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β-CYANOALANINE, AN INHIBITOR OF RAT LIVER CYSTATHIONASE*

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Abstract—β-Cyanoalanine, the neurotoxin naturally present in various species of Vicia (vetch), is shown to be an effective inhibitor *in vitro* of rat liver cystathionase. Inhibition was reversible with increasing amounts of substrate, and no evidence of an inhibitor–pyridoxal phosphate interaction was found. Structure–activity studies suggest that the cyano group and the *N*-unsubstituted amino acid of L configuration are probably essential structural features of the inhibition.

PREVIOUSLY we reported a relation in the rat between β-cyanoalanine, the neurotoxin in certain vetch plants, and pyridoxal.[1] Pretreating the rat with pyridoxal moderates the toxicity of a single, injected dose of β-cyanoalanine, and administering this amino acid at subtoxic levels results in cystathioninuria, a condition that occurs in the rat deprived of vitamin B_6.[2, 3] The results of a tracer experiment precluded the possibility of β-cyanoalanine as a direct precursor of cystathionine.[1] A tryptophane-load test and observations on the intact rat provided no tangible support for the suggestion that β-cyanoalanine might be acting as a vitamin B_6 inhibitor.[4] It seemed possible that β-cyanoalanine could be blocking the action of cystathionase (L-homoserine hydrolyase [deaminating] EC 4.2.1.15), a pyridoxal phosphate enzyme. In the present investigation the effect of β-cyanoalanine on this enzyme has been studied as a guide to the general mode of action of β-cyanoalanine.

METHODS

Materials. L- and D-β-Cyanoalanine,[4, 5] carbobenzoxy-L-β-cyanoalanine,[5] and sodium β-cyanopropionate hydrate[6] were synthesized as described. The synthesis of α-cyanoglycine is to be reported separately. γ-Glutamyl-β-cyanoalanine (LL) was natural material isolated from seeds of *Vicia sativa*.[7] 1,1,3-Tricyano-2-amino-1-propene was a gift of Dr. F. S. Eberts; 2-amino-3-phosphonopropionic acid and 2-amino-4-phosphonobutyric acid were gifts of Dr. A. F. Isbell. Other chemicals and reagents were obtained from commercial sources and used as supplied.

Animals and diets. These are described in the accompanying paper.[4]

Analyses. Absorptions at fixed wavelengths were measured with a Beckman DU

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or Coleman junior spectrophotometer; spectra were determined with the Bausch & Lomb Spectronic 505 recording spectrophotometer.

The presence of cyanide was investigated by the method of Epstein[8] as modified by Marsden.[9] The presence of α-cyanohydrins was investigated by treating the TCA-treated reaction mixtures with an excess of NaOH and analyzing for formed cyanide.[10] On paper chromatograms, cyanohydrins were investigated by a modified alkaline picrate method[11] by dipping the dried chromatogram through a saturated aqueous solution of picric acid, drying it at room temperature, and dipping it through 10% Na_2CO_3. The liberated cyanide formed a brown spot on a yellow background, which gave a level of detection of mandelonitrile of 0·08 μmole. A second method employed dipping the paper chromatogram through Epstein's pyridine–pyrazolone reagent[8] and damp-drying at room temperature, then dipping it through a solution of 1% chloramine T and 10% Na_2CO_3. A red spot formed on a white background, which gave a level of detection of mandelonitrile of 0·02 μmole.

Cystathionase preparations. Rats were decapitated and exsanguinated. Crude enzyme was prepared by homogenization of 5–10 g of washed rat liver in 50 ml of 0·2 M sodium borate buffer, pH 8·3, containing 5×10^{-4} M ethylenediamine tetra-acetic acid (EDTA) in a Waring–Blendor at 4°. The homogenate was centrifuged for 1 hr at 2620 *g*, and the precipitate was discarded. The supernatant fluid was adjusted to a protein concentration of 36 mg/ml (preparation A). For screening compounds as inhibitors, the 55% ammonium sulfate precipitate of a heat-treated crude liver homogenate[12] was suspended in 25 parts of 1% KCl in 10^{-4} M EDTA (preparation B). Rat liver cystathionase was purified by the method of Matsuo and Greenberg,[12] except that the second ethanol fractionation was omitted, and instead the product of the first ethanol fractionation was treated with protamine. Purification was carried out through the latter step (preparation C). Such preparations tended to fall in potency. Another cystathionase preparation was partially purified by the method of Gonzales and Horvath[13] (preparation D).

Protein concentrations were determined by a modified biuret method[14] with bovine serum albumin as standard.

Homoserine deaminase–cystathionase reactions. The cystathionase assay of Greenberg[15] was modified slightly. Enzymatic reactions usually were carried out in 1·5 ml in screw-cap vials at 37°. Reaction mixtures contained 0·2 M borate buffer, pH 8·3; 5×10^{-4} M EDTA; and $3{\cdot}5 \times 10^{-3}$ M mercaptoethanol (ME). Components dissolved in borate buffer were added. DL-Homoserine was used as substrate for routine determination of enzyme activity. Concentrations of substrate, pyridoxal phosphate (PALP), inhibitor, and enzyme preparation varied and are noted for each experiment. Buffer, EDTA, ME, PALP, and enzyme were mixed and incubated for 30 min at 37°. Addition of any inhibitor was followed by addition of substrate, and the reaction was allowed to proceed for 30 min at 37°. When cyanide and β-cyanoalanine (BCNA) were compared directly as inhibitors, mixtures were incubated in glass ampules, which were sealed immediately after addition of inhibitor and substrate. Reactions were stopped by adding 1·5 ml of 10% TCA, and the protein precipitate was removed by filtration. α-Keto acid in samples of the TCA filtrates was determined by the method of Friedemann and Haugen[16] as modified by Sayre and Greenberg.[17] One unit of enzyme is the amount that catalyzed production of 1 μmole of α-keto acid/hr.[15] Fractional inhibition was calculated directly from optical densities.

RESULTS

Screening β-cyanoalanine and relevant compounds as inhibitors of rat liver homoserine deaminase–cystathionase

β-Cyanoalanine and various nitriles and amino acids structurally related to it were tested as inhibitors of homoserine deaminase–cystathionase. For comparison, several known inhibitors of this enzyme, carbonyl reagents that bind the PALP cofactor, also were tested. Compounds were tested at the concentration and under conditions

TABLE 1. INHIBITION OF RAT LIVER HOMOSERINE DEAMINASE–CYSTATHIONASE BY β-CYANOALANINE AND OTHER COMPOUNDS*

Compound	Inhibition	Compound	Inhibition
Organic nitriles		Other amino acids	
L-β-Cyanoalanine	0·93	α,γ-Diaminobutyric acid	0
D-β-Cyanoalanine	0	α,β-Diaminopropionic acid	0
γ-Glutamyl-β-cyanoalanine(LL)	0·76	L-Aspartic acid	0·10
Carbobenzoxy-L-β-cyanoalanine	0	L-Asparagine	0
L-γ-Cyano-α-aminobutyric acid	0·88	L-Glutamic acid	0
α-Cyanoglycine	0·98	L-Glutamine	0
β-Aminopropionitrile	0	L-Isoasparagine	0
Sodium β-cyanopropionate	0	L-Serine	0
Succinonitrile	0	L-Threonine	0
Malononitrile	0·97	DL-β-Chloroalanine	0·29
1,1,3-Tricyano-2-amino-1-propene (malononitrile dimer)	0	2-Amino-3-phosphonopropionic acid	0
β-Hydroxypropionitrile	0	2-Amino-4-phosphonobutyric acid	0
2-Cyanoacetamide	0	L-Alanine	0
Nicotinonitrile	0	L-α-Aminobutyric acid	0
Indole-3-acetonitrile	0	L-Phenylalanine	0
Sulfur amino acids		γ-Aminobutyric acid	0
L-Cysteine	0·62		
DL-Homocysteine	0·23		
DL-Cystine	0·54		
DL-Homocystine	0	Miscellaneous compounds	
L-Cysteic acid	0	L-Malic acid	0
DL-Homocysteic acid	0	L-Thiomalic acid	0
S-Methyl-L-cysteine	0	L-Cycloserine	0
L-Methionine	0	DL-Penicillamine	0·47
DL-Methionine sulfoxide	0	Semicarbazide · HCl	0·87
S-Methyl-L-methionine chloride	0	2,4-Dinitrophenylhydrazine	0
L-Cysteinesulfinic acid	0	NaCN	0·53

* Reaction mixtures contained protein (enzyme preparation B), 0·4 mg; DL-homoserine, 4×10^{-2} M; PALP, 8×10^{-5} M; DL-compounds, 1×10^{-3} M; L-isomers and remaining compounds tested, 5×10^{-4} M. Assays as described under "Homoserine deaminase–cystathionase reactions". Four sets of reaction mixtures were run in duplicate, each containing buffer, EDTA, ME, PALP and in addition: A contained enzyme; B, enzyme and homoserine; C, enzyme and inhibitor; D, enzyme, homoserine, and inhibitor. O.D.$_{515}$ obtained in the analysis for α-ketobutyric acid for A was subtracted from that of B to give uninhibited activity; O.D.$_{515}$ of C was subtracted from that of D to give inhibited activity.

$$\text{Fractional inhibition} = \frac{\text{uninhibited activity} - \text{inhibited activity}}{\text{uninhibited activity}}.$$

established in preliminary experiments to be minimal for almost complete inhibition by BCNA. Table 1 shows results.

BCNA, α-cyanoglycine and γ-cyanoaminobutyric acid, malonitrile and γ-glutamyl-β-cyanoalanine were strongly inhibitory; cystine, penicillamine, and β-chloroalanine

inhibited somewhat less. The carbonyl reagents cyanide, semicarbazide, and cysteine were inhibitory in agreement with the findings of Greenberg *et al.*[15, 18] 2,4-Dinitrophenylhydrazine was not inhibitory, presumably because of its low basicity.[19]

Information as to whether an inhibitor had a direct action on PALP was obtained from the analyses for α-ketobutyric acid by observing the effect of the inhibitor on the reaction mixture in the absence of substrate, i.e. by comparing the optical densities of reaction mixtures A (buffer, EDTA, ME, PALP, and enzyme preparation) and C (buffer, EDTA, ME, PALP, enzyme preparation, and inhibitor) (see footnote to Table 1). In A, free PALP reacts with DPNH to give an $O.D._{515}$ higher than that of the reagent blank. A significantly low $O.D._{515}$ value for C was interpreted as evidence of reaction of inhibitor with PALP that might indicate inhibition by virtue of reaction of inhibitor with PALP or enzyme-bound PALP. Compounds which showed this effect markedly were malononitrile and, as expected from the literature, semicarbazide · HCl and NaCN. Malononitrile dimer and cycloserine also led to a reduced $O.D._{515}$ PALP in C, but did not inhibit at the concentration used. β-Cyanoalanine in C did not significantly affect the $O.D._{515}$.

Inhibition of purified homoserine deaminase–cystathionase by β-cyanoalanine and γ-glutamyl-β-cyanoalanine

Table 2 compares DL- + *allo*cystathionine with DL-homoserine as substrates for purified cystathionase and gives the degree of inhibition of each by BCNA. Inhibition by γ-glutamyl-β-cyanoalanine with DL-homoserine as substrate was also examined.

TABLE 2. INHIBITION OF PURIFIED HOMOSERINE DEAMINASE–CYSTATHIONASE BY β-CYANOALANINE*

Substrate (4×10^{-2} M)	Inhibitor (5×10^{-4} M)	$\Delta O.D._{515}$†	Fractional inhibition
DL-Homoserine		0·438	0
	BCNA	0·061	0·86
	γ-Glutamyl-BCNA	0·432	0
DL- + *Allo*cystathionine		0·235	0
	BCNA	0·096	0·59

* Reaction mixtures contained protein (enzyme preparation C), 0·14 mg, and PALP, $6{\cdot}4 \times 10^{-5}$ M; reactions and analyses were carried out as described under "Homoserine deaminase–cystathionase reactions".

† A measure of uninhibited or inhibited enzyme activity (see footnote to Table 1).

The ratio of enzyme activity with cystathionine to that with homoserine was 54 per cent, somewhat less than that observed by Matsuo and Greenberg[20] with DL-cystathionine and DL-homoserine (78 per cent). β-Cyanoalanine inhibited activity with both substrates, inhibition being somewhat less with cystathionine than with homoserine. However, evaluation of inhibition with cystathionine is complicated, since the four diastereomers are apparently cleaving differently,[21] possibly each with a separate degree of inhibition. The purified cystathionase was not inhibited by γ-glutamyl-β-cyanoalanine.

Inhibition of homoserine deaminase–cystathionase by β-cyanoalanine under various reaction conditions

Components of the assay mixture were preincubated individually with BCNA to determine conditions for maximal inhibition. Experimental details and results are outlined in Table 3. For each set of conditions, reaction mixtures were tested in the

TABLE 3. INHIBITION OF HOMOSERINE DEAMINASE–CYSTATHIONASE BY β-CYANOALANINE UNDER VARIOUS REACTION CONDITIONS*†

	Preincubation mixture (1)‡				Mixture (2)‡				Fractional inhibition
Expt.	Enz	Inh	Subs	PALP	Enz	Inh	Subs	PALP	
1	+	+					+	+	0·40
	+	−					+	+	
2	+			+		+	+		0·59
	+			+		−	+		
3		+		+	+		+		0·69
		−		+	+		+		
4‡		+	+		+			+	0·67
		−	+		+			+	

* Component present, +; absent, −.

† Enz = enzyme, 0·43 mg of protein (enzyme preparation D) per reaction mixture; Inh = inhibitor, 1×10^{-4} M BCNA; Subs = substrate, 1×10^{-1} M DL-homoserine; PALP = 2×10^{-6} M pyridoxal phosphate.

‡ Substances in (1) were preincubated at 37° for 30 min, then added to components in (2). The complete mixtures were incubated at 37° for 30 min before assay. In expt. 4, enzyme and PALP in (2) were also preincubated under the same conditions. Other components, total volume of reaction mixtures, and analyses as described.

presence and absence of BCNA. Experiments 1–4 investigate the preferential reaction of BCNA with apoenzyme, capacity of BCNA to inhibit holoenzyme, preferential binding of BCNA to the cofactor, and its reaction with the substrate, respectively. There was no striking difference in degree of inhibition under the various conditions. Most experiments described herein that investigate inhibition of enzyme activity use the conditions of experiment 2, i.e. test the capacity to inhibit holoenzyme.

Properties of β-cyanoalanine and cyanide as inhibitors of homoserine deaminase–cystathionase

The dependence of the inhibition by BCNA on substrate and coenzyme concentrations was determined. β-Cyanoalanine was used at a concentration effecting 50 per cent inhibition. When its concentration and that of DL-homoserine were held constant while that of PALP increased over a 40-fold range, fractional inhibition essentially remained unchanged (Fig. 1).

When the concentrations of BCNA and PALP were maintained constant and that of DL-homoserine varied, fractional inhibition by BCNA decreased with increasing concentrations of substrate (Fig. 2). Inhibition by 1×10^{-4} M BCNA was complete with 0·02 M DL-homoserine, but fell toward 53 per cent when the concentration of homoserine increased over a 10-fold range. In another experiment with $1{\cdot}2 \times 10^{-4}$ M BCNA and enzyme preparation C, inhibition fell from 44 toward 20 per cent when the homoserine concentration increased over the range 0·2–1 M.

By contrast, 25 per cent inhibition with 6×10^{-4} M KCN essentially remained unchanged with the same increase in homoserine concentration.

Figure 3 compares BCNA and KCN as inhibitors of homoserine deaminase–cystathionase. At all concentrations tested over the 20-fold increasing range, on a molar basis, BCNA was consistently more effective an inhibitor than KCN.

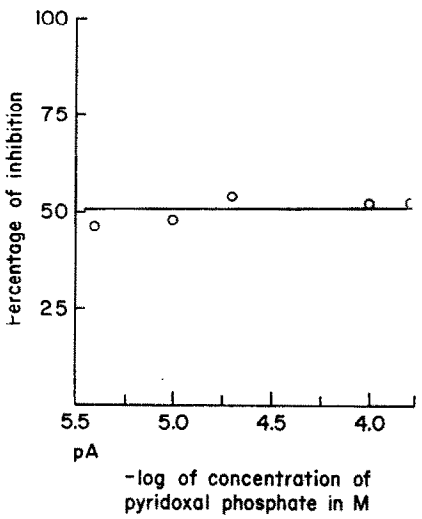

FIG. 1. Inhibition of rat liver homoserine deaminase–cystathionase by β-cyanoalanine. Variation of the fractional inhibition with the cofactor concentration. Reaction mixtures contained: protein (enzyme preparation D), 0·43 mg; DL-homoserine, 0·1 M; PALP, 4×10^{-6} to $1{\cdot}6 \times 10^{-4}$ M; BCNA, 1×10^{-4} M. (Enzymatic reactions and analyses carried out as described under "Homoserine deaminase–cystathionase reactions.")

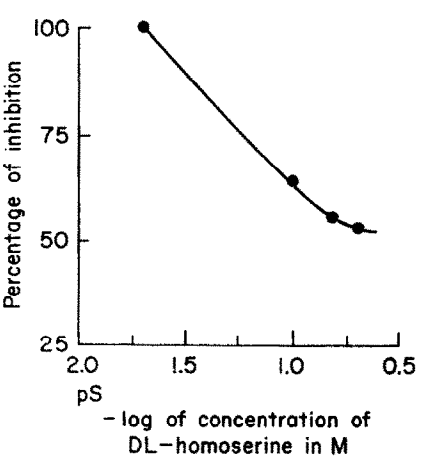

FIG. 2. Inhibition of rat liver homoserine deaminase–cystathionase by β-cyanoalanine. Variation of the fractional inhibition with the substrate concentration. Reaction mixtures contained: PALP, 2×10^{-5} M; enzyme preparation D and other components as in Fig. 1. (Reactions and analyses as described under "Homoserine deaminase–cystathionase reaction.")

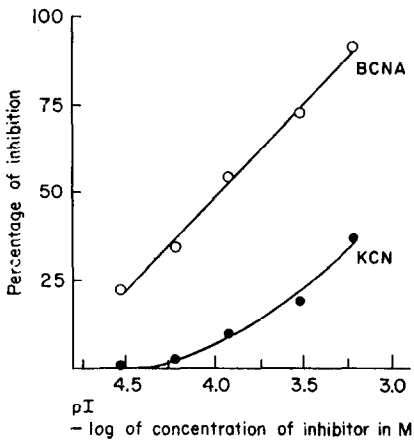

FIG. 3. Comparison of β-cyanoalanine and potassium cyanide as inhibitors of rat liver homoserine deaminase–cystathionase. Variation of the fractional inhibition with the inhibitor concentration. Reaction mixtures contained protein (enzyme preparation C), 0·14 mg; PALP, 2×10^{-5} M; DL-homoserine, 0·2 M; and KCN or BCNA (3×10^{-5} to 6×10^{-4} M) and were incubated for 5 min in sealed ampules. (Reactions and analyses otherwise as described under "Homoserine deaminase–cystathionase reactions.")

Examination for the formation of cyanide from β-cyanoalanine by rat liver

The cystathionase assay reaction was carried out in the outer well of closed Conway microdiffusion vessels with 0·5 ml of 1 N NaOH in the inner well to absorb liberated cyanide. Reaction mixtures in 1·5 ml of buffer contained ME, $1{\cdot}5 \times 10^{-3}$ M; PALP, 8×10^{-5} M; crude liver homogenate, 7 units; DL-homoserine, 4×10^{-2} M; and BCNA or NaCN, $5{\cdot}3 \times 10^{-4}$ M. After 2 hr at 37°, 0·5 ml of trichloroacetic acid was added, and the vessel was resealed and maintained for 2 hr at 37°.

Analysis for α-ketobutyric acid showed 26 per cent inhibition with NaCN and 86–92 per cent inhibition with BCNA. From NaCN, the recovery of cyanide from the inner well was 85 per cent or more. By contrast, no cyanide was detected from the reaction inhibited with BCNA, which indicates less than 5 per cent cleavage (if any) of BCNA to cyanide. To investigate the possibility that liberated cyanide might have been retained in the reaction mixture by reaction with the α-keto acid formed from the substrate, the reaction mixture inhibited with BCNA was analyzed for cyanohydrin (see "Analyses"), also with negative results.

Examination for nonenzymatic reaction between β-cyanoalanine and pyridoxal phosphate

Pyridoxal (PAL) and PALP can react nonenzymatically with amino groups of various amino acids to form imines (Schiff bases).[22, 23] This type of binding was investigated for BCNA. Pyridoxal phosphate, 1×10^{-5} M, was incubated in 0·1 M phosphate buffer, pH 7·5, for 30 min with BCNA at various concentrations up to 1×10^{-2} M, and the absorption spectrum was compared with that of PALP. No significant change appeared in the spectrum of PALP much below the concentration of 1×10^{-3} M BCNA. At the concentration ratio of BCNA to PALP of 1000:1, a large new peak was present at 255 mμ, the maximum of PALP at 388 mμ shifted to 410 mμ with a large decrease in intensity, and the maximum at 330 mμ shifted slightly to higher wavelength with some increase in intensity. These spectral changes differ from those reported

for the Schiff bases of a number of other amino acids and PALP at the concentration ratio of 2000:1,[23] and also seen here with 1×10^{-2} M lysine. (Such spectra typically show a new peak at 278 mμ, reduction of the 300 mμ maximum of PALP to an inflection, and a shift of the 388 mμ peak of PALP to 400–415 mμ with an increase in intensity.) It is not clear whether the differences are due to the presence of an additional chromophore (—C≡N) in the BCNA–PALP imine addition product or to the formation of a compound different from the typical Schiff base.

DISCUSSION

The present study shows that BCNA effectively inhibits rat liver homoserine dehydrase–cystathionase *in vitro*. Both these activities, which are recognized properties of the same enzyme,[20, 24] were inhibited. With 8×10^{-5} M PALP, 5×10^{-4} M BCNA, and 4×10^{-2} M DL-homoserine, i.e. at a concentration ratio of inhibitor to substrate of 1:40, the enzyme was inhibited approximately 90 per cent. The cystathioninuria previously observed in rats ingesting BCNA thus is probably a consequence of inhibition of cystathionase by this amino acid nitrile.*

The finding that inhibition of homoserine deaminase–cystathionase by BCNA can be reversed by increasing the concentration of substrate, but not of PALP, suggests that inhibition is directed toward the substrate rather than the coenzyme. In agreement is the observation that preincubation of the cofactor with BCNA followed by addition of the enzyme and substrate resulted in no significantly greater inhibition than when the inhibitor and substrate were added to a preincubated mixture of the enzyme and cofactor. Moreover, BCNA showed no unusual affinity for PALP when these were incubated together, as judged by the absence of effect on the absorption spectrum of PALP, until more than a 100-fold molar excess of BCNA had been reached.

The results of screening various substances structurally related to BCNA, with the exception of malononitrile, suggest that the L-amino acid nitrile configuration is probably essential for effective inhibition of homoserine deaminase–cystathionase by organic nitriles. The lower and higher homologs of BCNA, α-cyanoglycine and γ-cyano-α-aminobutyric acid, were highly inhibitory; however, it is not yet known whether their inhibition is of the same type as that of BCNA. Although the inhibitory effect of α-cyanoglycine was greater than that of an equimolar amount of NaCN, at least part of the effect may be due to free cyanide known to be liberated spontaneously to some extent from this amino acid nitrile. The enzyme was not inhibited by the D isomer of β-cyanoalanine nor by β-aminopropionitrile and sodium β-cyanopropionate lacking the carboxyl and amino group, respectively, of BCNA. Succinonitrile, β-hydroxypropionitrile, 2-cyanoacetamide, nicotinonitrile, and indole-3-acetonitrile, lacking both the carboxyl and amino group, and *N*-carbobenzoxy-L-β-cyanoalanine, an *N*-substituted derivative, were also non-inhibitory. γ-Glutamyl-β-cyanoalanine, another *N*-substituted derivative, inhibited a crude but not a purified preparation of cystathionase. The crude preparation presumably contained proteolytic enzymes that released BCNA. The only other inhibitory substances were: (1) compounds known to inhibit this enzyme by reacting as a substrate (cystine) or as a

* Two genetic disorders, showing cystathioninuria and homocystinuria are thought to involve an insufficient or defective enzyme of transulfuration (cystathionase and cystathionine synthetase, respectively).[25–27] β-Cyanoalanine might offer an experimental approach to examining the metabolic effects of impaired transulfuration in this general connection.

carbonyl reagent (semicarbazide, sodium cyanide); (2) penicillamine, known to inhibit certain other enzymes, e.g. transaminases, as a carbonyl reagent; (3) malononitrile, which appears to be a new carbonyl reagent presumably because of the presence of the activated methylene group; and (4) β-chloroalanine, which can be considered structurally similar to BCNA.

The possibility was considered that BCNA undergoes enzymatic cleavage to cyanide, which is the enzyme inhibitor. However, no experimental support has been obtained so far for this, since cyanide was found less inhibitory on a molar basis than BCNA and since it was not detected chemically in enzyme reaction mixtures 50–90 per cent inhibited by BCNA. Moreover, the nature of the inhibition of homoserine dehydrase by cyanide and BCNA differed in that the inhibition by added cyanide was independent of the concentration of the substrate. Although it is unlikely that cyanide is formed from BCNA by a separate enzyme, the possibility is not rigidly excluded that BCNA is cleaved by homoserine deaminase–cystathionase itself. Cyanide, if liberated in close proximity to the active center, could be more effective an inhibitor than added cyanide, and its formation and the resulting inhibition could then vary with the concentration of the substrate, homoserine.

Cystathionase from mammalian or microbial sources has been represented as an enzyme capable of effecting "γ-" or "β-elimination" from its substrates.[28] Thus, rat liver cystathionase converts L-cystathionine to cysteine, α-ketobutyric acid, and ammonia; L-homoserine to α-ketobutyric acid and ammonia; and L-cystine to S-thiocysteine, pyruvate, and ammonia. β-Cyanoalanine, bearing an electronegative substituent in the β-position, in general can be considered electrochemically similar to these substrates. This suggests an alternate explanation for the inhibitory action of BCNA, based on its competition with the substrate for the enzyme (such a concept of BCNA as a structural inhibitor of cystathionase being consistent with the inability to detect cyanide in inhibited mixtures and the observed variation of the inhibition with the concentration of homoserine). This type of inhibition, well recognized with some other enzymes,[29] appears not to have been encountered with cystathionase before. It would be of interest to examine the effect of BCNA on other enzymatic γ- or β-elimination reactions dependent on pyridoxal phosphate, such as the dehydration of serine and threonine and the cleavage of tryptophane to indole, as well as on synthetase or replacement reactions similarly catalyzed at the γ- or β-position.

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