine into new GSH in conceptuses previously treated with DEM was made through addition of the selective γ-glutamylcysteinylsynthetase inhibitor, L-buthionine[*S,R*]sulfoximine (BSO). Simultaneous addition of BSO (1 mM) with [^{35}S]cysteine (Table 2) shows that BSO treatment caused a significant decrease in total con-

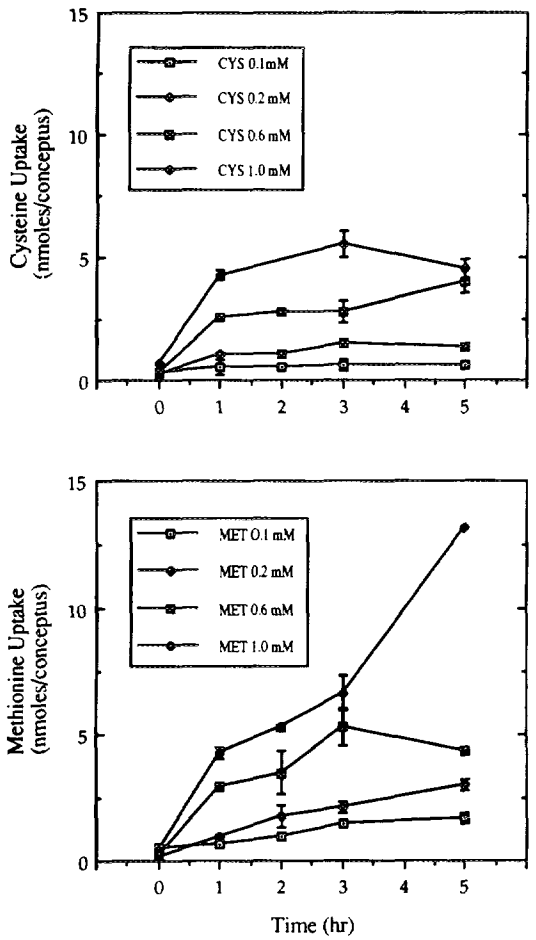

FIG. 2. Uptake of cysteine and methionine by intact Day 10 rat conceptuses in whole embryo culture. Individual conceptuses were removed from culture at the indicated times and sampled for uptake of [^{35}S]cysteine or [^{35}S]methionine as described under Materials and Methods. Conceptuses were treated with DEM (500 μM) for 2 hr prior to the addition of labeled amino acid. Each series of radiolabeled cysteine or methionine concentration was accompanied by the addition of the corresponding unlabeled amino acid (0.2 mM) as described under Materials and Methods. Each point represents the mean of three to five separate determinations and the error bars indicate the SEM.

ceptal GSH in 3 hr, but more importantly, decreased incorporation of labeled cysteine into GSH to 6% of the corresponding control. A large reduction in normal cysteine utilization was also made evident by the 30% increase in free intracellular cysteine seen under these same conditions.

In order to determine label incorporation into total GSH, it was also necessary to account for labeled GSH lost through efflux into the culture medium (including oxidized and reduced forms). No net efflux of [^{35}S]GSH into the culture media was detected during the first 2 hr following addition of labeled cysteine. Thereafter, [^{35}S]GSH was released into the culture media at a rate of 8 pmol/hr/conceptus over the subsequent 3 hr (Fig. 5).

When [^{35}S]methionine was used as a source of sulfur amino acid for GSH synthesis, no incorporation whatsoever was seen into new GSH in Day 10 conceptuses, indicating that the conceptuses are incapable of converting methionine to cysteine via transulfuration at this stage of development. A radioactive chromatographic peak, identified as homocysteine, was consistently found in methionine-treated conceptuses, but represented a small proportion of the total radioactivity in the conceptus (data not shown).

DISCUSSION

Alterations of intracellular GSH status have been clearly shown to affect the embryotoxicity elicited by chemical agents and as a result of exposure of the developing conceptus to environmental extremes (Harris *et al.*, 1987; Slott and Hales, 1987; Wong *et al.*, 1989; and Harris *et al.*, 1988). The ultimate survival and continued normal development of the conceptus may depend on its ability to quickly restore GSH pools lost through turnover and consumption of the tripeptide in detoxication and antioxidant reactions. The purpose of the current study was to describe the capacity of the organogenesis-stage rat conceptus to synthesize GSH and restore intracellular GSH pools *in vitro* using the rat whole embryo culture system. Evaluations of GSH synthesis were designed to demonstrate the net temporal restoration of GSH pools, to determine whether the embryo and VYS behave differently with respect to their respective abili-

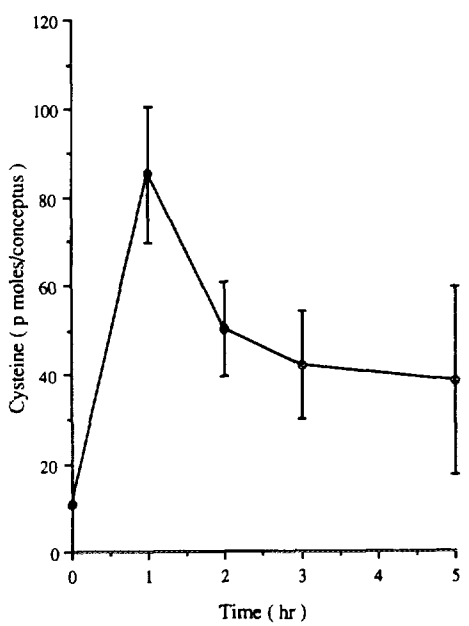

FIG. 3. Intracellular free cysteine concentrations in the Day 10 rat conceptus, determined by HPLC analysis of [^{35}S]cysteine under the conditions described under Materials and Methods. Each point represents the mean of three to five separate determinations and the error bars indicate the SEM.

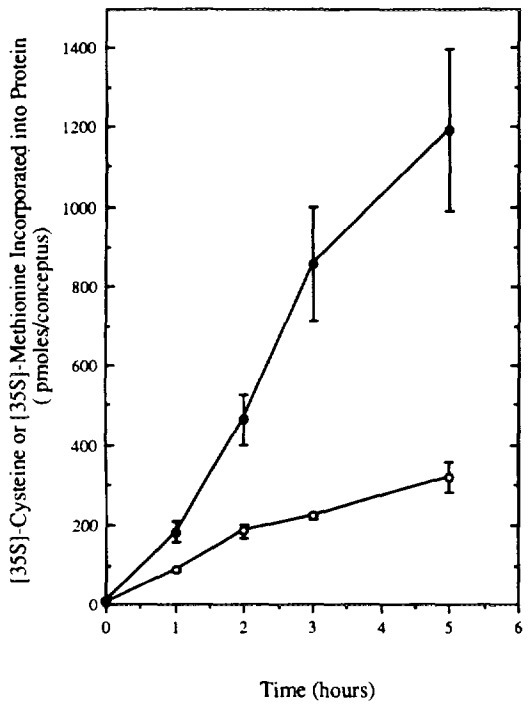

FIG. 4. Incorporation of [^{35}S]cysteine and [^{35}S]methionine into total protein of whole Gestational Day 10 rat conceptuses grown *in vitro*. Data points represent total picomoles of [^{35}S]amino acid incorporated into protein and are shown as the mean ± SEM of three or four independent determinations. ●, methionine; ○, cysteine.

ties to maintain intracellular GSH status, and to provide some clues as to the source of amino acid precursor used in the restoration of GSH pools. The results of this work demonstrate an essential requirement for free intracellular cysteine as the sulfur amino acid precursor required for new GSH synthesis in the developing conceptus. Adult tissues, such as the liver, are able to convert methionine to cysteine via transsulfuration in the cystathionine pathway, obtaining a significant portion of the cysteine needed for GSH synthesis from methionine (Reed and Orrenius, 1977). No evidence of ^{35}S incorporation into GSH was observed at this stage of rat development when [^{35}S]methionine was used as the sulfur amino acid source, even though methionine was found to be taken up by the conceptus at a two- to threefold greater rate than cysteine over the 5-hr period. A higher relative rate of methionine uptake was also reported by Horton *et al.* (1987) using a similar approach to study GSH synthesis in isolated lung cell populations. Absence of a cystathionine pathway in the early rat conceptus is consistent with a report by Gaull *et al.* (1972) where they show that the human fetus, even at a later comparative stage of development, also lacks cystathionase activity. HPLC analysis of conceptuses given [^{35}S]methionine show no incorporation of radioactivity into GSH but do produce a peak of radioactivity that coelutes with an authentic homocysteine standard. Methionine, normally utilized in the formation of *S*-adenosylmethionine for cellular transfer of methyl groups, is likely to be metabolized to homocysteine but not further converted to cysteine via the cystathionine pathway. Our results suggest that reports of improved growth and development of rat conceptuses obtained from supplemental methionine in media prepared from sera of species other than rat do not likely involve a requirement to utilize methionine sulfur in synthesis of GSH (Flynn *et al.*, 1987; Coelho *et al.*, 1989; Coelho and Klein, 1990). Equimolar concentrations (0.2 mM) of unlabeled methionine were included with all [^{35}S]cysteine additions to ensure a consistent turnover of GSH in experiments and to allow for direct comparisons between cultures receiving labeled cysteine or methionine. This precaution was prompted by reports that methionine is able to inhibit efflux of GSH from hepato-

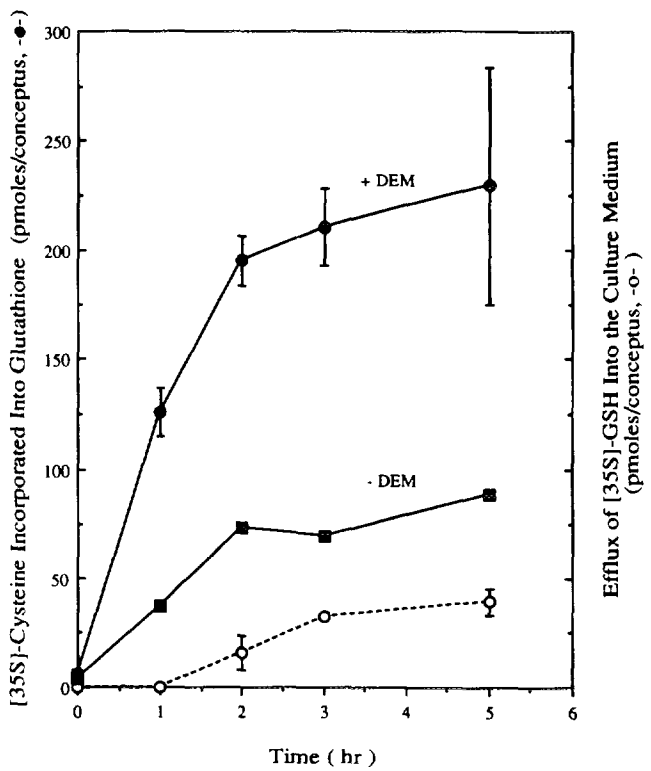

FIG. 5. Intracellular synthesis of GSH and its efflux into the culture media in Day 10 rat conceptuses grown in whole embryo culture. Rates of synthesis were determined by the incorporation of [^{35}S]cysteine into new GSH. Conceptuses were either depleted of GSH with DEM (500 μM) 2 hr prior to the addition of radiolabeled cysteine (0.2 mM) or incubated under the same conditions without DEM. The relative GSH status in the embryo and VYS at the time of cysteine addition (Time 0 in this figure) is indicated in Fig. 1 by an arrow. Recovery and quantitation of new GSH was as described under Materials and Methods. ●, DEM-treated conceptuses; ■, control (no DEM) conceptuses; ○, efflux of GSH into the culture medium. Error bars represent the SEM of three to six separate determinations, except for conceptuses cultured without DEM which are the mean of two separate determinations.

TABLE 2

Effects of Buthionine[*S,R*]sulfoximine (BSO) on Intracellular Glutathione (GSH) and Cysteine Contents and on the Incorporation of [^{35}S]Cysteine into GSH of the Gestational Day 10 Rat Conceptus *in Vitro*

Treatment	GSH (pmol/conceptus)	Cysteine (pmol/conceptus)	[^{35}S]Cysteine incorporated (pmol/conceptus)
Control	3276[a]	309	246[b]
BSO (1 mM)	1125	402	14

[a] Values shown represent the mean of two separate determinations made using whole rat conceptuses treated with DEM and grown in whole embryo culture. GSH and cysteine were determined using HPLC as described under Materials and Methods.

[b] [^{35}S]Cysteine incorporation represents the total amount of labeled amino acid into new GSH in 3 hr.

cytes in the dose range chosen for evaluations of GSH synthesis in this report (Aw *et al.*, 1984).

Exposure of Day 10 rat conceptuses to DEM *in vitro* resulted in the expected depletion of GSH, both in the embryo and VYS. After depletion had reached its maximum, recovery of GSH levels in the VYS commenced immediately and reached levels found in untreated controls by 4 hr. The delayed onset of recovery in the embryo proper and subsequent slow rate of replenishment suggest that the embryo proper may remain susceptible to chemical and environmental insult for a much longer period than the VYS, due to prolonged reduction of the GSH pools. The data presented (Fig. 1 and Table 1) show that during the first hour after addition of [^{35}S]cysteine (arrow in Fig. 1) no recovery is evident in the embryo, indicating that optimal rates of [^{35}S]cysteine incorporation measured in the whole conceptus are due exclusively to synthesis in the VYS. Indeed, initial rates of GSH synthesis in the VYS (263 pmol/VYS/hr, calculated for the interval of 1.25 hr after optimal depletion was reached at 45 min, was nearly identical to increases in GSH seen for the whole conceptus (288 pmol/conceptus/hr) during the same interval. Optimal rates of GSH synthesis determined from [^{35}S]cysteine incorporation studies (126 pmol/conceptus/hr) were found to be considerably slower than comparable rates of GSH replenishment determined for the whole conceptus during a slightly earlier time interval.

Comparison of net rates of GSH synthesis, determined by HPLC analysis of total GSH, with studies using incorporation of [^{35}S]cysteine in the whole conceptus show that the initial rates of [^{35}S]cysteine incorporation account for less than 20% of the total GSH synthesized by the conceptus. This may be expected because further comparisons of total, free intracellular cysteine levels in the whole conceptus with the proportion of amino acid taken up from the culture medium ([^{35}S]cysteine) shows that only 10–20% of the intracellular cysteine pool available for GSH synthesis is obtained from the media via uptake mechanisms. This should not be surprising, since it has been reported that the embryo obtains 86 and 99% of the amino acids serine and leucine, respectively, which are necessary for new protein synthesis, from proteolytic degredation of maternal histiotroph in the VYS (Rowe and Kalaizis, 1985; Beckman *et al.*, 1990). Reed and Orrenius (1977) have, likewise, proposed that a significant proportion (50%) of the amino acid precursor used for GSH synthesis in the liver comes from intracellular proteolytic activity. Using assays selective for the determination of activities for selected cathepsins, we have determined that rates of proteolysis are very low in the embryo proper when compared to those in the VYS and are not likely to provide sufficient free amino acid to independently support new GSH synthesis (Ambroso and Harris, unpublished data). Chemical agents that inhibit or interfere with processes of histiotrophic nutrition may, therefore, attenuate the ability of the conceptus to synthesize GSH due to reductions in amino acid precursor supply.

Determination of [^{35}S]cysteine utilization shows that nearly equal amounts of cysteine were incorporated into new GSH (247 ± 35 pmol/conceptus) as into protein (318 ± 37 pmol/conceptus) during a comparable 5-hr period. This may account for a portion of the apparent dilution of labeled cysteine that is taking place over time. Oxidation and utilization of cysteine in other metabolic and biosynthetic pathways is also likely. Methionine incorporation into protein occurs at higher rates than with cysteine, essentially following the pattern of total conceptal uptake. Intracellular cysteine is normally maintained at low concentrations (30–200 μM) and is often incorporated into GSH as a means of maintaining intracellular cysteine pools in a stable, but readily available form (Griffith, 1987). The similar accumulations of cysteine into protein and GSH pools indicate that under conditions of severe GSH depletion, protein synthesis could also be compromised due to competition with GSH synthesis for the available amino acid precursors.

The onset of GSH recovery in the embryo seen at 2 hr after cysteine addition was correlated directly in time to both the initial observed release of labeled GSH from the conceptus into the culture media and the near complete recovery of GSH pools in the VYS. The inability of the embryo proper to restore depleted GSH until the VYS becomes replete and begins to release newly synthesized GSH, raises several questions about the possible regulation of GSH synthesis in the embryo and the availability of suitable GSH precursors. An absence of GSH replenishment in the embryo immediately following depletion with DEM indicates that the embryo either lacks the inherent ability to synthesize GSH or the VYS is not providing the appropriate supply of necessary precursor. If cells of the embryo

are unable to synthesize GSH at this stage of development, irregardless of adequate precursor availability, direct transport of GSH would be required to maintain intracellular GSH levels. Some tissues, such as the intestinal epithelium and kidney proximal tubules, possess specific GSH transporters which are capable of moving intact GSH into cells, thus bypassing the exclusive need for *de novo* synthesis via the γ-glutamyl pathway (Hagen and Jones, 1987; Lash and Jones, 1983). No evidence is yet available to show that the developing conceptus is capable of direct GSH transport. If GSH replenishment in the embryo required transport of GSH, accumulation should occur at rates directly proportional to the quantity of GSH provided to the embryo from the VYS. Our data on GSH repletion profiles in embryo and VYS are consistent with this possibility. We do, however, consider the hypothesis that multiple differentiating cell types in the embryo are unable to synthesize GSH untenable. Nonetheless, investigations have been initiated to determine whether GSH transporters can be found in the conceptus at this stage of development. The most plausible mode of sulfur amino acid supply to the embryo would be via GSH, followed by glutathionase activity and the incorporation of precursors. The liver of mature animals is able to provide GSH to other organs, such as the kidney, through synthesis, efflux, and transport through the bloodstream (Griffith and Meister, 1979). Embryonic blood levels of GSH have not yet been determined in the organogenesis-stage conceptus so it will require additional investigations to determine whether embryonic intracellular GSH levels are regulated by circulating free cyst(e)ine or by GSH. At slightly later stages of rat development, tissue and circulating levels of cysteine were found to be low in the conceptus due, perhaps, to its known embryotoxicity (Malloy *et al.*, 1983). Once reaching the embryo, active GGT or direct GSH transport would be required to provide the amino acids needed for *de novo* synthesis. Inhibition of GGT with acivicin or anti-GGT have previously been shown to elicit dysmorphogenesis in the rat conceptus *in vitro* (Stark *et al.*, 1987). Inhibition of GGT with acivicin at doses of 5–10 μM resulted in significant reductions in embryonic, but not VYS, GSH levels after 24 hr. These observations favor GSH as a more stable and less reactive transport form and is in agreement with our efflux data showing that the onset of GSH recovery in the embryo began only after GSH release from the conceptus was first observed.

The patterns of differential depletion and repletion of GSH described in this study for the developing rat conceptus suggest that disturbances in the ability of the VYS to provide the developing embryo with an adequate supply of GSH may be very important in determining the overall level of cellular protection in the embryo. We have shown that the VYS requires over 3 hr to replace GSH lost after exposure to DEM and that the embryo proper has failed to completely replenish its depleted GSH pool at times in excess of 4 hr. These data indicate that as a consequence of a single chemical exposure the embryo may remain susceptible to chemical and environmental insults through relatively long, critical periods of embryogenesis. A complete understanding of how intracellular thiol status is maintained and regulated in the conceptus during development and how the homeostasis is altered by chemical and environmental stresses will be important factors in elucidating mechanisms of abnormal development and embryotoxicity.

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