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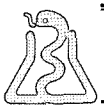

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Establishment of New Method for the Assay of Glutamate-cysteine Ligase Activity in Crude Liver Extracts

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ABSTRACT. As the antioxidant and free radical scavenger, glutathione (GSH) participates in the preservation of cellular redox status and defense against reactive oxygen species and xenobiotics. Glutamate-cysteine ligase (GCL; also known as γ-glutamylcysteine synthetase, EC 6.3.2.2) is the rate-limiting enzyme in GSH synthesis. In the present study, the accurate method for determination of GCL activity in crude liver extracts was developed by measuring both γ-glutamylcysteine and GSH from cysteine in the presence of glutamate, glycine and an ATP-generating system. We added glycine to promote the conversion of γ-glutamylcysteine to GSH, and to minimize the possibility of γ-glutamylcysteine metabolism to cysteine and oxoproline by γ-glutamylcyclotransferase. We established optimal conditions and substrate concentrations for the enzyme assay, and verified that inhibition of GCL by GSH did not interfere with this assay. Therefore, this assay of hepatic GCL under optimal conditions could provide a more accurate measurement of this enzyme activity in the crude liver extracts.

Keywords: Glutamate-cysteine ligase, GSH, Enzyme assay, Liver extracts.

INTRODUCTION

Glutathione (GSH) is the main nonprotein thiol in all mammalian cells and it is present in millimolar concentrations. It plays an important role in protecting cells against toxins and free radicals via GSH *S*-transferases and GSH peroxidases, maintains the cellular redox state via NADPH-dependent GSSG reductase and the thiol-disulfide balance by nonenzymatic or thiol-transferase-catalyzed reduction of disulfides of GSH, and is involved in the transport and storage of cysteine (Meister, 1985). The tripeptide GSH (γ-L-glutamyl-L-cysteinylglycine) is synthesized in the cytosol by sequential action of two ATP-requiring enzymes, glutamate-cysteine ligase (GCL) and GSH synthetase with formation of the intermediate dipeptide, γ-glutamylcysteine. The first step of GSH biosynthesis is rate-limiting, and the activity of GCL is regulated by the availability of cysteine and by feedback inhibition by GSH over cysteine and GSH ranges that are similar to those found *in vivo* (Richman and Meister, 1975; Huang *et al.*, 1993b).

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List of Abbreviations: BCS, bathocuproine disulfonate; DNFB, 1-fluoro-2,4-dinitrobenzene; GCL, glutamate-cysteine ligase; GSH, glutathione

glutamate + cysteine + ATP
$\rightarrow$ γ-glutamylcysteine + ADP + P_i
γ-glutamylcysteine + glycine + ATP $\rightarrow$ GSH + ADP + P_i

To study the regulation of GSH synthesis in the liver, an accurate measurement of GCL activity that could be used to assay crude liver samples is needed. Several approaches have been used to assay GCL activity in crude liver extracts, but none of these has emerged as a widely accepted method. A broad range of activities have been obtained with various assays, underscoring the lack of validity of some of these assays; the reported level of GCL activity in rat liver ranges from about 3 to 100 $nmolmin^{-1}mg\ protein^{-1}$ (Hunter and Grimble, 1997; Lu *et al.*, 1991; White *et al.*, 1987; Yan and Huxtable, 1995), but the validity and suitability of various assays for analysis of crude tissue samples have not been stringently established.

Some investigators measured only γ-glutamylcysteine or only GSH as the reaction product (Borroz *et al.*,

1994; Lu *et al.*, 1991; Yan and Huxtable, 1995), but we noticed that both products accumulated when liver extracts were incubated with cysteine and glutamate, due to the presence of glycine and GSH synthetase in crude liver extracts. Hence, measurement of only one product would underestimate GCL activity. Another approach has been to measure ADP or P_i production in the GCL reaction (Hunter and Grimble, 1997; Seelig and Meister, 1985; White *et al.*, 1987), but further metabolism by GSH synthetase also produces ADP and P_i and the presence of other ADP- or P_i-forming systems, particularly ATPases, would likely result in the overestimation of GCL activity. Several investigators have used an alternative substrate α-aminobutyrate instead of cysteine to avoid potential concerns of cysteine oxidation to cystine, which is not a substrate of GCL (Hunter and Grimble, 1997; Seelig and Meister, 1985; Shi *et al.*, 1994a,b; White *et al.*, 1987). Although GCL utilizes L-cysteine and L-α-aminobutyrate about equally well as a substrate (Orlowski and Meister, 1971), this does not allow the study of the interaction of the physiological substrate with the enzyme. Also either γ-glutamylcysteine or γ-glutamyl-α-aminobutyrate can be metabolized by GSH synthetase or γ-glutamylcyclotransferase (Meister, 1989), resulting in the loss of γ-glutamylcysteine or γ-glutamyl-α-aminobutyrate and the underestimation of GCL activity. Another concern with the assay of crude tissue samples is that endogenous tissue GSH concentrations may be high enough to feedback inhibit GCL.

In the present study, we developed and assayed GCL activity in crude liver extracts by measuring both γ-glutamylcysteine and GSH in a reaction mixture that contains cysteine, glutamate, glycine and an ATP-generating system. We added glycine to promote the conversion of γ-glutamylcysteine to GSH, and to minimize the possibility of γ-glutamyulcysteine metabolism to cysteine and oxoproline by γ-glutamylcyclotransferase. We also examined the effects of GSH concentration in crude liver extracts on GCL activity, and established optimal assay conditions and substrate concentrations for the assay.

MATERIALS AND METHODS

Materials

L-Cysteine, L-glutamate, glycine, GSH, γ-glutamylcysteine, γ-L-glutamylglutamate, 1-fluoro-2,4-dinitrobenzene (DNFB), sodium salt of bathocuproine disulfonate (BCS), iodoacetate, bovine serum albumin, sodium salt of bathophenanthroline disulfonate (BPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). All other reagents were of analytical grade and were obtained from commercial sources.

L-[^{35}S]Cysteine was purchased from Amersham (Product #SJ141, Arlington Heights, IL, USA) 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. The eluate was aliquoted into portions (50 or 100 μCi/tube), lyophilized and stored at -70°C until used.

Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) were housed in stainless steel mesh cages in a room maintained at 20°C and 60~70% humidity with light from 08:00 to 20:00 hr. Rats were given ad libitum access to water and a nonpurified diet (Prolab RMH 1000, Agway, Syracuse, NY, USA). Rats weighed approximately 250~300 g when they were used to obtain liver. The care and use of animals was approved by the Cornell University Institutional Animal Care and Use Committee.

Procedure for assay of GCL activity

Minced liver was homogenized in ice-cold 50 mM Epps [N-(2-hydroxyethyl)piperazine-N'(3-propanesulfonic acid)] buffer (pH 8.5) to prepare homogenate that contained 100 or 200 g (wet weight) of liver per liter. The activity of GCL was measured by incubating ~0.07 or 0.14 g (wet weight) of liver homogenate (0.7 ml) with 1 mM L-cysteine, 20 mM L-glutamate, 10 mM glycine, 0.05 mM BCS, 5 mM $MgSO_4$, 50 mM KCl, 10 mM ATP, 10 mM phosphocreatine, 37.5 units creatine phosphokinase, and 100 mM Epps buffer (pH 8.5) in a final volume of 2.5 ml for 15 min (0.14 g homogenate) or 30 min (0.07 g homogenate) at 37°C except as indicated in the figures. Reactions were started by the addition of cysteine and the final pH of the complete incubation mixture was 8.1. to terminate the reaction, 0.5 ml of reaction mixture was transferred to a tube containing 0.2 ml of 6.2 M perchloric acid, 1.25 mM γ-L-glutamylglutamate (internal standard for HPLC), 3.75 mM BCS, and 3.75 mM BPS. The tube was centrifuged at 2000 ×g for 10 min, and 0.5 ml of acid supernatant was removed and stored at -20°C until HPLC analysis of reaction products. Zero-time incubations were used as blanks.

For determination of the inhibition of GCL activity by GSH, L-[^{35}S]cysteine was used as substrate, and different concentrations of GSH were added to the incubation mixture. Use of radiolabeled substrate and detection of radiolabeled products increased the sensitivity of the assay procedure so that the lower inhibited levels of

GCL activity could be quantitated.

Measurement of GSH and γ-glutamylcysteine

The measurement of the products, γ-glutamylcysteine and GSH, was based on anion-exchange HPLC of dinitrophenyl derivatives using the method of Farris and Reed (1987) as modified by Stipanuk *et al.* (1992b) using a 22 cm × 4.6 mm 3-aminopropyl column (Spheri-5 Amino, 5-mm particles; Brownlee Labs, Santa Clara, CA, USA). A binary gradient was used for the mobile phase. Eluant A was 80% (v/v) methanol and eluant B was 0.5 M sodium acetate in 64% (v/v) methanol. Eluants were filtered and degassed under vacuum prior to use.

Acid supernatant fractions from the stopped reaction mixture were neutralized, treated with dithiothreitol to reduce disulfides, reacted with iodoacetate to block free thiol groups, and derivatized with DNFB. Standard solutions were treated similarly. DNFB reacts with primary amines to form relatively stable products (2,4-dinitrophenyl *S*-carboxymethyl derivatives) that absorb light at 350 nm. Aliquots (100 μl) of derivatized samples or standards were injected onto the HPLC column. Following an injection of sample, the mobile phase was maintained at 91% eluant A/9% eluant B (v/v) for 5 min. Over the next 18 min, eluant was linearly changed to 67% eluant A/33% eluant B. Then, over the next 1 min, eluant was linearly changed to 50% eluant A/50% eluant B and the column was washed for 5 min at a flow rate of 1.3 ml/min. Finally, the column was brought back to initial conditions (91% eluant A) over the next 1 min. The total run time including reequilibration of the column was 37 min and the flow rate was 1.2 ml/min except as indicated.

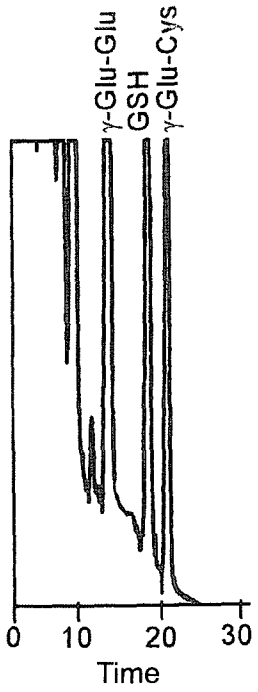

Fig. 1. Anion exchange HPLC of GSH and γ-glutamylcysteine (γ-Glu-Cys) produced during a GCL assay of liver homogenate. Acid supernatant fractions from the stopped reaction mixture were neutralized, treated with dithiothreitol to reduce disulfides, reacted with iodoacetate to block free thiol groups, and derivatized with DNFB before they were injected onto the HPLC column. The S-carboxymethyl derivatives of GSH and γ-glutamylcysteine were detected by their absorbance at 350 nm. γ-Glutamylglutamate (γ-Glu-Glu) was added as an internal standard.

A sample chromatogram for analysis of GSH and γ-glutamylcysteine produced during a GCL assay of liver homogenate is shown in Fig. 1. The elution times for the 2,4-dinitrophenyl *S*-carboxymethyl derivatives of GSH and γ-glutamylcysteine were approximately 17.5 and 20 min, respectively. Standard curves were generated by linear regression of the peak area and the concentration of GSH or γ-glutamylcysteine in the standard solutions. The values for the samples were obtained using the regression equation.

When [^{35}S]cysteine was used as the substrate, the eluate was collected in fractions encompassing the GSH and the γ-glutamylcysteine peaks for measurement of [^{35}S]GSH and γ-glutamyl[^{35}S]cysteine production. Then, Ecoscint liquid scintillation cocktail (National Diagnostics, Atlanta, GA, USA) was added, and radioactivity was measured by liquid scintillation spectrometry. The molar quantity of GSH and γ-glutamylcysteine produced was calculated by dividing the amount of [^{35}S]GSH and γ-glutamyl[^{35}S]cysteine produced by the specific activity of L-[^{35}S]cysteine used as substrate.

Enzyme activity was expressed as nmol of GSH plus γ-glutamylcysteine formed per minute per mg protein. Protein was determined by a modification of the Lowry method with bovine serum albumin as the standard (Markwell *et al.*, 1978).

Kinetic data were fitted to the Michaelis-Menten equation using the Leonora program (Oxford University Press, Oxford, UK) to obtain the apparent K_m and K_i values.

RESULTS

Standard Curves of GSH and γ-glutamylcysteine

As shown in Fig. 2, standard curves of 2,4-dinitrophenyl S-carboxymethyl derivatives of GSH and γ-glutamylcysteine were linear in the range 0~500 μM (concentrations in incubation mixture) and $r^2 \geq 0.99$ for the lin-

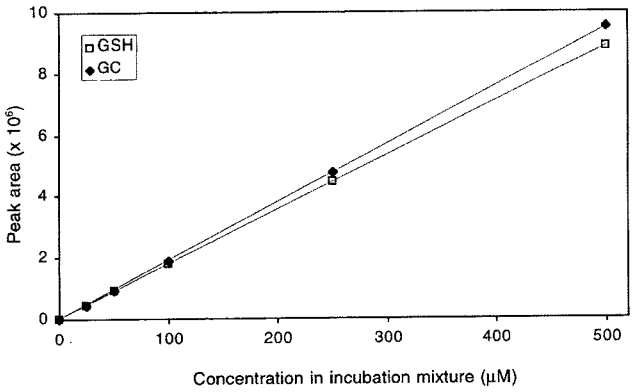

Fig. 2. Standard curves of GSH and γ-glutamylcysteine (GC).

ear regression. This range corresponded to 0~2.7 nmol/100 μl injection volume. The peak areas of the GSH and γ-glutamylcysteine standards were equal up to 100 μM, which was the upper limit of concentrations of γ-glutamylcysteine in the samples. Therefore, a standard curve for GSH can be routinely used for quantification of the concentrations of both GSH and γ-glutamylcysteine.

Linearity of GCL assay with enzyme concentration and time

The GCL assay was routinely run with enzyme × time duplicates to check on linearity with both enzyme concentration and time. In the standard assay, duplicates consisted of the incubation mixture containing 0.7 ml of 10% (w/w) liver homogenate (~5.6 mg liver protein/ml incubation mixture) that was incubated for 30 min or of the incubation mixture containing 0.7 ml of 20% homogenate (~11.2 mg liver protein/ml incubation mixture) that was incubated for 15 min. As shown in Fig. 3, the enzyme assay was linear with respect to enzyme concentration up to 11.2 mg liver protein/mL incubation mixture (Fig. 3A) and was linear with respect to incubation time for at least 45 min (Fig. 3B) with $r^2 \geq 0.99$ for the relationship of GSH plus γ-glutamylcysteine with both enzyme concentration and incubation time.

When the proportions of the two products, GSH and γ-glutamylcysteine, are considered, the proportion of GSH to γ-glutamylcysteine was increased by time and by tissue (crude enzyme) concentration (Table 1). This suggests that γ-glutamylcysteine concentration in the incubation mixture limits the rate of GSH synthesis.

Effects of pH and substrate concentrations on GCL activity

The pH optimum for GCL has been reported to be in the range of 8.3~8.8 (Mooz and Meister, 1971) for the hog liver enzyme and in the range of 8.0~8.4 for the rat kidney enzyme (Seelig and Meister, 1985). The maximum activity was found at pH 8.1 for the incubation mixture with a broad pH optimum (data not shown).

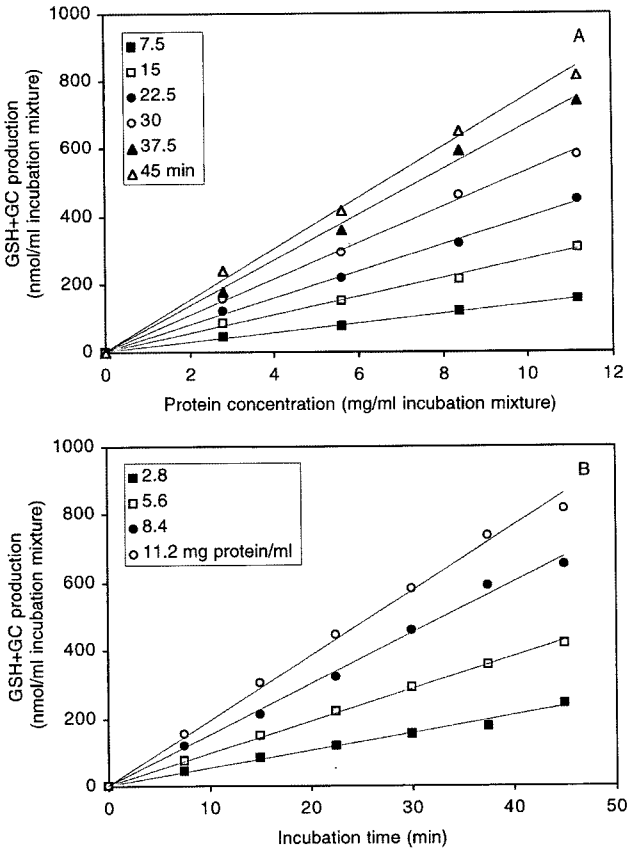

Fig. 3. GSH and γ-glutamylcysteine (GC) production with various concentrations of liver homogenate (A) or with various incubation times (B). Liver homogenates, containing 2.8, 5.6, 8.4 or 11.2 mg protein/ml incubation mixture, were incubated for 7.5, 15, 22.5, 30, 37.5 or 45 min for measurement of GCL products (GSH + γ-glutamylcysteine) under standard assay condition. Results are expressed as means for three experiments.

Table 1. The ratio of GSH to γ-glutamylcysteine production (GSH/γ-glutamylcysteine) with various concentrations of liver homogenate or with various incubation times

Incubation time (min)	Protein concentration (nmol·min^{-1}·mg protein^{-1})			
	2.8	5.6	8.4	11.2
7.5	0.52 ± 0.08	0.91 ± 0.04	0.93 ± 0.09	1.09 ± 0.06
15	0.74 ± 0.12	1.20 ± 0.14	1.29 ± 0.10	1.04 ± 0.06
22.5	0.94 ± 0.11	1.30 ± 0.07	1.46 ± 0.07	1.55 ± 0.16
30	0.99 ± 0.16	1.30 ± 0.05	1.39 ± 0.11	1.65 ± 0.17
37.5	1.18 ± 0.15	1.37 ± 0.09	1.51 ± 0.09	1.61 ± 0.03
45	1.05 ± 0.22	1.38 ± 0.15	1.61 ± 0.16	1.82 ± 0.08

Each value represents as means ± SEM for three experiments.

Table 2. The activity of GCS with various concentrations of cysteine, glycine, or glutamate in the incubation mixture

Concentration (mM)	GCS activity (nmol·min^{-1}·mg protein^{-1})		
	Cysteine	Glycine	Glutamate
0.5	1.63 ± 0.08	-	-
1	1.72 ± 0.09	-	-
2.5	1.57 ± 0.10	1.57 ± 0.02	1.22 ± 0.07
5	1.26 ± 0.10	1.50 ± 0.05	1.18 ± 0.12
10	1.11 ± 0.02	1.57 ± 0.10	1.45 ± 0.08
20	0.66 ± 0.01	1.56 ± 0.03	1.57 ± 0.10
50	-	1.63 ± 0.06	1.23 ± 0.18

Each value represents as means ± SEM for three experiments.

As shown in Table 2, maximal GCL activity occurred with substrate concentrations of between 0.5 and 2.5 mM cysteine and between 10 and 20 mM glutamate. The activity of GCL was maintained over various concentrations of glycine in the range between 2.5 and 50 mM. The proportion of GSH to γ-glutamylcysteine produced during the assay was about 1.2~1.3 and was not changed by the concentration of glycine, suggesting that 2.5 mM or less glycine is enough to saturate GSH synthetase and to permit further metabolism of γ-glutamylcysteine to GSH in the incubation mixture.

We observed a notable inhibition of GCL by cysteine when it was present at concentrations of 5 mM or higher (Table 2). To further assess the effect of cysteine concentration on the activity of GCL, the enzyme kinetics with respect to cysteine concentration were determined at saturating concentrations of other substrates (20 mM glutamate, 10 mM glycine). The apparent K_m of GCL for cysteine was determined by analyzing the initial velocity of the reaction with different concentrations of cysteine up to 1 mM (Fig. 4). By fitting the data to the Michaelis-Menten equation, an apparent K_m of 0.10 mM for cysteine was obtained.

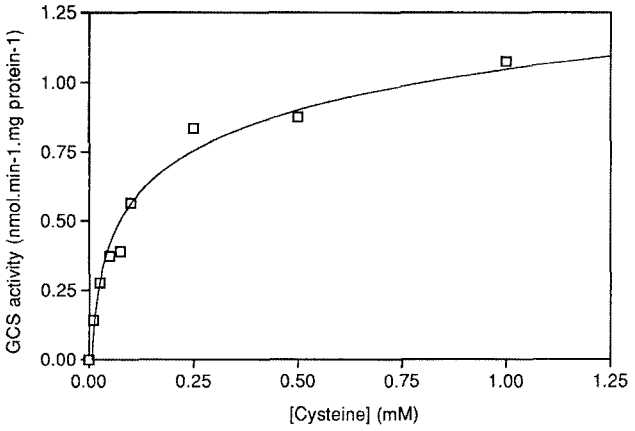

Fig. 4. The effect of cysteine concentration on the activity of GCL. The activity of GCL was measured in rat liver homogenates using the standard assay conditions except the concentration of cysteine was varied from 0.01 to 1 mM. Results are expressed as means for two experiments.

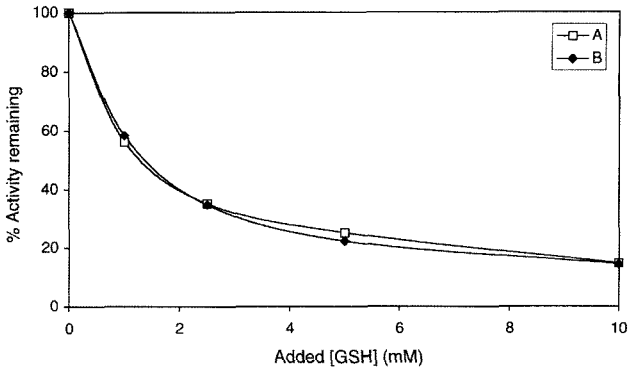

Fig. 5. The inhibition of GCL activity by various concentrations of GSH. **A** (□) 1~10 mM GSH was added directly into the standard assay mixture and the activity of GCL was determined. **B** (◆) 10 mM GSH was added directly to the liver homogenate and the concentration of GSH was diluted to 1 mM without any dilution of homogenate by addition of homogenate without GSH. The activity of GCL was determined under standard assay condition. Results are expressed as means for two experiments.

Inhibition of GCL activity by GSH

To examine the feedback inhibition of hepatic GCL by GSH, liver homogenate was incubated in a standard assay mixture in the presence of additional GSH at concentrations up to 10 mM. The activity of GCL was inhibited by additional GSH at concentrations of 1 mM, suggesting that the GSH concentration contributed by use of liver homogenate (crude enzyme) in the assay mixture (~0.05-0.3 mM) would not inhibit GCL substantially and hence would not interfere with the assay of GCL activity in crude liver tissues (Fig. 5: Curve A). With 10 mM added GSH, GCL exhibited ~15% of control activity, *i.e.* ~85% inhibition, which is similar to that observed by Eaton and Hamel (1994) for hepatic GCL. The apparent K_i for GSH was ~1.4-1.7 mM, which is much lower than the reported value (8.2 mM) for GSH inhibition of the isolated kidney GCL holoenzyme (Huang *et al.*, 1993b).

The inhibition of GCL by GSH involves the binding of GSH to the glutamate site of GCL (Richman and Meister, 1975). It is possible that dilution of tissue homogenate (prepared with liver with a relatively high GSH concentration) may not eliminate the inhibition of GCL if a high affinity form of the GCL-GSH complex exists. To test this possibility, 10 mM GSH was added directly to liver homogenate, allowed to sit on ice for 10~15 min,

and then the concentration of GSH in the homogenate was diluted to 1 mM by addition of homogenate that did not contain added GSH. As shown in Fig. 5, the curve for inhibition of GCL by GSH using this second approach (Curve B) was almost identical with Curve A, indicating that the GSH-GCL enzyme complex has a rapid off-time compared to the incubation time for the enzyme assay. Thus, the low concentration of GSH added to the assay mixture along with the tissue preparation should not inhibit the activity of GCL at V_{max} conditions. Using our standard assay conditions, the concentration of GSH in the incubation mixture would be only 2.8 or 5.6% of the concentration present in liver itself due to dilution in homogenate and in the incubation mixture.

DISCUSSION

The product of GCL, γ-glutamylcysteine, can be metabolized either to GSH by GSH synthetase or to 5-oxo-L-proline and cysteine by γ-glutamylcyclotransferase in the liver. Therefore, in crude tissue samples, it may be necessary to measure more than one product for determination of GCL activity. Glycine, which is a required substrate for GSH synthetase, is abundant in crude tissue extracts, and it has been shown that some type of coordination or linkage may exist between the two GSH-synthesizing enzymes (Meister, 1989), suggesting that γ-glutamylcysteine may be more readily available to GSH synthetase rather than to γ-glutamylcyclotransferase *in vivo*.

We included 10 mM glycine in the incubation mixture to allow maximal metabolism of γ-glutamylcysteine to GSH, but a glycine concentration of 2.5 mM or somewhat less was sufficient to yield maximal rates of further metabolism of γ-glutamylcysteine to GSH. This resulted in an approximately 1.2~1.3 : 1 distribution of product between GSH and γ-glutamylcysteine. Under standard assay conditions, synthesis of GSH was probably limited by γ-glutamylcysteine concentration and/or GSH synthetase activity. Regardless, it is clear that not all γ-glutamylcysteine is converted to GSH and that both products must be measured for assay of GCL activity in crude tissue samples. However, in tissues with a high activity of γ-glutamyltranspeptidase such as kidney, lung and intestine, GSH could be degraded into γ-glutamyl amino acid and cysteinylglycine by this enzyme. Therefore, an inhibitor of γ-glutamyltranspeptidase may be required if the assay were applied to kidney, lung or intestine samples.

The DNFB-derivatization and HPLC method employed in this study is efficient for separation and subsequent measurement of GSH and γ-glutamylcysteine without prior separation from substrates. Also the 2,4-dinitrophenyl S-carboxymethyl derivatives are stable for about three weeks in the freezer, which makes the assay convenient to use. The limit of detection of GSH or γ-glutamylcysteine in this assay was about 10 pmol, which is close to the reported values of fluorometric detection; 6.25 pmol for monobromobimane (mBBr) derivatives and 12.50 pmol for *O*-phthalaldehyde (OPA) derivatives (Yan and Huxtable, 1995).

Although this method using DNFB is widely used for determination of GSH derivatives, mBBr or OPA could be used as alternative derivatization compounds to analyze both GSH and γ-glutamylcysteine using HPLC. However, mBBr is relatively expensive compared to DNFB and OPA. ^{35}S-labeled cysteine also could be used in cases where greater specificity and sensitivity are needed.

We observed an inhibition of GCL activity by L-cysteine, one of its substrates. Richman and Meister (1975) did not report inhibition of renal GCL by cysteine in their studies using up to 16 mM cysteine. We suspect that they did not observe this inhibition and also obtained a relatively high apparent K_m (0.35 mM) for cysteine due to autooxidation of cysteine to cystine in their incubation mixture such that the true cysteine concentration was lower than reported. However, it is also possible that kidney GCL has a higher apparent K_m for cysteine and it is not inhibited by cysteine at concentrations > 10 mM. A more recent study from the same laboratory reported an apparent K_m for cysteine in isolated renal GCL holoenzyme to be 0.2 mM (Huang *et al.*, 1993b). Lu *et al.* (1992) reported the apparent K_m for cysteine in cell extracts of cultured hepatocytes to be 0.08 mM, and they included dithiothreitol in their GCL assay mixture to reduce disulfides to thiols. In our assay, we added BCS, a copper-specific chelator, to prevent oxidation of cysteine to cystine (Coloso *et al.*, 1990), and estimated the apparent K_m of GCL for cysteine to be 0.10 mM.

The concentration of cysteine in the GCL assay mixtures used in several published methods (> 5 mM) is high relative to the concentration we found to be inhibitory (Ochi, 1996). Although the concentration of cysteine that shows a significant inhibition of GCL is above the physiological range and possibly not a significant factor *in vivo*, using a high concentration of cysteine as a substrate for *in vitro* assay of GCL activity can result in a significantly decreased value for GCL activity.

A concern about assay of GCL activity using crude liver extracts is that GSH, which is present in liver extracts and is synthesized during the assay itself,

could inhibit the activity of GCL. However, in our standard assay, the tissue GSH level (2~6 mM) was diluted 1 : 18 or 1 : 36 (weight to volume) to yield GSH concentrations (~0.05-0.3 mM) too low to cause a significant inhibition of GCL. The total concentration of GSH at the end of the standard assay period had increased to ~0.2-0.5 mM, which was below but close to the concentration observed to produce inhibition of GCL.

In addition, the formation of products was proportional to the amount of enzyme and to time of incubation during the first 45 min of reaction, suggesting that there is no possible inhibition of GCL by the initial or produced GSH in the incubation mixture. And in enzyme × time duplicates, a higher enzyme concentration duplicate that has twice as much as high GSH concentration had the same enzyme activity as a lower enzyme concentration duplicate. And the interaction of GSH with GCL seems rapid and readily reversible and inhibition was not observed under our standard assay conditions.

In summary, the developed method for the assay of GCL activity in crude liver extracts measures both GC and GSH as products under conditions in which inhibition of GCL by the initial or produced GSH in the incubation mixture was not observed. This method was found to be sensitive and simple, and has been employed successfully in measuring the activity of GCL in crude liver extracts from rats. Therefore, this assay of hepatic GCL under optimal conditions could provide a more accurate measurement of this enzyme activity in the crude liver extracts.

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