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MODULATION OF CYSTEINE METABOLISM IN MICE— EFFECTS OF PROPARGYLGLYCINE AND L-CYST(E)INE-DEGRADING ENZYMES*

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Abstract—In an attempt at evaluating the therapeutic effects of cysteine depletion on the growth of cysteine-dependent L1210 leukemia, the effects *in vivo* of a cysteine- and cystine-degrading enzyme as well as the combined use of an inhibitor of cysteine biosynthesis with cystine-free diets, were measured in mice. The kinetic properties of rat liver γ-cystathionase (EC 4.2.1.13) and *Enterobacter cloacae* cysteine desulfhydrase (EC 4.4.1.1) toward the catabolism of cystine and cysteine, as well as the inhibitory properties of some possible physiological components, are described. A novel and sensitive assay for the detection of cysteine in biological fluids was used to measure the ability of these enzymes to deplete plasma cysteine levels when injected into normal mice. Cysteine desulfhydrase caused no alteration in mouse plasma cysteine concentrations, presumably due to rapid clearance ($T_{1/2}$ less than 10 min) of the enzyme. γ-Cystathionase caused a 60 per cent drop in plasma cysteine concentrations which returned to normal with the clearance of the enzyme ($T_{1/2}$ = 2 hr). The properties and limitations of these enzymes are discussed within the context of their ability to deplete plasma cys(e)ine *in vivo*. When propargylglycine, an effective covalent inhibitor of γ-cystathionase, both *in vitro* (K_i = 0.1 mM, saturating $T_{1/2}$ = 0.5 min) and *in vivo*, was combined with diets lacking cystine, no reduction in plasma cysteine or increase in survival of mice bearing L1210 leukemia could be observed. The compound would induce a cystathioninemia and a cystathioninuria when given to mice and was not toxic in cell culture or in animals when tested with an adequate source of cystine, but was highly toxic in the absence of cystine.

Nutritional and enzymatic studies at the Sidney Farber Cancer Institute have suggested that alterations in cysteine metabolism seem to be associated with lymphoid oncogenesis [1–4]. Lymphoid cell lines of malignant origin derived from rodents or humans will not grow in cell culture media which lack cystine but contain its precursor cystathionine, whereas lymphoid cell lines derived from normal donors will proliferate under such conditions. This difference in growth requirements has been correlated with a decreased activity of γ-cystathionase (the last enzyme in cysteine biosynthesis) in the malignant cell lines [2–4]. The activity of cystathionase in thymocytes *in vivo* decreased with the development of thymic tumors induced by Moloney type C virus [3]. Ohnuma *et al.* [5] have also confirmed the cystine requirement for cells in short-term culture derived from patients with acute lymphocytic and myelocytic leukemia. Of six hepatomas of varying growth characteristics, all showed reduced γ-cystathionase activity as compared to control livers irrespective of the protein content of their diet [6]. Previous to these reports, Weisberger *et al.* [7–9] had demonstrated that the selenium analog of cystine, selenocystine, would inhibit the influx of cystine into leukemic cells. Administration of this analog showed an antileukemic response in man as well as rodents.

Due to the apparent demand for preformed cyst(e)ine by malignant lymphoid cells, we have investigated various ways to create plasma cyst(e)ine depletion in mice. This report examines the combined use of propargylglycine, an effective inhibitor of cysteine biosynthesis, and cystine-free diets. In addition, the effects of the injection of a large amount of purified cyst(e)ine-degrading enzyme were also determined, this approach being analogous to the treatment of asparagine-dependent leukemic cells with the enzyme L-asparaginase [10]. An earlier attempt by Bergel [11] at enzymatic cysteine depletion failed to produce an enzyme of suitable activity. This report also examines the substrate and inhibitory properties of various amino acids toward the cystine-degrading enzyme γ-cystathionase (EC 4.2.1.13) and toward cysteine desulfhydrase (EC 4.4.1.1). In addition, the effects of the injection of a large amount of these purified enzymes on mouse plasma cysteine levels were determined. The ability of these enzymes to inhibit growth of leukemic cells in tissue culture has been previously reported in preliminary form by this laboratory [12]. The limitations of these approaches and enzymes for therapy *in vivo* are discussed.

In addition to oncogenesis, aberrations in cysteine metabolism are associated with a number of genetic disorders, usually with some form of mental or physical impairment [13]. Diagnostically, these conditions may result in homocystinuria, cystathioninuria, or cystinosis. This report describes the ability of low levels of propargylglycine to cause a cystathionin-

* All amino acids are of the L-configuration unless otherwise stated.

uria in mice. If these animals were fed a diet with sufficient cystine, no manifestations of toxicity were evident. A long-term study of these conditions may serve as an animal model for cystathioninuria.

MATERIALS AND METHODS

Chemicals. L(+)Cystathionine, β-cyano-L-alanine, L-homoserine, and β-nicotinamide adenine dinucleotide (reduced form) were purchased from Sigma Chemical Co., St. Louis, MO. 5,5-Dithiobis-2-nitrobenzoic acid was obtained from Nutritional Biochemical Corp., Cleveland, OH. D,L-Propargylglycine was synthesized by the methods of Gershon *et al.* [14] and D,L-2,7-diaminooct-4-ynedioic acid by the method of Schlögl [15]. [U-^{14}C]glyoxylic acid, Na^+ salt (5.8 mCi/m-mole) and L-[U-^{14}C] homoserine (39 mCi/m-mole) were obtained from the Amersham/Searle Corp., Arlington Heights, IL. [1-^{14}C]iodoacetic acid (13.3 mCi/m-mole) and tritiated water (0.1 and 5 Ci/g) were purchased from New England Nuclear Corp., Boston, MA. Tritiated propargylglycine was prepared by precipitating 50 mg propargylglycine dissolved in 1 ml water with 75 mg $AgNO_3$. The washed and dried silver acetylide was decomposed in 0.05 ml of tritiated water (0.1 or 5 Ci/g) by the addition of 0.05 ml of concentrated HCl. After 15 min, 1 ml H_2O was added and the AgCl was removed by centrifugation. The supernatant solution was dried and redissolved in water three times. The products obtained with different batches of tritiated H_2O demonstrated specific activities of 0.04 and 1.2 mCi/m-mole respectively. The radioactivity comigrated with the ninhydrin reactive material when chromatographed on cellulose thin-layer plates (*n*-butanol–acetic acid–water, 10:7:3). If during the preparation procedure, D_2O replaced the tritiated water, the nuclear magnetic resonance (n.m.r.) spectrum of the deuterated product showed a loss of the acetylenic proton.

Enzymes. γ-Cystathionase was isolated from rat liver by a modification of the procedure described by Greenberg [16]. The procedure consisted of centrifuging the blended rat liver at 23,000 *g* and precipitating the protein from the supernatant with ammonium sulfate between 45 and 70% saturation. The precipitate was dissolved and reprecipitated with 40% ethyl alcohol at −5°. The dissolved precipitate was first chromatographed on Ultrogel ACA 34 and then on Whatman CM32 cellulose. The enzyme preparation showed a single band by sodium dodecylsulfate–polyacetylamide gel electrophoresis with the methods of Weber and Osborn [17]. The yields and purification achieved with this procedure are depicted in Table 1.

Cysteine desulfhydrase was isolated from *Enterobacter cloacae*. The organism was grown on minimal salts media (Difco 0817-01-2) to which 0.1 g/liter of cystine was added to induce the enzyme activity. The cells were ruptured with a French pressure cell, and the 40,000 *g* supernatant was precipitated with ethyl alcohol between 55 and 75 per cent at −5°. The precipitate was dissolved and chromatographed on Whatman DE52 cellulose. The active fractions were pooled and precipitated with ammonium sulfate at 45% saturation. The precipitate was dissolved and rechromatographed on Whatman DE 52 cellulose. The yields and purification achieved with this procedure are shown in Table 2. Lactic dehydrogenase isolated from beef heart was purchased from Sigma Chemical Co., St. Louis, MO.

Diets. The normal diet consisted of Purina Mouse Chow, obtained from Ralston Purina Co., St. Louis, MO. Cystine-free diets were a Rogers–Harper amino acid-defined diet (pelleted form) from which cystine

Table 1. Purification of rat liver γ-cystathionase

Sample	Volume (ml)	Total activity (μmoles/min)	Total protein* (mg)	Sp. act. (μmoles/min/mg)	Yield (%)	Purity
23,000 *g* Fraction†	600	192	18,000	0.0107	100	1
45–70% $(NH_4)_2SO_4$	48	181	1,262	0.143	94	13.3
0–40% EtOH	45.5	131	91	1.44	68	135
Ultrogel	48	67.2	43.2	1.55	35	145
CM-cellulose	32	49.6	32.0	1.55	26	145

* Methods of Lowry, as cited in Ref. 31.
† From 200 g of rat liver.

Table 2. Purification of *E. cloacae* cysteine desulfhydrase

Sample	Volume (ml)	Total activity* (μmoles/min)	Total protein† (mg)	Sp. act. (μmoles/min/mg)	Yield (%)	Purity
40,000 *g* Fraction‡	146	2463	3650	0.67	100	1
55–75% EtOH	11	926	825	1.12	38	1.6
DEAE-cellulose-1	102	833	147	5.67	34	8.4
0–45% $(NH_4)_2SO_4$	3	643	20	32.2	26	48
DEAE-cellulose-2	10.5	514	4.5	114.5	21	170

* Assay for H_2S uncorrected for H_2S inhibition.
† Methods of Lowry as cited in Ref. 31, corrected for buffer blank.
‡ From 50-g cells.

was omitted and methionine increased on a per sulfur basis. The Rogers–Harper amino acid-defined diet (Catalog No. 170010) was a control diet in these experiments. Both diets were purchased from Teklad Test Diets, Madison, WI.

Enzyme assays. A continuous assay for γ-cystathionase-catalyzed decomposition of cystathionine, homoserine or cystine, and for cysteine decomposition by cysteine desulfhydrase was obtained by measuring pyruvate or α-ketobutyrate released with a coupled enzyme assay procedure as described by Flavin and Slaughter [18]. The appearance of a mercaptoamino acid from either cystathionine or cystine could be measured by the aryl disulfide procedure of Flavin and Slaughter [18]. A colorimetric assay for H_2S released from cysteine by cysteine desulfhydrase was performed as described by Kredich *et al.* [19]. γ-Cystathionase activity in crude liver homogenates was measured by the addition of the homogenate to 0.1-ml of 0.1 M phosphate buffer, pH 8.0, containing 10^{-4} M pyridoxal phosphate and 25,000 cpm [U-^{14}C]L-homoserine. The reaction was terminated by the addition of 0.02 ml of 1 N HCl, and the radioactive α-ketobutyrate was determined by the methods of Livingston *et al.* [3]. Inhibition kinetics for γ-cystathionase using homoserine as substrate were performed by diluting 1 ml of 100 mM L-homoserine with 0.02 ml L-[U-^{14}C]homoserine (39 mCi/m-mole). After the addition of the enzyme to various concentrations of the substrate and inhibitor in 10^{-4} M pyridoxal phosphate, 0.1 M phosphate buffer, pH 8.0, aliquots of the reaction were terminated at 0, 10 and 20 min by the addition of 0.02 ml of 1 N HCl. The radioactive α-ketobutyrate formed was measured as above.

Plasma cyst(e)ine levels. The total concentration of cysteine and cystine in plasma was measured by a modification of the procedure described by Ball [20]. Mixed disulfides bound to serum albumin and cystine in 0.1 ml plasma were reduced by the addition of 0.02 ml of 1 M phosphate buffer containing 10 mM dithioerythritol, pH 6.8. The plasma sample was deproteinized by the addition of 0.2 ml of absolute ethyl alcohol and centrifuged. To the supernatant solution (0.2 ml) was added 0.005 ml of 50 μCi/ml of [U-^{14}C]glyoxylic acid, and the mixture was incubated at 60° for 5 min. The reaction was stopped by the addition of 0.02 ml of 1 N HCl and the thiazolidine formed was isolated by chromatography on a BioRad AG50W-X2 column (0.4 cm × 8 cm) which had been equilibrated with 0.05 N HCl. After the addition of the sample, the column was eluted with 4 ml of 0.05 N NaOH. Each 4-ml fraction was collected in a scintillation vial and mixed with 10 ml of New England Nuclear Formula 950A fluor. The samples were assayed for radioactivity in a Beckman liquid scintillation counter using a full tritium and carbon channel. The radioactivity contained in the acid and base fractions were summed and the total was normalized to 400,000 cpm to correct for losses during sample application on the column. The addition of either a known amount of cysteine or a cysteine-degrading enzyme to the plasma gave the expected increase or loss in the recovery of the radioactive thiazolidine in the basic fractions. This assay would not detect glutathione but homocysteine would form a radioactive thiazane.

A procedure which would differentiate between cysteine and homocysteine was developed based on the preparation and isolation of their carboxymethyl derivatives. Plasma (0.1 ml) was reduced with 0.004 ml of 0.14 M β-mercaptoethanol in 0.5 M phsophate buffer, pH 8.0. After 5 min the sample was deproteinized by the addition of 0.2 ml of absolute ethanol. To 0.2 ml of the supernatant were added 0.004 ml of the β-mercaptoethanol–phosphate buffer and 0.025 ml [1-^{14}C]iodoacetic acid (50 μCi/ml). After 1 hr at room temperature, the radioactive carboxymethyl cysteine formed was isolated by chromatography of 0.2 ml of the sample on a Hamilton HP-B80 resin (1 cm × 20 cm) at 55° with 0.066 N, pH 3.28, Hamilton buffer (1.5 ml/min, 100 psi). With 1.5-ml fractions, carboxymethyl cysteine eluted in fractions 13–17 which were assayed for radioactivity and totaled. Carboxymethyl homocysteine did not elute in this region. The assay was linear with cysteine concentration between 1 and 20 nmoles. Figure 1 shows a comparison between the standard curves for both of these assay procedures.

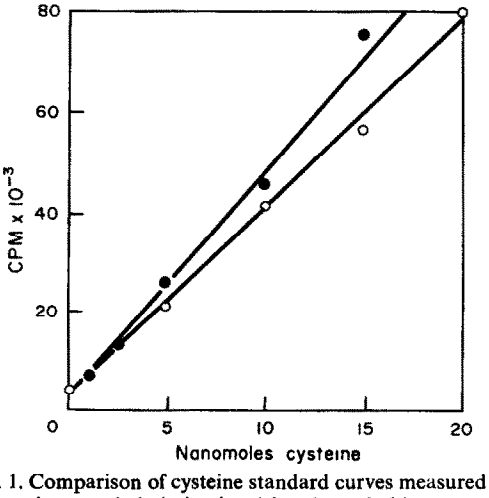

Fig. 1. Comparison of cysteine standard curves measured as the carboxymethyl derivative (closed symbols) or as the thiazolidine (open symbols). For details see Materials and Methods.

RESULTS

Properties of propargylglycine in vitro. Propargylglycine has been reported by Abeles and Walsh [21] to inhibit γ-cystathionase in an irreversible covalent manner presumably via the generation of an allene intermediate on the enzyme surface. We chose to determine the kinetics of the inactivation and to reconfirm the covalent nature of the product. Figure 2A shows the time-dependent inhibition of purified rat liver γ-cystathionase activity with varying inhibitor concentration. When the half-time ($T_{\frac{1}{2}}$) of enzyme activity is replotted versus the reciprocal of the inhibitor concentration, a binding constant (K_I) of 0.1 mM and a saturating inactivation half-time of 0.5 min were observed (Fig. 2B). The covalent nature of this inactivation was investigated by inhibition of the enzyme with propargylglycine in which the acetyl-

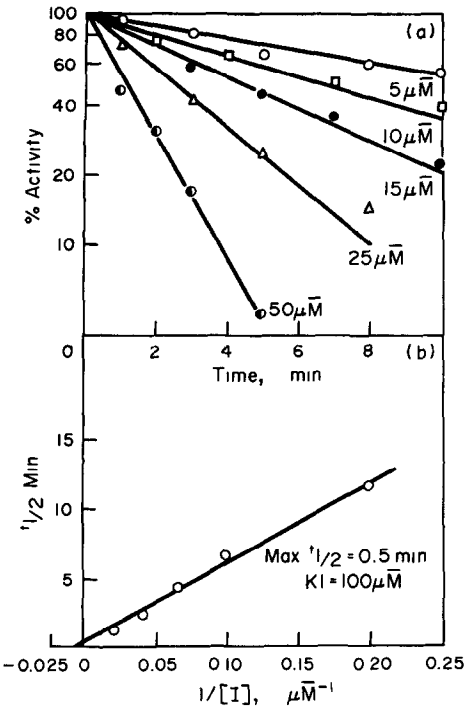

Fig. 2. (A) Kinetics of inactivation of purified rat liver γ-cystathionase by propargylglycine. Rat liver γ-cystathionase (0.05 mg/ml) was incubated with various concentrations of propargylglycine in 10^{-4} M pyridoxal phosphate, 0.1 M phosphate buffer, pH 7.5, at room temperature. Aliquots were withdrawn at various times and assayed for residual cystathionase activity by the aryl disulfide procedure [18]. (B) Half-times of inactivation are plotted vs the reciprocal of the inhibitor concentration.

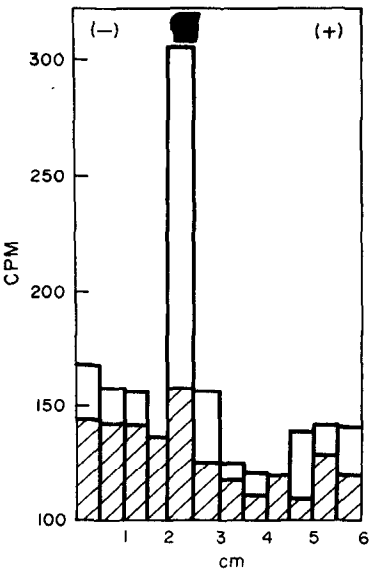

Fig. 3. Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis of γ-cystathionase reacted with tritiated propargylglycine. γ-Cystathionase (50 μg) which had been previously incubated with 0.1 mM tritiated propargylglycine (1.2 mCi/m-mole) for 10 min was electrophoresed (open bars). Gel slices (0.5 cm) were dissolved in 0.5 ml of 30% H_2O_2 at 60° overnight. The dissolved gels were diluted with 10 ml of New England Nuclear 450A fluor and assayed for radioactivity. The above enzyme solution was preincubated with SDS at 100° prior to the incubation with tritiated propargylglycine and was then electrophoresed and assayed for radioactivity as described above (shaded bars). The top figure presents a Coomassie Blue stained gel. Electrophoresis was from left to right.

enic proton had been exchanged with tritium. Figure 3 shows that the radioactive inhibitor will electrophorese with the inactivated enzyme even under the conditions of denaturation with sodium dodecyl sulfate (SDS). If SDS denaturation preceded incubation with the radiolabeled inhibitor, little, if any, radioactivity will electrophorese with the protein. This experiment demonstrates the covalent nature of the inactivation product as well as the necessity for a catalytically active enzyme in order to get a reaction with the inhibitor.

2,7-Diaminooct-4-ynedioic acid, a symmetrical acetylenic analog of cystathionine, did not inhibit rat liver γ-cystathionase when preincubated with the enzyme up to 1 mM for 15 min.

Effects of propargylglycine in vivo. Tritium-labeled propargylglycine was injected intraperitoneally into a BDF_1 mouse, and plasma was collected at various times to determine the rate of clearance. The radioactive material in the plasma did not chromatograph on cellulose thin-layer plates in the same position as propargylglycine nor did it inhibit γ-cystathionase under sufficient conditions for inhibition by starting material. Thus, the compound was either rapidly metabolized or the tritium, which is stable in solution, was exchanged under the biological conditions. The half-life of the radioactivity in the plasma was the same as that for tritiated water, suggesting a biologically catalyzed exchange of the acetylenic tritium with water.

The time course of loss and recovery of the liver γ-cystathionase activity in mice was determined (Fig. 4) after a single injection of propargylglycine (14 mg/kg). A similar experiment with one-fifth of the dose indicated esssntially the same time course of loss and recovery. At a 5-fold higher dose, a longer time (2–3 days) was necessary for the recovery of enzyme activity. The recovery of enzyme activity after inhibition with propargylglycine presumably results from continued synthesis of the enzyme. Daily injections of 14 mg/kg were sufficient to prevent recovery of liver cystathionase activity during a 5-day period. In order to maximize the selectivity of propargylglycine *in vivo*, studies with the higher dosages were avoided.

With daily injections of 14 mg/kg of propargylglycine, the plasma cysteine levels were measured in mice which were fed either normal mouse chow, or a synthetic amino acid-defined diet devoid of cystine, or a synthetic amino acid-defined diet containing cystine. Employing this treatment over a 4-day period showed no statistical difference between the plasma cysteine concentrations among the three diets, although other manifestations of the treatment were evident. Table 3 shows the cystathionine levels in plasma and urine when mice were treated with

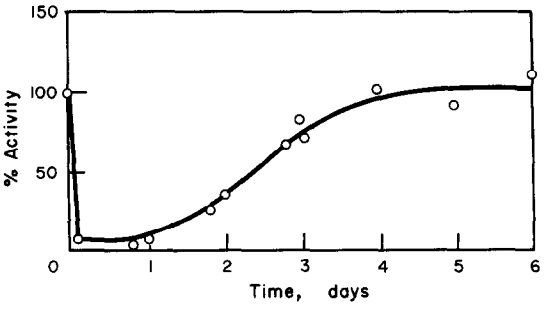

Fig. 4. γ-Cystathionase activity in the livers of BDF_1 mice injected with a single dose of 14 mg/kg of propargylglycine. At the times indicated, animals were sacrificed and their livers extracted and homogenized with equal parts of 0.1 M phospate buffer, pH 7.5. γ-Cystathionase activity was assayed by the [^{14}C]homoserine assay procedure described in Materials and Methods. A similar inhibition and recovery profile could be observed with cystathionine as substrate and the aryl disulfide assay procedure [18].

this protocol. Both a cystathioninemia and a cystathioninuria were created by the procedure, particularly on the cystine-free diet. Homocystinemia and homocystinuria were not observed.

Weight loss caused by propargylglycine when given to mice on the various diets is shown in Fig. 5. By this criterion, propargylglycine (14 mg/kg/day) was toxic only when combined with diets from which cystine was omitted. A similar pattern of toxicity was observed with normal lymphoid cells in culture. When "normal" [4] lymphoid cells (SLT) were grown in modified Eagle's medium which lacked cystine but contained cystathionine, the ID_{50} for propargylglycine was 1 μM, whereas if the cell line was grown in media containing cystine, the compound was not toxic at concentrations as high as 500 μM.

No increases in survival were observed when mice previously adapted to the cystine-free diet were given 10^5 L1210 cells i.p. on day 0 and treated qd 1–4 or qd 1–8 with propargylglycine (3, 7 or 14 mg/kg/day).

Table 3. Cystathionine content of plasma and urine from mice injected i.p. with propargylglycine (14 mg/kg/day) for 4 days*

	Plasma†		Urine‡	
Diet	Control	Treated	Control	Treated
Normal	0.00	11 ± 3	0.54 ± 0.13	5.1 ±
Cystine free	0.00	69 ± 15	0.83 ± 0.08	15.0 ± 10.2

* Cystathionine levels were measured with the use of a Beckman model 121 M amino acid analyzer. Creatinine measurements were performed by the Clinical Chemistry Laboratory of the Sidney Farber Cancer Institute.

† Average values for four mice; nmoles/ml on day 4.

‡ Average values for days 1–4; μmoles/mg of Creatinine.

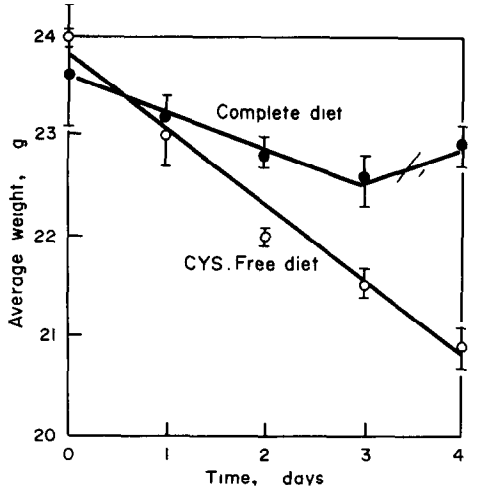

Fig. 5. Effects of propargylglycine on the weight of normal mice. Ten BDF_1 mice were fed a synthetic amino acid-defined diet for 4 days which either contained cystine (filled symbols), or which did not contain cystine (open symbols). After this dietary adjustment period, propargylglycine (14 mg/kg) was injected daily i.p. and the mouse weight were recorded. In the absence of propargylglycine, mice gained weight on both diets. The difference between the means at day 4 is significant at the 1 per cent level of confidence as determined by a *t*-test.

Properties of rat liver γ-cystathionase in vitro. Rat liver γ-cystathionase degrades cystathionine to α-ketobutyrate, ammonia and cysteine, and also degrades homoserine to α-ketobutyrate and ammonia, as well as cystine to thiocystine, pyruvate and ammonia [22, 23]. Thiocysteine, the product of cystine degradation, is unstable and will spontaneously oxidize cysteine to cystine with the elimination of H_2S [23]. The kinetic constants of the enzyme toward these three substrates are shown in Table 4. Although cystine has the poorest V_{max}, it has the most favorable K_m and at low substrate concentrations it appears to be the substrate toward which the enzyme is most efficient. It is the ability of the enzyme to degrade cystine that we wish to exploit therapeutically.

Since the effective activity of cystathionase in a therapeutic situation also depends on the levels of inhibitors which may be present in a physiological fluid, various amino acids were examined for their inhibitory properties (Table 5). The competitive inhibition of cystathionine cleavage by cystine with a K_I of 0.1 mM (similar to its K_m of 0.067 mM) gives further support to the proposal that the enzyme decomposes both substrates [23]. Cysteine was a very effective inhibitor, having a K_I of 0.3 mM, which is considerably lower than the K_m of cystathionine (8.3 mM). β-Cyanoalanine, a structural analog of pro-

Table 4. Substrate specificity of γ-cystathionase

Substrate	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m
Cystathionine*	3.5	4.6	1.3
Homoserine†	15.4	7.8	0.51
Cystine†	0.067	0.29	4.3

* Kinetic constants were determined from Lineweaver–Burk plots of the rates of formation of a sulfhydryl group at varying concentrations of cystathionine (1.0 to 8.0 mM) (see Methods).

† Kinetic constants were determined from Linewaever–Burk plots of the rates of formation of α-keto acid with varying homoserine (2.0 to 20 mM) and cystine (0.1 to 1.0 mM) concentrations (see Methods).

pargylglycine, showed competitive inhibition with a very low K_I of 0.011 mM. The ability, although not the magnitude, of this compound to inhibit γ-cystathionase has been previously reported [24]. Propargylglycine demonstrated inhibition kinetics that were consistent with the irreversible titration of enzyme activity (Fig. 2).

Evaluation of rat liver γ-cystathionase in vivo. When purified rat liver γ-cystathionase (3 mg, 4.8 units) was injected intraperitoneally into a BDF_1 mouse, the enzyme activity increased and reached the maximum level in 2 hr and then declined (Fig. 6). In a similar experiment a semilogarithmic plot of this activity has demonstrated a half-life of about 2 hr. The minimum cysteine concentration (40% of the zero time) coincided with the maximum enzyme concentration, and the cysteine levels returned to normal with the disappearance of the injected enzyme. When this experiment was repeated and cysteine measured as the carboxymethyl derivative, a similar loss and recovery of plasma cysteine were observed.

Properties of cysteine desulfhydrase from E. cloacae in vitro. Cysteine desulfhydrase isolated from *E. cloacae* degrades cysteine to hydrogen sulfide, pyruvate and ammonia. Like other cysteine desulfhydrases [25, 26], the amount of pyruvate formed was less than the amount of H_2S produced, presumably due to the release of unstable and reactive 2-aminoacrylate. The enzyme had no activity toward cystine. The velocity of the reaction as a function of substrate concentration is presented in Fig. 7. The enzyme showed sigmoidal kinetics with half-maximal velocity at a cysteine concentration of approximately 0.3 mM. The enzyme was capable of degrading cysteine in blood in the presence of a reducing agent. The enzyme was not inhibited by either propargylglycine or β-cyanoalanine but was inhibited by H_2S, the product of its reaction with cysteine.

Evaluation of E. cloacae *cysteine desulfhydrase.* in vivo. No measurable cysteine desulfhydrase activity could be detected in the plasma of BDF_1 mice after the intraperitoneal injection of 10 units enzyme. Intravenous injections demonstrated, however, that the half-life of enzyme activity in plasma was less than 10 min. In case active sequestered enzyme may have existed other than in the plasma, cysteine concentrations were measured after 12 units (0.1 mg) of i.p.-administered enzyme. No drop in plasma cysteine concentration could be detected between 15 min and 4.5 hr.

DISCUSSION

The formation of a radioactive thiazolidine, from the condensation of an α-amino, β- or γ-thiol with a radioactive aldehyde, proved to be a reliable and rapid technique for the determination of cysteine levels in bological fluids such as tissue culture media or samples of plasma. Due to the high sensitivity of the assay (1–10 nmoles), determinations of the cysteine level in the plasma of mice could be undertaken by the sequential samplings of 0.2 ml blood from one mouse. This sensitivity reduced the number of animals and consequently the amount of cyst(e)ine-degrading enzyme necessary to evaluate *in vivo* the effects of enzyme injection.

Table 5. Inhibitors of γ-cystathionase*

Inhibitor	Substrate	K_I (mM)	Type
Cystine	Cystathionine	0.10	Competitive
Cysteine	Homoserine	0.30	Competitive
Homocysteine	Homoserine	2.5	Competitive
β-Cyanoalanine	Cystathionine	0.011	Competitive
β-Cyanoalanine	Homoserine	0.011	Competitive
Propargylglycine†	Cystathionine	0.10	Irreversible

* Kinetic constants were determined from Dixon plots of the effects of inhibitor concentration on the rate of production of sulfhydryl groups (cystathionine as substrate) or radioactive α-ketobutyrate (homoserine as substrate). A minimum of three different substrate concentrations was used (see Methods).

† Data from Fig. 2.

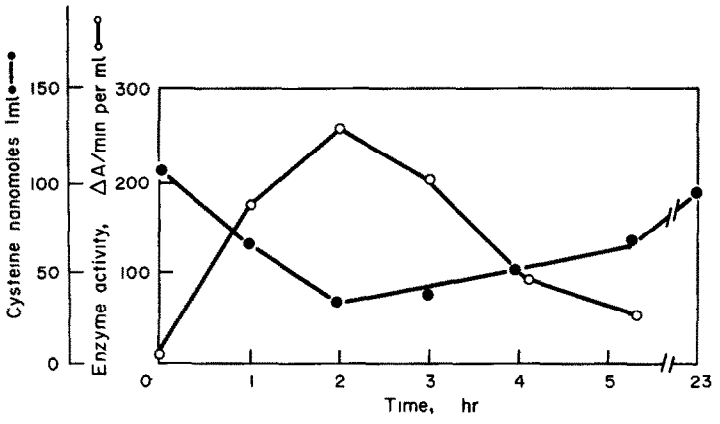

Fig. 6. Plasma cysteine levels and γ-cystathionase activity in a BDF_1 mouse after an i.p. injection of 4.8 units of purified rat liver γ-cystathionase. Blood (0.25 ml) was collected by orbital bleeding every hr. After removing the red cells by centrifugation (2 min at 8,000 *g*), 0.01 ml plasma was assayed for cystathionase activity by the aryl disulfide procedure [18], and 0.1 ml was rapidly deproteinized with 0.2 ml of absolute ethanol and assayed for cysteine by the thiazolidine formation procedure (see Materials and Methods).

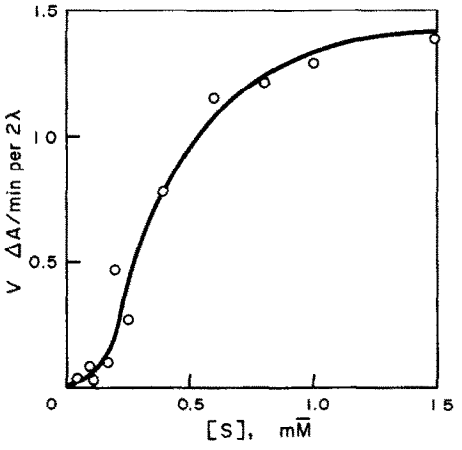

Fig. 7. Effects of cysteine concentration on *E. cloacae* cysteine desulfhydrase activity. The product, H_2S, was measured by a colorimetric procedure described by Kredich *et al.* [19].

Suppression of the level of cysteine in mouse plasma was attempted by giving an inhibitor of cysteine biosynthesis (propargylgycine) to animals placed on diets free of cysteine. Propargylglycine was a very efficient inhibitor of rodent liver γ-cystathionase both *in vitro* and *in vivo* (Figs. 2 and 4). The compound was very selective in that it took 1000 times greater concentration to inhibit glutamic pyruvic transaminase [27] and 100 times greater concentration to inhibit cystathionine synthetase [27] to the same extent in a given amount of time as compared to γ-cystathionase in this report. This selectivity probably relates to the mechanism of inactivation by the inhibitor. Only enzymes which can labilize a proton on the β-carbon can generate the reactive allene intermediate which is the inactivating species [28]. Propargylglycine was largely unreactive with denatured γ-cystathionase and was not toxic in either tissue culture or whole animal studies when tested with adequate sources of cystine. Its high toxicity in the absence of cystine was also consistent with the selective inhibition of γ-cystathionase *in vivo*. Although propargylglycine was active *in vivo*, as judged by the loss of liver cystathionase activity, cystathioninemia, and synergistic toxicity with dietary cystine reduction, no reduction in the levels of cysteine in the plasma could be measured when the compound was given to animals on diets deficient in cysteine.

Table 6. Comparative properties of cysteine desulfhydrase and γ-cystathionase from various sources

Enzyme	Cysteine desulfhydrase		γ-cystathionase	
Source	*E. cloacae**	*S. Typhimurium*†	Rat liver*	*Neurospora*‡
Substrate	Cysteine	Cysteine	Cystine	Cystine
K_m	0.3 mM	0.2 mM	0.07 mM	0.03 mM
Specific activity (units/mg)	114	451	0.28	0.10
pH optimum	8.5 to 9.0	8.5 to 9.0	7–8	7–8
Mol. wt	230,000	230,000	190,000	—
Plasma T1/2	10 min	10 min§	1.7 hr	—

* This work.
† Data from Kredich *et al.* [19, 25].
‡ Data from Flavin and Segal [30].
§ Unpublished observations.

Apparently, the levels of cysteine in the plasma are maintained at the expense of substantial breakdown of tissue as indicated by the weight loss of the animals when treated with this combination. No increase in survival was observed when mice bearing L1210 leukemia were treated with this combination.

The injection of an enzyme which degrades cyst(e)ine has the capability of creating a depletion of cysteine in the animal, much as the injection of *E. coli* asparaginase depleted asparagine from the plasma of man and other animals [10]. In order for an enzyme to achieve this goal, it must satisfy a number of enzymatic and biologic criteria [29], some of which will be discussed for γ-cystathionase and cysteine desulfhydrase.

Both enzymes have K_m values of about 0.1 mM which are close to the concentration of most amino acids in blood plasma. The maximum velocity with which cysteine was degraded by cysteine desulfhydrase (114 units/mg) was much greater than the rate of decomposition of cystine by γ-cystathionase (0.3 units/mg). The low turnover number of γ-cystathionase requires that a large amount of enzyme must be given to obtain depletion *in vivo* of cysteine (Fig. 6).

The half-life of injected enzyme in plasma greatly affects *in vivo* the activity of an enzyme as indicated by the correspondence between the recovery of cysteine concentration with the decrease of γ-cystathionase activity in the plasma (Fig. 6). The ineffectiveness of cysteine desulfhydrase *in vivo* in reducing the levels of cysteine in plasma can be explained by its extremely short half-life in plasma (less than 10 min). Chemical and physical modification of the enzyme might offer a means of prolonging the half-life. If a form of this enzyme can be made, or isolated, which has a reasonable plasma half-life, cysteine desulfhydrase might produce the desired depletion of plasma cysteine such that the toxicity and the therapeutic efficacy of this form of therapy can be evaluated.

A comparison between the properties of the *Enterobacter* and *Salmonella* cysteine desulfhydrases and rat liver and *Neurospora* γ-cystathionases is given in Table 6. Very similar properties between enzymes isolated from both species were observed including the very short plasma half-life in mice of the cysteine desulfhydrase enzymes and the very low turnover number toward cystine of the γ-cystathionase. In terms of the amount of enzyme produced per gram of cells. *E. cloacae* and rat liver were the preferred starting materials.

The combined use of propargylglycine to reduce host synthesis of cysteine and a cysteine-degrading enzyme to destroy the dietary intake of cysteine can be considered. This approach should reduce the amount of enzyme necessary to deplete cysteine in plasma by decreasing the input of this amino acid from organs which have a large synthetic capacity. The combined therapy may also prevent organs which have a high synthetic rate for cysteine from acting as a sanctuary for leukemic cells which would otherwise have died from lack of cysteine. It should be noted that γ-cystathionase should not be used in combination with propargylglycine, not only because it is inhibited by the compound but also because it would convert the high levels of cystathionine in the blood back to cysteine. Neither of these problems exists with cysteine desulfhydrase. If a form of this enzyme can be made, or isolated, which has a reasonable half-life in plasma, these combination studies can be considered. This report suggests that substantial host toxicity may accompany the combined therapy, such that particular attention to dosages may be necessary in order to reduce the ability of host tissues to "feed" leukemic cells and not totally destroy their ability to make cysteine such that they can survive extracellular cysteine depletion.

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