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γ-THIOCYANOAMINOBUTYRIC ACID, A CYSTATHIONASE SUBSTRATE*

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Abstract—Purified rat liver γ-cystathionase catalyzed the decomposition of γ-thiocyanoaminobutyrate (γ-SCNabu) into α-ketobutyrate, ammonia and thiocyanate in stoichiometrically equivalent amounts. K_m values and velocities of the enzyme were comparable for γ-SCNabu and homoserine. Addition of a variety of inhibitors affected both activities to similar degrees, thereby suggesting a common catalytic site on the enzyme. The major γ-thiocyanolyase activity of a cell-free extract of rat liver was associated with γ-cystathionase. γ-SCNabu appears to be metabolized by a cystathionase-like reaction. DL-γ-SCNabu was six-tenths as toxic as cyanide and 35-fold more toxic than thiocyanate. Rats poisoned by γ-SCNabu had toxic blood cyanide concentrations. γ-SCNabu had negligible antimicrobial action *in vitro* against a variety of organisms.

γ-Thiocyanoaminobutyric acid thiocyano-lyase (adding CN) (EC 4.4.1), a cyanide-metabolizing enzyme recently isolated from *Chromobacterium violaceum* D 341, catalyzes the synthesis of γ-cyanoaminobutyric acid (γ-CNabu) and thiocyanate from cyanide and homocystine. γ-Thiocyano-α-aminobutyric acid (*S*-cyanohomocystine, γ-SCNabu), which forms by a nonenzymatic cyanolysis of homocystine, effectively replaces homocystine as co-substrate and is a likely intermediate in the biological utilization of homocystine [1]. During a study of the distribution of this new enzyme, we observed γ-thiocyano-lyase activity to be present in crude extracts of rat liver (16 nmoles thiocyanate/min/mg of protein formed from 25 mM γ-SCNabu). The liver and bacterial thiocyano-lyases differed, since the liver enzyme was inhibited by cyanide and it catalyzed, in addition to thiocyanate, the formation of α-ketobutyrate and ammonia rather than γ-CNabu. Both enzymes were inhibited by thiocyanate.

Enzymatic cleavage of a carbon–sulfur bond is a known property of γ-cystathionase (L-homoserine hydro-lyase [deaminating] EC 4.2.1.15), which degrades the amino acid thioethers cystathionine, djenkolic acid and lanthionine [2], and the disulfide cystine [3] as well as the hydroxyamino acid homoserine [2]. The bacterial thiocyano-lyase (adding CN) has little cystathionase activity [1]. In this study γ-cystathionase has been isolated in purified form from rat liver and found to have significant γ-thiocyanolyase activity. This finding has been correlated with observations made *in vivo* in the young rat on the metabolism and toxicity of γ-thiocyanoaminobutyrate.

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MATERIALS AND METHODS

Thiocyanate was determined as its red ferric complex [1, 4]. α-Ketobutyrate was determined as its 2,4-dinitrophenylhydrazone [5]. Ammonia was determined on the amino acid analyzer [6]. Blood cyanide was determined on samples taken immediately following cessation of respiration after administration of DL-γ-SCNabu (5 mg/100 g) or NaCN (1 mg/100 g) to 250–350 g rats. A mixture of 2 ml blood, 3 ml water, and 3 drops of octanol was acidified with 1 ml of 6 N H_2SO_4 and swept at 90° for 10 min with an N_2 stream into 4 ml of 0·1 N NaOH. After neutralization of the contents of the receiver with 0·4 ml of 1 N acetic acid, cyanide was determined [7]. Recoveries of added cyanide were 87 per cent. Liver samples were minced and then treated similarly.

Thiocyanate excretion after a single subcutaneous dose of L-γ-SCNabu [1] (20 μmoles/100 g of body weight) to young male Wistar rats was determined on 24-hr urine specimens collected in the presence of thymol. Control rats were pair-fed standard laboratory Chow. Thiocyanate values for test animals were corrected by the values for control animals.

γ-Cystathionase was purified from rat liver and assayed by described procedures [5, 8] as modified [9]. The enzyme was taken through the first crystallization step. Crystallization did not succeed, but some less pure material precipitated and was removed, which resulted in a 3-fold purification of the enzyme remaining in solution. The specific activity, 4·4 U/mg, was close to that reported for cystathionase purified by one and two crystallizations: 4·7 and 5·8 U/mg [5], and 4·5 and 5·2 U/mg [9]. Gel electrophoresis in glycine–Tris buffer [10] containing 10^{-5} M pyridoxal-P, pH 8·1, indicated that activity and protein were associated with one major band, R_f 0·75 toward the cathode, that represented a purity of 92 and 85 per cent respectively. Before use, the crystallization solution of the enzyme was passed over a Sephadex G-25 column in 0·2 M KCl–5 × 10^{-4} M EDTA–5 × 10^{-5} M pyridoxal-P, pH 7·5.

Apo-γ-cystathionase was prepared by treatment of the holoenzyme with cysteine followed by Sephadex filtration as described for the bacterial thiocyanolyase (adding CN) [1].

Pharmacological studies. Toxicity was determined in male Sherman rats (35–65 g). Compounds were given in water. The toxic dose is weight in mg per 100 g of body weight which consistently caused death in three or four animals, and was usually not lethal at a 25 per cent lower level. Administered i.p., the toxic dose of DL-γ-SCNabu was 3·5; *per os*, it was >18. Toxic dosages produced hyperventilation, convulsions and labored breathing preceding death, which occurred in 5–30 min. At the dosage 2·5, slight ataxia, decreased touch response and convulsive activity resulted. Comparative toxic dosages (in μmoles/100 g) of DL-γ-SCNabu, NaCN and KSCN were 22, 13 and 770 respectively.

Toxic dosages of γ-SCNabu, i.p., led to blood cyanide concentrations of 4·37 and 4·9 μg/ml; liver CN^- was 3·45 μg/g. A toxic dosage of NaCN led to blood CN^- of 3·83 μg/ml. Blood CN^- concentrations as low as 2·6 and 3·2 μg/ml have been observed in cyanide poisoning, *per os*, in man and as low as 5 and 7·1 μg/ml in dogs [11].

The protective effects of L-homocystine and L-cystine (as their sodium salts in 0·25 M aqueous solution) against the toxicity of NaCN (1 mg/100 g) in rats (105–125 g) were evaluated as described for cystine [12] except that amino acids were administered i.p. and NaCN was given s.c. At the ratio to NaCN of 10:1, both amino acids afforded 100 per cent protection; at a 5:1 ratio, 25 per cent protection resulted.

Antimicrobial action against various gram-positive and gram-negative bacteria, fungi and trichomonas was tested *in vitro* by the tube-dilution technique by Panlabs, Inc., Fayetteville, N.Y. At 20 μg/ml, DL-γ-SCNabu exhibited no significant inhibitory activity against *S. aureus*, *E. coli*, *M. ranae*, *Ps. aeruginosa*, *C. albicans*, *T. mentagrophytes* and *T. foetus* (10 μg/ml).

RESULTS AND DISCUSSION

During the purification of γ-cystathionase, both homoserine dehydratase and γ-SCNabu γ-thiocyanolyase activity were determined at each step. Like cystathionase [5, 8], the γ-thiocyano-lyase activity precipitated mainly in the 50–75% $(NH_4)_2SO_4$ fraction, had an optimal rate near pH 8 in Tris-HCl buffer, and required pyridoxal 5′-phosphate (pyridoxal-P), as evidenced by the preparation of an inactive apoenzyme and its reactivation by pyridoxal-P. Moreover, during each step, the ratio of the velocity of the decomposition of DL-homoserine (at pH 7·5) to that of γ-thiocyanoaminobutyric acid (at pH 8·1) remained constant at 0·75 ± 0·05, except for the first step when the ratio was 1·05. It therefore seemed likely that the major γ-thiocyano-lyase activity of the crude rat liver extract was a property of cystathionase.

The purified cystathionase, with a homoserine dehydratase specific activity of 4·4 U/mg [5], catalyzed the following reaction:

γ-thiocyanoaminobutyric acid →

$$SCN^- + \alpha\text{-ketobutyric acid} + NH_3.$$

Table 1. Effect of inhibitors of various types on the dehydratase and thiocyano-lyase activities of hepatic cystathionase

Inhibitor*	Substrate: DL-Homoserine, 40 mM I_{50} (mM)	Substrate: L-γ-Thiocyanoaminobutyric acid, 20 mM I_{50} (mM)
Sodium cyanide	0·8	1
Potassium thiocyanate	100	35
D-Cycloserine	6·5	5·5
L-β-Cyanoalanine	0·085	0·1

* Compounds were tested at five concentrations with the indicated substrate, 5×10^{-4} M pyridoxal-P, and 108 or 216 mU enzyme (sp. act., 1·08 U/mg) respectively, in 1 ml of 0·2 M Tris-HCl buffer, pH 8·1. Mixtures were assayed for α-ketobutyrate and thiocyanate respectively. Curves were plotted relating concentration of inhibitor to percentage inhibition. From each of these, I_{50} was derived as the concentration of additive inhibiting the enzyme by 50 per cent.

Thiocyanate, α-ketobutyrate and ammonia were present in the ratios of 1·00:1·04:1·05.

Further evidence for the identity of the γ-thiocyano-lyase activity with cystathionase was obtained by observing the effect on both activities of inhibitors of various types. These included: (1) cyanide and (2) D-cycloserine, which interact with the pyridoxal-P cofactor of cystathionase [5, 13]; (3) thiocyanate, which appears to be an end-product inhibitor of the γ-thiocyano-lyase, and (4) β-cyanoalanine, a substrate-analog inhibitor of γ-cystathionase [14]. All four compounds were inhibitory to both the homoserine dehydratase and the γ-thiocyano-lyase actions of the purified cystathionase. Table 1 gives the I_{50} values toward both activities. Figure 1 shows the competitive nature of β-cyanoalanine's inhibition of the dehydratase, $K_i = 8$ μM, and the γ-thiocyanolyase, $K_i = 15$ μM, activities. γ-Cystathionase is even somewhat more active in liberating thiocyanate than in cleaving thioethers: the relative rates of this enzyme with γ-thiocyanoaminobutyric acid, cystathionine, djenkolic acid and homoserine (all L-) were 22:18:12:100 [each at 50 mM with $2·5 \times 10^{-5}$ M pyridoxal-P and 50 mM Tris-HCl (pH 8·1) when incubated with 200 mU enzyme at 30° for 10 min]. The K_m values were similar for L-γ-SCNabu ($K_m = 20$ mM) and for DL-homoserine ($K_m = 17$ mM), as derived from the experiment in Fig. 1. These findings incidentally provide the basis for a very convenient alternative colorimetric assay for cystathionase.

A cystathionase-type of cleavage may have some significance for the metabolism of γ-SCNabu *in vivo*, inasmuch as rats excreted approximately 50 per cent of a single dose of this amino acid as inorganic thiocyanate within the following 24 hr. Under similar conditions, 93 per cent of administered thiocyanate was excreted unchanged.

Being more than half as lethal as cyanide, DL-γ-SCNabu showed an unexpected high degree of toxicity for an amino acid. Acute poisoning by γ-SCNabu

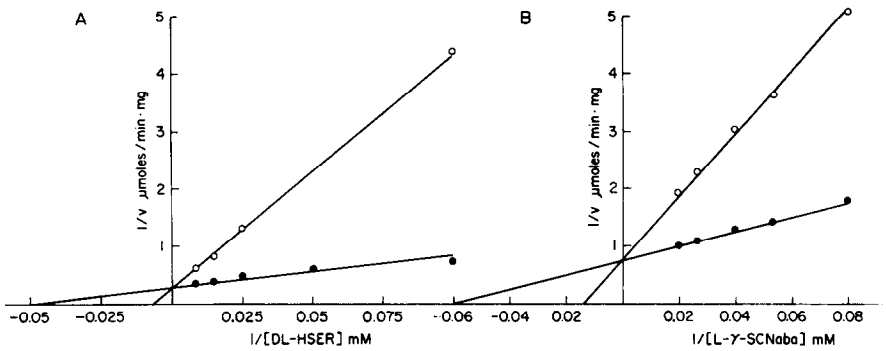

Fig. 1. Kinetics of inhibition of the homoserine dehydratase and γ-thiocyanoaminobutyrate γ-thiocyanolyase activities of rat liver cystathionase by L-β-cyanoalanine (β-CNala). Lineweaver–Burk diagrams of the velocities of the reactions in the presence (○) and in the absence (●) of β-CNala are shown. Curve A, reaction mixtures in 1 ml contained 10–120 mM DL-Hser, 5×10^{-4} M pyridoxal-P, 50 μM β-CNala, 0·2 M Tris-HCl (pH 8·1) and 108 mU enzyme, sp. act., 1·08 U/mg, and were incubated at 30° for 10 min. α-Ketobutyric acid was determined. Curve B, mixtures contained 12·5 to 50 mM L-γ-SCNabu, 216 mU enzyme, and other components and procedure as for curve A. Thiocyanate was determined.

can probably be attributed to cyanide intoxication, since the blood of rats given lethal dosages of γ-SCNabu and cyanide had comparable cyanide levels that, moreover, were close to those observed in cyanide poisoning in man and dogs [11]. In addition, the signs of γ-SCNabu and cyanide intoxication were similar, and cyanide and thiocyanate intoxications and some of their pharmacological actions have been observed to be similar [15]. The conversion of thiocyanate into cyanide *in vivo* has been attributed to the action of erythrocytic thiocyanate oxidase [16–18]. Recently this reaction has been shown to be a property of the peroxidase action of hemoglobin [19]. Several other tissue peroxidases also can catalyze the reaction [19]. In the current study, presumably thiocyanate is liberated from γ-SCNabu by the action of γ-cystathionase and then in part is converted by peroxidase action into cyanide. Like other anions, administered thiocyanate is thought to be located mostly in extracellular fluid. If γ-SCNabu is taken up by tissues in the same manner as other amino acids, it may serve as a means of introducing thiocyanate and its metabolites into certain cells.* The observation that γ-SCNabu is 35 times more toxic than thiocyanate might reflect such a process. The additional possibility exists that some cyanide is liberated enzymatically from γ-SCNabu without requiring the formation of thiocyanate, in analogy with the metabolic route considered likely for certain organic thiocyanate insecticides [21].†

Thiocyanate in mammalian tissues is generally attributed to diet and to cyanide detoxication as catalyzed by rhodanese [18]. The present findings might suggest an alternative route to thiocyanate in which homocystine, cyanide and cystathionase participate. As the first part of such a route, the cyanolysis of homocystine to γ-SCNabu would be analogous to an auxiliary pathway for cyanide detoxication whereby cystine undergoes cyanolysis to form 2-imino-4-thiazolidine carboxylic acid via the unstable β-thiocyanoalanine (β-SCNala) [18, 22]. Homocystine afforded the young rat only a similar degree of prophylaxis against a minimal lethal dosage of cyanide as did cystine. Moreover, inasmuch as γ-SCNabu was found to be not much less toxic than cyanide and to be far more toxic than thiocyanate, it seems unlikely that a homocystine-γ-SCNabu-thiocyanate pathway could be a major mechanism for the mammalian detoxication of large amounts of cyanide.

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* Pharmacologic interest in γ-SCNabu may derive from its conversion to thiocyanate and cyanide, especially in view of current interest in the antitumor potential of cyanide. Moreover, since thiocyanate is both a product and inhibitor of cystathionase, γ-SCNabu may perhaps represent an example of a 'k_{cat} inhibitor' [20].

† As suggested by a reviewer.

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