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METABOLISM OF β-CYANOALANINE*†

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Abstract—The cystathioninuria induced in the rat by feeding it L-β-cyanoalanine (BCNA) has been investigated further. The excreted cystathionine was isolated in crystalline form and established as having the LL configuration. The protective effect of pyridoxal also was examined, especially in relation to evaluating BCNA as an inhibitor of vitamin B_6. β-Cyanoalanine and a series of relevant compounds, including known inhibitors of vitamin B_6 and structurally related lathyrus toxins, were fed to rats for prolonged periods. Of these, BCNA alone elicited cystathioninura, and this was not reversed by the administration of pyridoxal. β-Cyanoalanine produced no physical signs of B_6 deficiency or of osteolathyrism. In contrast to B_6 deficiency, the tryptophane-load test was negative and tissue concentrations of cystathionine were low. Brain, liver, blood, and muscle contained free BCNA and a new bound form of it, γ-glutamyl-β-cyanoalanylglycine, which may be a detoxication product.

SEEDS of certain species of lathyrus and vetch, used as forage or consumed in excess as food by humans in times of shortage, have been known to cause neurotoxicity.[1, 2] In connection with a series of investigations of the toxic principles of such legumes, β-cyanoalanine and the dipeptide, γ-glutamyl-β-cyanoalanine, were isolated from seeds of *Vicia sativa* (common vetch) and found to account for the high toxicity of these vetch seeds to the chick.[3, 4]

β-Cyanoalanine produces convulsions and rigidity when fed to or injected into the rat or chick. Attempts to reverse or prevent the convulsions in the rat with anticonvulsants of known central nervous system activity have been ineffective.[5] Injected pyridoxal, however, does afford some protection against a single dose of β-cyanoalanine.[6] Moreover, the rat that has been fed β-cyanoalanine excretes cystathionine,[6] a condition previously induced experimentally only by the administration of a vitamin B_6-deficient diet.[7, 8] These observations led us to suggest in a preliminary communication that β-cyanoalanine may be a naturally occurring vitamin B_6 antagonist or an antienzyme, possibly in the transulfuration reaction that converts methionine to cystine via cystathionine.[6] Inhibition of the B_6-requiring step[9] that converts cystathionine to cystine could lead to accumulation of cystathionine. It seemed of interest to investigate the action of β-cyanoalanine, especially since excessive excretion of cystathionine has been observed in patients with neural adrenal tumors[10] and in individuals with the inborn error of metabolism, cystathioninemia.[11, 12] The present

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paper reports more fully the earlier observations and a further investigation of the metabolism and properties of β-cyanoalanine. The companion paper[13] reports an investigation of the effect of β-cyanoalanine on rat liver cystathionase *in vitro*.

Previously identified chromatographically, the excreted cystathionine has now been isolated in crystalline form and shown to have the LL configuration. The possibility that the D-*allo*diastereomer of cystathionine, which has an optical rotation similar to that of L-cystathionine,[14] was being excreted after administration of β-cyanoalanine was eliminated on the basis of the ion-exchange chromatographic pattern.

The protective effect of pyridoxal has been examined, including its effect on the LD_{50} of injected β-cyanoalanine and its efficacy in feeding experiments. The rat fed β-cyanoalanine also has been examined with respect to several parameters characteristic of vitamin B_6 deficiency, including response to the tryptophane-load test, concentration of cystathionine in tissues, and reversibility of the cystathioninuria with B_6. A number of relevant substances including the synthetic vitamin B_6 antagonists, penicillamine and semicarbazide, known to act by binding the B_6 cofactor of certain enzymes, have been compared with β-cyanoalanine in long-term feeding experiments for ability to induce cystathioninuria. Also presented are other properties of β-cyanoalanine including toxic symptoms and distribution of this amino acid in tissues, hydrolysis of γ-glutamyl-β-cyanoalanine *in vivo*, and the distribution in tissues and possible significance of a new tripeptide metabolite of β-cyanoalanine, γ-glutamyl-β-cyanoalanylglycine.

METHODS

Chemicals. Mann Research Laboratories, Inc., New York, N.Y., supplied pyridoxal·HCl, M. A.; pyridoxine·HCl, U. S. P.; DL-glyceraldehyde, CP; thiamine·HCl, U. S. P.; sodium riboflavin-5-phosphate; DL-penicillamine, M. A.; and cycloserine. 2,4-Diaminobutyric acid dihydrochloride from the same source was converted to the monohydrochloride that was recrystallized twice from aqueous ethanol. Potassium cyanide, reagent, and semicarbazide·HCl were obtained from Matheson Coleman and Bell, East Rutherford, N. J. L-Cystathionine, used as reference, was purchased from Cyclo Chemical Corp., Los Angeles, Calif. Reference samples of D-cystathionine and D-*allo*cystathionine were gifts of Dr. V. du Vigneaud, and L-*allo*cystathionine was a gift of Dr. M. D. Armstrong. β-Aminopropionitrile·HCl, B grade, was supplied by Calbiochem, Los Angeles, Calif. L-β-Cyanoalanine (BCNA) was prepared as described[15] with the modification that the protecting group of carbobenzoxy-β-cyanoalanine was removed by hydrogenolysis in the presence of palladium black.[16]

Animals and diets. Sherman strain male weanling rats, obtained from Camm Research Farms, Wayne, N. J., were used. Chicks were white leghorns of unspecified sex, 1 day-old, obtained from Shamrock Farms, North Brunswick, N. J. Rats were housed singly in metal box cages for the LD_{50} determinations and in cages with raised floors for feeding experiments. They were maintained 1–3 days on the basal diet before experimentation. Compounds were dissolved in distilled water and injected s.c. unless indicated otherwise. Food and water were taken *ad libitum*. Urine was collected in the presence of either toluene or thymol in metabolism cages equipped with stainless steel funnels and mesh grids. It was stored frozen until analyzed.

The basal rations for the rat[6] and chick[4] were commercial diets described previously. Methionine, cystine, and pyridoxine contents of the rat ration were 0·46%, 0·41%, and 3·9 mg/lb; of the chick ration, 0·21%, 0·2%, and 2·5 mg/lb (private communications from the supplier). The pyridoxine-deficient diet was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

LD_{50} Values of Table 1 were based on mortality within 2 days after treatment. Each LD_{50} was obtained from a dose–mortality plot evaluated according to the procedure of Litchfield and Wilcoxon.[17]

Chemical determinations. Excreted xanthurenic acid in the tryptophane-load test was determined by the method of Wachstein and Gudaitis.[18] Urine was collected for 48 hr after dosing with tryptophane.

Tissue amino acids and peptides, including BCNA, γ-glutamyl-β-cyanoalanine, and cystathionine, were determined on the Beckman/Spinco automatic amino acid analyzer, model 120, by the quantitative chromatographic-ninhydrin procedure of Spackman *et al.*[19] The 150-cm resin column was used with 0·2 N sodium citrate buffer at pH 3·25 and 30°, with a change to pH 4·25 and 50° at 13 hr.[19]

RESULTS

Toxic symptoms of β-cyanoalanine in the chick and rat

In the chick, acute toxic symptoms of BCNA, fed or injected, resemble the description of thiamine deficiency in pigeons,[20] or of the strychnine-like convulsive state with opisthotonus that precedes death in some turkey poults with B_6 deficiency.[21] Retraction of the head and arching of the back, with pectoral muscles predominating, are pronounced (Fig. 1). The convulsive state may last 6–15 hr and may be followed by recovery.

The effect of subcutaneously injected BCNA in the rat is highly reproducible with respect to toxic dosage and the time of onset, nature, and duration of symptoms. Generally 30–40 min after injection of toxic doses, preconvulsive behavior starts (hyperkinesis, tail twitching followed by tail rigidity and extension), and in about 10 min, intermittent convulsions with depression follow. Rats assume a rigid opisthiotonic position with back muscles predominating. Death results usually within 4 hr. Occasionally a rhythmic feeding motion is observed with sublethal doses.

Rats were fed high subacute levels of BCNA for prolonged periods (0·45 per cent and 0·5 per cent for 2 months; 0·6 per cent for 1 month followed by 0·75 per cent for 3 1/2 months). Survivors developed no skeletal abnormalities or other gross symptoms of osteolathyrism, nor did they show acrodynia, ring tail, unthriftiness, or severe malnutrition as signs of vitamin B_6 deficiency. The only effects noted were hyper-irritability during the initial weeks, some retardation of growth, high mortality, and an elevated level of excreted cystathionine.

Effect of pyridoxal on the toxicity of β-cyanoalanine in the rat and chick

Injected pyridoxal·HCl lessens the severity and significantly delays the onset of the symptoms that follow a single toxic dose of BCNA in the rat. Table 1 shows the effect of injected pyridoxal·HCl on the mortality of rats injected with BCNA. When rats were pretreated with pyridoxal·HCl, LD_{50} of BCNA rose from 13·4 to 18·9 mg/100 g. When a second dose of pyridoxal·HCl was given at the onset of preconvulsive behavior, LD_{50} of BCNA rose to 22·5 mg/100 g.

The following compounds afforded no protection to weanling rats when injected 20 min before BCNA (22 mg/100 g) (dosages are in mg/100 g body wt. and the number of rats used is in parentheses): pyridoxine·HCl, 22 mg (8); sodium riboflavin phosphate, 15 mg (4); pyridoxine·HCl, 22 mg + sodium riboflavin-5-phosphate, 15 mg (5); thiamine·HCl, 5·4 mg (5); glyceraldehyde, 23 mg (3).

TABLE 1. EFFECT OF PYRIDOXAL · HCl ON THE LD_{50} OF β-CYANOALANINE

Treatment*	Body wt. Range	Body wt. Avg.	No. of animals treated	LD_{50}† (mg/100 g body wt.)	Ratio† LD_{50}/LD_{50} BCNA alone
β-Cyanoalanine	32–41	(36)	20	13·4 (12·6–14·3)	
Pyridoxal‡ + β-cyanoalanine, 20–40 min later	33–39	(36)	19	18·9 (16·7–21·4)	1·4 (1·3–1·6)
Pyridoxal‡ + β-cyanoalanine, 25 min later + pyridoxal,‡§ 1½–2½ hr later	32–37	(35)	19	22·5 (21·6–23·4)	1·7 (1·6–1·8)

* The dosage range of β-cyanoalanine was 11–25 mg/100 g.

† Confidence limits (19/20) given in parentheses.

‡ Administered as pyridoxal · HCl, 22 mg/100 g body weight.

§ The second dose given at onset of hyperactivity.

Pyridoxal·HCl, incorporated as 0·2 per cent of the diet, afforded no protection to rats (40 g) against the hyper-irritability, retardation of growth, and high mortality of BCNA when incorporated as 0·62 per cent of the diet, after a week's pretreatment with the pyridoxal·HCl alone.

Pyridoxal·HCl was administered as daily 5-mg supplements to chicks (90 g) fed a diet incorporating BCNA (0·1 per cent for 3 days, then 0·15 per cent), after 8 days' pretreatment with the pyridoxal supplements only. Mortality was 100 per cent in both the pyridoxal-supplemented and unsupplemented groups (average survival, 9·8 and 8 days, respectively). Appropriate control subjects, fed the basal diet and the diet supplemented with pyridoxal, were maintained in both feeding experiments.

Excretion of β-cyanoalanine and γ-glutamyl-β-cyanoalanine

Hydrolysis of γ-glutamyl-β-cyanoalanine to β-cyanoalanine by the rat. When BCNA was administered subcutaneously or intraperitoneally to young rats in large single doses, 38–55 per cent of it was excreted unchanged in the next 48 hr, most of this appearing within 24 hr (Table 2, expts. 1 and 2). When γ-glutamyl-β-cyanoalanine was administered in similar molar dosage, 36 per cent was excreted as BCNA and 3·5 per cent as unchanged dipeptide within 24 hr (expt. 3).

Cystathioninuria in the rat fed β-cyanoalanine

1. *Identification of excreted* L-*cystathionine.* (a) *Chromatographic evidence.* Fig. 2, upper chromatogram, reproduces the amino acid analysis of the neutral and acidic ninhydrin-positive substances in a sample of urine collected on day 32 from a rat receiving a diet incorporating 0·5% BCNA. The lower chromatogram shows the analysis on day 46, 8 days after the rat had been placed back on the basal ration. In the upper curve, large amounts of BCNA are seen to be excreted. Also present is a large, unusual peak (15 μmole), 8 ml after the indication of the pH change, which is

FIG. 1. The convulsive state with opisthotonus induced in the chick by β-cyanoalanine.

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virtually absent (0·4 μmole) in the lower curve. The chromatographic identification of this material as cystathionine was described in the preliminary report.[6] Chromatography on the amino acid analyzer in a modified system (the buffer change is omitted) now permits excluding the L-*allo* and D-*allo* configurations of cystathionine. (The *allo* diastereomers emerge as a single peak near 600 ml, 28 ml before the combined

TABLE 2. EXCRETION OF β-CYANOALANINE AND CYSTATHIONINE BY THE RAT GIVEN SINGLE DOSAGES OF β-CYANOALANINE OR γ-GLUTAMYL-β-CYANOALANINE

Expt.	Compound*	Administered (mg)	Administered (μmole)	Administered (μmole/100 g)	Excreted: Collection period (hr)	Excreted: BCNA (μmole)	Excreted: Cysta† (μmole/rat)
1	BCNA	16	140	132	0–48	78	1·0
	control				0–48		0·2
2	BCNA	23·6	207	131	0–24	60	0·3
					24–48	18	0·1
	control				0–24		0·2
3	γ-GluBCNA	23·1	94·9	138	0–24	34 + 3·2 μmole γ-GluBCNA	0·5

* Abbreviations: BCNA, β-cyanoalanine; γ-GluBCNA, γ-glutamyl-β-cyanoalanine; Cysta, cystathionine. Compounds injected subcutaneously in expt. 1 and intraperitoneally in expts. 2 and 3. BCNA was dissolved in 0·9% sodium chloride; controls received doses of saline. Average body wt. was 51–53 g in expts. 1 and 2, and 34 g in expt. 3. Groups contained 2 or 3 rats.

† Some error is inherent in these values owing to the low experimental figures and the large factors used for calculation.

peak of L-cystathionine and D-cystathionine[22] with which the excreted material was co-eluted.)

(b) *Isolation and crystallization of excreted cystathionine.* Urine from two rats receiving a 0·45% BCNA diet was pooled and centrifuged (Table 3, days 25–38; sp. gr., 1·04). One-fifth (containing 82 μmole cystathionine) was chromatographed in six portions on the 150-cm resin column of the amino acid analyzer in the system described above ("Chemical determinations"). The buffer and temperature were changed manually, and 200 ml of eluate was collected in a graduated cylinder, then in 1-ml fractions with a fraction collector. To locate the position of the pH change, ΔB, pH of fractions 70–120 was determined. Samples (10 μl) of fractions after ΔB were spotted on filter paper and sprayed with 0·15% ninhydrin in acetone. The contents of fractions in the ninhydrin-positive area 7–9 ml after ΔB were pooled and concentrated. The solution was adjusted to pH 2·2 and freed of buffer salts on an 8-ml column of Dowex 50-X8 (H^+) resin, 200–400 mesh. The dry residue was taken up in a minimum of water, and the solution was adjusted to pH 5·5 and diluted with ethanol to turbidity. After the mixture was cooled, the crystals were collected by centrifugation. Two recrystallizations from water–ethanol furnished 9·4 mg (52 per cent) cystathionine that was homogeneous on the amino acid analyzer: $[\alpha]_D^{25}$ + 23·8° (*c* 0·25, 1 N HCl); reported $[\alpha]_D^{20}$ + 23·7° (*c* 1) for synthetic L-cystathionine.[23]

2. *Excretion pattern of cystathionine.* Eight young rats were fed diets containing BCNA in concentrations of 0·3–0·75 per cent for periods up to 8 weeks, and 24-hr specimens were collected at various intervals. After 2–3 weeks, cystathioninuria was pronounced in each case (Tables 3, 4). The excretion pattern for two rats on a 0·45

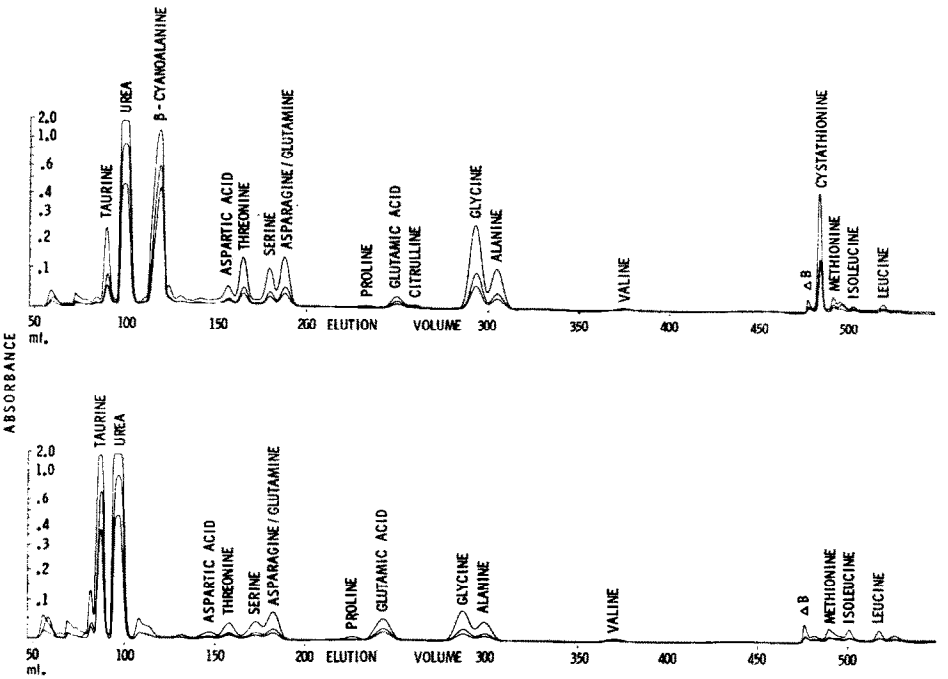

FIG. 2. Amino acid analyses showing the cystathioninuria in a rat ingesting β-cyanoalanine. The column of Amberlite IR-120 (0·9 × 150 cm) of the automatic amino acid analyzer was used (see "Chemical determinations"). The buffer change, ΔB, took effect at 476 ml. The curves are tracings of the absorbance of the ninhydrin reaction product. The upper chromatogram was obtained on analysis of 1·8 per cent of the volume excreted on day 32 by the rat on the diet incorporating 0·5% β-cyanoalanine. The lower chromatogram was obtained on analysis of 1·6 per cent of the volume excreted on day 46 by the same rat placed back on the basal diet (see "Identification of excreted cystathionine").

per cent diet is shown in Table 3. Average daily excretion (pooled sample) from day 25–38 was 16 μmole. Rats fed a 0·15% BCNA diet excreted only 0·7 μmole on day 27. Controls on the basal diet uniformly showed only trace amounts (0·1–0·2 μmole; Tables 2, 3). Single large injections of BCNA or of γ-glutamyl-β-cyanoalanine usually elevated the excretion of cystathionine slightly (Table 2).

Distribution of cystathionine, free β-cyanoalanine, and γ-glutamyl-β-cyanoalanylglycine in various tissues of the rat fed β-cyanoalanine

Concentrations in brain, kidney, muscle, blood, liver, and urine are given in Table 4. Except for urine, there is little accumulation of cystathionine.

γ-Glutamyl-β-cyanoalanylglycine, a recently identified metabolite of β-cyanoalanine,[24] is found in highest concentration in liver (0·2 m-mole/100 g). This bound form

of BCNA is present also in blood, muscle, and brain, but is absent from kidney and urine. The two last, compared to other tissues, have high concentrations of free BCNA.

TABLE 3. EXCRETION OF CYSTATHIONINE BY THE RAT FED A 0·45% β-CYANOALANINE DIET*

Day†	Body wt. (g)	Cystathionine (μmole/rat) (24 hr)
0	40	0·2
10	55	2·2
17	91	6·7
24	134	16·3
34	190	25·0
39	211	17·0
46	228	15·0
49	220	13·5
54	243	13·1
59	256	9·2

* Cystathionine excretion by controls on days 14 and 32 was 0·1 μmole.

† On days 40–54, rats were injected subcutaneously with 50 mg pyridoxal · HCl in 2 doses.

TABLE 4. DISTRIBUTION OF CYSTATHIONINE, β-CYANOALANINE, AND γ-GLUTAMYL-β-CYANOALANYLGLYCINE IN VARIOUS TISSUES OF THE RAT FED β-CYANOALANINE

Diet	Day	Tissue*	Cysta**	BCNA	γ-GluBCNAGly
			(μmole†)		
0·3% β-Cyanoalanine (37–121 g)‡	24	Urine	7·5	218	
	28	Liver	4·2		318
0·45% β-Cyanoalanine (40–250 g)‡	59	Urine	9·2	433	§
	60	Blood‖	0·1	< 4¶	24
		Liver	0·7	64	212
		Muscle		26	28
		Brain	1·2	66•	52
		Kidney	1·2	146	§

* Trichloroacetic acid extracts of organs and blood.

** Some error is inherent in most values owing to the small observed peaks and the large factors used for calculation.

† Micromoles per 100 g wet tissue or per 100 ml blood, or excreted per rat in 24 hr.

‡ Values in parentheses are initial and final body weights.

§ None detected; small quantities could be obscured by the adjacent large BCNA peak.

‖ γ-Glutamyl-β-cyanoalanine, 9 μmole, was also present.

¶ Presence of overlapping material prevented its direct chromatographic determination. Approximate value obtained by elecrophoresis at pH 5·65 followed by re-electrophoresis of neutral material at pH 8·5.

• Identity confirmed by electrophoresis as in ¶.

Tryptophane-load test in the rat with cystathioninuria induced by β-cyanoalanine

Four rats fed diets incorporating 0·5–0·6% BCNA for 17–21 days showed retardation of growth and hyper-irritability, especially when approached or touched. They

weighed 55–65 g and excreted 3·6–5·4 μmole cystathionine the day before being dosed with tryptophane. Subcutaneous injections of 40 mg of L-tryptophane resulted in a 24-hr increase in excretion of xanthurenic acid of 0·5 mg (0·36–0·56 mg). Controls showed an increase of 0·4 mg. Porter *et al.*,[25] using adult rats maintained on a pyridoxine-deficient diet for 30 days and given similar dosages of tryptophane, reported a 4-fold increase in excretion of xanthurenic acid over that of animals on the stock diet. Kuchinskas and du Vigneaud, using[26] a young rat fed a diet incorporating 0·35% penicillamine, observed a 24-hr increase of 9·5 mg in excretion of xanthurenic acid over that of the same rat on the basal diet.

Attempted reversal of cystathioninuria with injected pyridoxal

Two rats showing pronounced cystathioninuria on a diet incorporating 0·45% BCNA were injected daily with 50 mg pyridoxal·HCl for 15 days, starting on day 40. From day 34, before the pyridoxal had been administered, excretion of cystathionine declined somewhat, possibly due to decreased food consumption or to age. It is clear from the results shown in Table 3 that the large supplements of pyridoxal do not readily reverse the cystathioninuria. A similar lack of effect of pyridoxal on excretion of cystathionine was observed with a rat fed a 0·5% BCNA diet.

Effect of various vitamin B_6 antagonists and other substances on cystathionine excretion

Compounds were fed at levels sufficiently high to affect rate of growth. Included were the synthetic vitamin B_6 antagonists, penicillamine, cycloserine, and semicarbazide, and the lathyrus toxins, β-aminopropionitrile and 2,4-diaminobutyric acid. None of these, with the exception of BCNA and the pyridoxine-deficient diet, induced significant cystathioninuria (Table 5). The level of excretion of cystathionine on the pyridoxine-deficient diet (4·2 μmole/24 hr after 55 days) is in general agreement with the results of Hope,[8] who found 6 μmole/day with rats of another strain after 6–8 weeks on a B_6-deficient diet. BCNA, fed at levels of 0·3 and 0·45 per cent, is apparently far more effective in inducing excretion of cystathionine (7·5 and 16·3 μmole/24 hr, respectively, after 23 days) than is the pyridoxine-deficient diet.

DISCUSSION

In the rat, the protection afforded by pyridoxal against a single, toxic, injected dose of BCNA is reflected in the increase of the LD_{50} of this amino acid from 13·4–22·5 mg/100 g of body weight on administration of pyridoxal. This effect suggests a possible use for pyridoxal in acute poisoning by vetches or other natural materials containing BCNA. However, continuous supplementation of the diet with high levels of pyridoxal along with BCNA was not protective.* Moreover, pyridoxine, generally considered nutritionally equivalent to pyridoxal in the rat,[27] was not a substitute in protecting it under conditions in which pyridoxal was fully protective.

β-Cyanoalanine appears to be a specific agent for inducing cystathioninuria in the rat. β-Aminopropionitrile, the structurally related lathyrus principle derived from sweet peas, which produces skeletal (collagen) abnormalities in the rat,[28] and 2,4-diaminobutyric acid, a toxic factor in various other lathyrus peas, which induces hyper-irritability and delayed convulsions in the rat,[29] when ingested had little effect

* One possible explanation for the varying degrees of protection afforded by pyridoxal against BCNA toxicity is that a large dose of pyridoxal could mobilize inactive apoenzyme into active enzyme, an effect that should be less significant on repeated administration of inhibitor and pyridoxal.

on excretion of cystathionine (Table 5). In agreement is the recent observation that these factors, in contrast to BCNA, do not inhibit cystathionase in rat liver *in vitro*.[13] Penicillamine and semicarbazide, convulsants like BCNA, are known to inhibit certain transaminase, decarboxylase, or dehydrase activities by combining as carbonyl reagents with the pyridoxal cofactor or by inhibiting its formation.[30–32]

TABLE 5. EXCRETION OF CYSTATHIONINE BY THE RAT FED VARIOUS VITAMIN B_6 ANTAGONISTS AND OTHER SUBSTANCES

Substances fed	No. of rats	Day	Body wt.		Body wt. of controls	Cystathionine excretion (μmole/rat) (24 hr)
			Initial	Final	Final	
β-Aminopropionitrile · HCl, 0·38%	2*	17	35	69	91	0·4
L-2,4-Diaminobutyric acid · HCl, 0·25%	2	56	56	265	327	0·2
KCN,† 6 mg daily	1	6	334	309	339	0·2
DL-Penicillamine, 0·4%	2	29	130	115		0·6
Semicarbazide · HCl, 0·15%	1‡	29	53	124	203	0·1
Cycloserine, 0·75%	2	46	35	236	264	1·1
Pyridoxine-deficient diet	7	56	50	115		4·2
+ 0·0015% pyridoxine · HCl	7	57	50		282	<0·1
L-β-Cyanoalanine, 0·3%	2	24	37	101	152	7·5
0·45%§	2	24	40	134		16·3

* Rats had pronounced scoliosis at 3 weeks; they died on day 22 and day 26.

† Injected subcutaneously in 2-mg doses 3 times daily.

‡ Specimen taken 2 days before death. A second rat died on day 55.

§ Data of Table 3.

Penicillamine[13] and semicarbazide,[13, 33] acting on the cofactor, also inhibit rat liver cystathionase *in vitro*,but after having been ingested for almost 1 month, they induced little cystathioninuria. Although these data make it unlikely that BCNA is acting as an antagonist of B_6, they do not rule out that possibility since it has been recognized that vitamin B_6 inhibitors can selectively impair reactions *in vivo* that are dependent upon the cofactor.[32–34] But it does seem clear that cystathionase in the rat is not readily deprived of its B_6 cofactor by antagonists known to be able to bind that cofactor. Likewise, it is unlikely that BCNA is creating a deficiency of B_6 cofactor specifically in the cystathionine-cleaving reaction in view of the lack of response of the cystathioninuria of the treated rat to administration of pyridoxal.

The B_6-deficient rat characteristically has high levels of tissue cystathionine. Thus, for rats on B_6-deficient diets the following concentrations have been noted: 274 μmole cystathionine/100 g liver after 6–8 weeks (reported by Swendseid *et al.*[35]) 390 μmole/100 g brain after 4 weeks (reported by Hope[36]) and 175 μmole/100 g liver (observed here for the rats excreting 4·2 μmoles cystathionine after 8 weeks on the deficient diet). Since BCNA induces cystathioninuria in the rat more readily than does a diet deficient in vitamin B_6, it was interesting that there were only low concentrations of cystathionine in the tissues of the treated rat (0·1–1 μmole/100 g for rats excreting 9 μmole cystathionine after 8 weeks on the diet incorporating 0·45% BCNA). A further finding that differentiated the rat treated with BCNA from the B_6-deficient

rat was the failure to respond to the tryptophane-load test with an increased excretion of xanthurenic acid.†

The various results thus provide no tangible support for the concept of action of BCNA as an inhibitor of vitamin B_6.[6] They suggest perhaps a secondary function for pyridoxal in connection with BCNA that still remains to be explained. The cystathioninuria and its irreversibility by pyridoxal, the negative tryptophane-load test, and the inability of agents capable of binding pyridoxal phosphate to induce significant excretion of cystathionine can all be reconciled with the recent finding that BCNA is an inhibitor, probably of the structural type, of rat liver cystathionase.[13]

In addition to its unexpectedly low content of cystathionine, the liver of the rat fed BCNA was characterized by the presence of a prominent new component recently identified as γ-glutamyl-β- cyanoalanylglycine.[24] This bound form of BCNA, which is an analog of glutathione in which a β-cyanoalanine residue replaces the cysteine residue, constituted about 70 per cent of the BCNA present in the liver (212–318 μmole/100 g, Table 4). The tripeptide was also present, although in smaller amount, in all other tissues examined with the exception of the kidney and urine. Appreciable concentrations of free BCNA and cystathionine in the presence of each other were found only in urine. β-Cyanoalanine ingested by the rat is apparently converted into the γ-glutamyl tripeptide, which in turn is hydrolyzed, probably by the kidney, to BCNA for excretion. (Consistent with this is the finding that administered γ-glutamyl-β-cyanoalanine is excreted largely as BCNA. Moreover, in the rat and a number of other mammals, kidney, compared to other organs, is a relatively rich source of γ-glutamyl transpeptidase and enzymes capable of hydrolyzing the analogous glutathione.[39,40]) In contrast to BCNA, γ-glutamyl-β-cyanoalanine does not inhibit purified rat liver cystathionase *in vitro*.[13] If, as expected, the behavior of γ-glutamyl-β-cyanoalanylglycine toward cystathionase is similar to that of the dipeptide, incorporation by the rat of dietary BCNA into γ-glutamyl-β-cyanaoalanylglycine might then be a detoxicating mechanism, with only the unbound BCNA causing cystathionine to accumulate as the result of inhibiting cystathionase. The relative distribution in the various tissues of cystathionine, free BCNA, and the bound form of BCNA suggests that, at the levels of BCNA studied, inhibition of cystathionase by BCNA might take place in the kidney chiefly to a significant degree.

† The tryptophane-load test is negative also in human cystathioninuria,[12] in which the defect apparently lies in the B_6-requiring cystathionase reaction.[37, 38]

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