Toxic Principle in Vetch. Isolation and Identification of γ-L-Glutamyl-L-β-cyanoalanine from Common Vetch Seeds. Distribution in Some Legumes[1,2]

Charlotte Ressler, S. N. Nigam, and Y.-H. Giza

Contribution from the Division of Protein Chemistry, Institute for Muscle Disease, Inc., New York, New York 10021. *Received October 16, 1968*

Abstract: γ-Glutamyl-β-cyanoalanine has been identified in seedlings and seeds of common vetch and isolated from the seeds in crystalline form after electrophoresis or anion-exchange chromatography. Its structure has been confirmed by synthesis. For this a mixed anhydride procedure and protecting groups that were removed by hydrogenolysis were used. The α isomer, α-glutamyl-β-cyanoalanine, also has been synthesized and differs in its properties from the naturally occurring peptide. γ-Glutamyl-β-cyanoalanine is neurotoxic to the young rat and chick. Together with the β-cyanoalanine present in the free state, it can account for the toxicity of common vetch seeds to the chick. Among seeds of 41 various legumes that have been surveyed quantitatively, β-cyanoalanine is present in every variety of common vetch and in approximately one-third of the vetch species and is accompanied by β-cyanoalanine in bound form.

During a study of a metabolic relationship between β-cyanoalanine and asparagine in certain species of *Lathyrus* and vetch, L-β-cyanoalanine-4-^{14}C was administered to seedlings of *Vicia sativa* (common vetch).[3,4] Radioactivity accumulated as unidentified, soluble, acidic material. Since all other plants examined converted β-cyanoalanine-4-^{14}C into asparagine, the ^{14}C-labeled vetch material was investigated with the hope of identifying a biological intermediate in the synthesis of asparagine. With the Birch reagent the material was found to be reducible to bound 2,4-diaminobutyric acid.[5] Such behavior suggested the presence, for the first time, of a natural bound form of β-cyanoalanine. The labeled, reducible material was purified by electrophoresis and chromatography on paper. Hydrolysis converted it into glutamic acid and aspartic acid, which suggested that the administered β-cyanoalanine had been incorporated as a peptide of glutamic acid. Bound β-cyanoalanine was then detected in seedlings that had received no β-cyanoalanine-^{14}C, and the β-cyanoalanine peptide was identified electrophoretically in seeds of the same plant in which it accompanies β-cyanoalanine in the free state.

Identification of a second form of β-cyanoalanine in common vetch seeds had further interest in view of the neurotoxicity of this amino acid.[6] In addition to their use as cover crops, vetches have had use in livestock and human nutrition[7] and represent a potential source of food in times of shortage. Moreover, common vetch seeds of a closely related variety contaminating wheat and lathyrus peas have been associated with several outbreaks of lathyrism in man.[6,8,9] This paper presents in detail the isolation from common vetch seeds of γ-L-glutamyl-L-β-cyanoalanine in crystalline form, its characterization, and its chemical synthesis, as well as the preliminary evidence leading to its detection. Feeding experiments carried out with the chick suggest that β-cyanoalanine and γ-glutamyl-β-cyanoalanine can account for the toxicity of common vetch seeds to this species. The role of γ-glutamyl-β-cyanoalanine (or β-cyanoalanine) in certain legumes as a major product of cyanide fixation and as an intermediate in the biosynthesis of asparagine from cyanide is presented in the accompanying paper.[4] Part of this work has been previously reported.[10]

Results and Discussion

Although young seedlings of common vetch were far superior with regard both to the concentration of glutamyl-β-cyanoalanine and the number of contaminants that had to be removed, seeds were chosen as the starting material for the isolation of the dipeptide[11] because they were available commercially. The distribution of acidic material was noted with the R_f and electrophoretic mobility established for glutamyl-β-cyanoalanine-^{14}C in the preliminary metabolic experiment. Presence of bound β-cyanoalanine was confirmed by reduction.[5]

Material in aqueous ethanol seed extracts was adsorbed on Amberlite CG-120 (H^+ cycle) cation-exchange resin. That which eluted early with 3 *N* NH_3 was electrophoresed on blocks of Solka-floc in pyridinium acetate buffer, pH 5.7. An acidic fraction was then reelectrophoresed at pH 3.5; this enabled the glutamyl-β-cyanoalanine to be separated from aspartic acid and glutamic acid, the chief acidic contaminants. The eluted peptide was isolated and characterized as a crystalline dicyclohexylammonium salt. Amino acid analysis[12] showed 1 mol each of aspartic acid, glutamic acid, and NH_3. In the analysis for bound β-cyanoalanine[5] 1 mol of glutamic acid was present, and

(1) Aided by Grant NB 04316 from the U. S. Public Health Service and by Muscular Dystrophy Associations of America.

(2) Presented (a) at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept 1963 (Abstracts, p 4A), at the Symposium on Deleterious Compounds of Natural Origin in Foods and Feeds, and at the Third Conference on Toxicity of Cycads, Chicago, Ill., April 1964.

(3) C. Ressler, Y.-H. Giza, and S. N. Nigam, *J. Am. Chem. Soc.*, **85**, 2874 (1963).

(4) C. Ressler, Y.-H. Giza, and S. N. Nigam, *ibid.*, **91**, 2766 (1969).

(5) C. Ressler and D. V. Kashelikar, *ibid.*, **88**, 2025 (1966).

(6) C. Ressler, *J. Biol. Chem.*, **237**, 733 (1962).

(7) J. A. Harper and G. H. Arscott, *Poultry Sci.*, **41**, 1968 (1962).

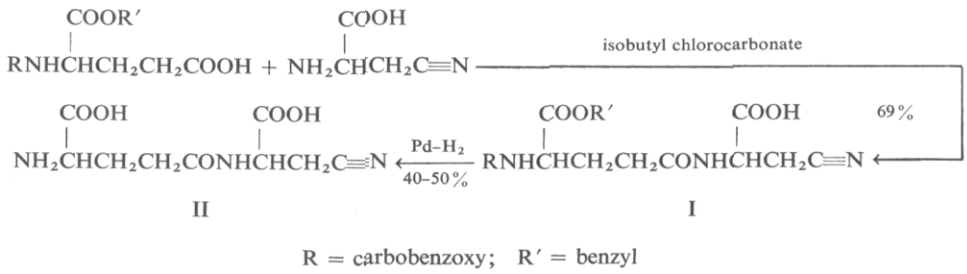
(8) S. R. A. Shah, *Indian Med. Gaz.*, **74**, 385 (1939).

(9) L. A. P. Anderson, A. Howard, and J. L. Simonsen, *Indian J. Med. Res.*, **12**, 613 (1925).

(10) (a) See ref 2a; (b) C. Ressler, *Federation Proc.*, **23**, 1350 (1964); (c) C. Ressler, S. N. Nigam, Y.-H. Giza, and J. Nelson, *J. Am. Chem. Soc.*, **85**, 3311 (1963).

(11) Occurrence of glutamyl-β-cyanoalanine in common vetch seeds was subsequently observed electrophoretically by B. Tschiersch, *Tetrahedron Letters*, **13**, 747 (1964).

(12) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).



R = carbobenzoxy; R′ = benzyl

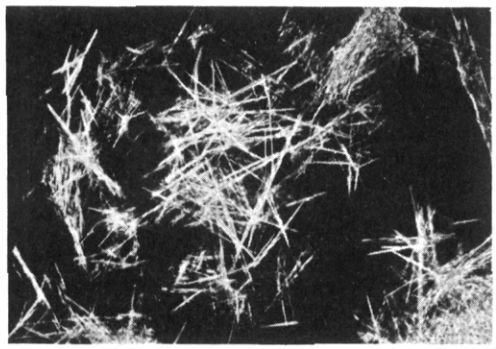


Figure 1. Photomicrograph of crystals of γ-L-glutamyl-L-β-cyanoalanine isolated from seeds of *Vicia sativa*.

1 mol of 2,4-diaminobutyric acid appeared in place of aspartic acid.

γ-Glutamyl-β-cyanoalanine was isolated more directly and conveniently by passing crude seed extracts through a column of Dowex 1-X4 (acetate cycle) anion-exchange resin and eluting the adsorbed material with two concentrations of pyridinium acetate, pH 4.1, which also afforded good separation from aspartic and glutamic acids. The chromatographed peptide was precipitated from water with alcohol, then crystallized from water. To obtain large amounts of γ-glutamyl-β-cyanoalanine, crude extracts of 250 g of seeds were chromatographed in batches on a resin column, 4.5 × 55 cm. For convenience crude dipeptides from two or three such batches were combined before crystallization. Isolation yields of the dipeptide were calculated on the basis of bound β-cyanoalanine, expressed as γ-glutamyl-β-cyanoalanine, in the crude aqueous ethanol seed extracts. In nine chromatographic runs with a total of 1.8 kg of seeds, the yield was 55–67% of dipeptide melting 3–6° below the purest material. Further recrystallization afforded 34–38% of γ-glutamyl-β-cyanoalanine of highest purity or melting within 2° of it. The isolated dipeptide was homogeneous on the amino acid analyzer and on paper chromatography. It had a correct elementary analysis for γ-glutamyl-β-cyanoalanine and the expected amino acid and ammonia composition after hydrolysis. Crystals of natural γ-glutamyl-β-cyanoalanine are shown in Figure 1.

The sequence glutamyl-β-cyanoalanine was established by applying the DNP technique.[13] That the glutamic acid residue was linked through its γ-carboxyl group to the amino group of β-cyanoalanine was established by synthesis of γ-L-glutamyl-L-β-cyanoalanine and its isomer α-L-glutamyl-L-β-cyanoalanine and by a close comparison of these with the natural peptide and with each other.

(13) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

Synthetic peptides having β-cyanoalanine in a position other than N-terminal were unknown. The route used to γ- and α-glutamyl-β-cyanoalanines involved the intermediate protected dipeptides α-benzyl-N-carbobenzoxy-γ-L-glutamyl-L-β-cyanoalanine (I) and γ-benzyl-N-carbobenzoxy-α-L-glutamyl-L-β-cyanoalanine (III), respectively. I was prepared by coupling α-benzyl-N-carbobenzoxy-L-glutamate with synthetic β-cyanoalanine in 96% tetrahydrofuran by the mixed anhydride procedure with isobutyl chlorocarbonate[14] as shown in the scheme. It was unnecessary to protect the carboxyl group of β-cyanoalanine as the product was isolated readily as its recrystallized dicyclohexylammonium (DCHA) salt in 69% yield. A similar attempt to prepare III by a mixed anhydride procedure coupling γ-benzyl-N-carbobenzoxy-L-glutamate with β-cyanoalanine resulted in very low yield. III was synthesized in somewhat better yield by a modified *p*-nitrophenyl ester procedure.[15] γ-Benzyl-α-*p*-nitrophenyl-N-carbobenzoxy-L-glutamate was treated with β-cyanoalanine in 86% tetrahydrofuran at 5°. The product, obtained in 21% yield as the purified DCHA salt, agreed in properties with the product of the mixed anhydride coupling.

Protecting groups were removed from I by hydrogenolysis in the presence of palladium black catalyst after removal of DCHA with Amberlite CG-120 (H^+ cycle) resin. The resulting γ-glutamyl-β-cyanoalanine (II) was obtained as the crystalline free dipeptide and, in 40–50% yield, as the DCHA salt, the superior crystallizing properties of which appreciably facilitated isolation and purification. Hydrogenolysis of the DCHA salt of I afforded γ-glutamyl-β-cyanoalanine-DCHA directly.

Synthetic α-L-glutamyl-L-β-cyanoalanine, obtained as the major product of hydrogenolysis of III, was readily distinguishable from natural glutamyl-β-cyanoalanine by its behavior on paper electrophoresis and on column chromatography before and after reduction with sodium–methanol–ammonia. Synthetic γ-L-glutamyl-L-β-cyanoalanine agreed well with the natural peptide in all chemical and physical criteria examined [see Identification of N-(γ-Glutamyl)-β-cyanoalanine in Seeds and Seedlings . . . .]. These also showed similar toxicity in the weanling white rat; dosages of 46 and 50 mg per 100 g of body weight administered subcutaneously resulted in convulsions and death.

In feeding experiments with the several-day-old chick γ-glutamyl-β-cyanoalanine and free β-cyanoalanine had similar toxicities on a molar basis. Lethal toxicity develops at a dietary level of β-cyanoalanine near 0.075%. The rat excretes γ-glutamyl-β-cyanoalanine largely as β-

(14) J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).
(15) M. Bodánszky, M. Szelke, E. Tömörkény, and E. Weisz, *Acta Chim. Acad. Sci. Hung.*, **11**, 179 (1957).

cyanoalanine,[16] and it seems likely that the toxicity of the peptide is that of the free amino acid. Diets of common vetch seeds have been reported toxic to the chick,[7] duck,[9] and monkey,[9] but not to the pony[17] and rat.[18] This striking species difference has been confirmed for the chick and the rat and can be reconciled with the species difference in susceptibility to dietary β-cyanoalanine and the content of free and bound β-cyanoalanine in the seeds. Studies appear elsewhere of the metabolism and action of β-cyanoalanine in experimental animals, especially as an inhibitor of rat liver cystathionase.[16,19] A possible role in this for γ-glutamyl-β-cyanoalanine and γ-glutamyl-β-cyanoalanylglycine, another new natural bound form of β-cyanoalanine, has been suggested.[16,20]

Because of their toxic properties and their widespread role in cyanide metabolism in plants,[3,4] an attempt was made to obtain additional information on the distribution of β-cyanoalanine and γ-glutamyl-β-cyanoalanine. Seeds of 14 varieties of *V. sativa*, 13 *Vicia*, and 7 *Lathyrus* species, and a limited number of other members of the *Leguminosae*, including seeds that contain cyanogenetic glycosides and several peas that serve as common foods for man, were examined for free, and, in many cases, for either γ-glutamyl- or bound β-cyanoalanines. All the varieties of *V. sativa* had significant amounts of free β-cyanoalanine. Since in two of these γ-glutamyl-β-cyanoalanine was considerably less than in the commercial variety, substituting one of these for commercial culture might be advantageous. Four of the vetch species had free and large amounts of bound β-cyanoalanine. Twelve additional vetch species have since been found to have β-cyanoalanine and γ-glutamyl-β-cyanoalanine.[21] In each seed investigated for both, free β-cyanoalanine was always accompanied by bound β-cyanoalanine. γ-Glutamyl-β-cyanoalanine, however, was present in a small quantity in a species of *Lathyrus* in which no β-cyanoalanine was detected, and trace amounts of bound β-cyanoalanine were found in a number of species of *Lathyrus* and vetch having no free β-cyanoalanine. No correlation was observed between the presence of cyanogenetic glycoside and β-cyanoalanine in the two cyanogenetic glycoside-bearing plants, *V. angustifolia* and *Lotus corniculatus*; only the former contains significant β-cyanoalanine. This amino acid was not detected in any of the common foods examined. So far, β-cyanoalanine appears to accumulate only in some species of *Vicia*,[10,21] in *Lathyrus sylvestris* W. seedlings,[4,10b] and in *Chromobacterium violaceum*.[22]

Experimental Section[23]

Sources of Seeds. Common and hairy vetch seeds were purchased from Craver-Dickinson Seed Co., Buffalo, N. Y. Seeds of the varieties of *Vicia sativa* and of some of the other species of vetch were obtained as specimen packets from the Instituto Botanico Dell' Universita, Siena, Italy. Seeds of the vetches, *V. angustifolia*, *V. monantha*, *V. bengalensis*, *V. dasycarpa*, *V. pannonica*, *V. cracca*, were obtained from the U. S. Department of Agriculture; caley peas, from Sawan, Inc., Columbus, Mo.; alfalfa seed, lima bean, soybean, sweet pea, perennial pea, lentil, and broad bean, from Vaughan's Seed Co. and Herbst Brothers Seedsmen, Inc., New York, N. Y., or W. Atlee Burpee Co., Philadelphia, Pa.; lupin and bird's foot trefoil, from Fredonia Seed Co., New York, N. Y.

Scanning of Seeds of Various Legumes for Free and Bound β-Cyanoalanine. Extraction. Pulverized, hexane-extracted seeds (0.1–1 g) of varieties of *V. sativa* were extracted with methanol overnight under anhydrous conditions in a micro-Soxhlet extractor. Preliminary experiments with *V. sativa* seeds had shown this procedure to lead to almost the same concentration of β-cyanoalanine (β-CNAla) as extraction with 30% ethanol overnight at 5°. However it was unsuitable for γ-glutamyl-β-cyanoalanine (γ-Glu-β-CNAla), which lost potential ninhydrin color when heated in methanol in a sealed tube at 100° overnight. To determine bound β-CNAla, the hexane-extracted material was stirred at 5° overnight with 30% ethanol, 5 ml/g. Extraction with aqueous alcohol was repeated, and the combined extracts were concentrated. The residues were taken up in water and clarified by centrifugation.

Determinations. β-Cyanoalanine was identified by its unusual green color with ninhydrin in acetone and its characteristic electrophoretic mobility in systems 2 and 4.[6] In several instances, identity was confirmed and concentration determined on the amino acid analyzer with system A. The absorbance of the ninhydrin product at 570 and 440 mμ had the ratio 2.2. **γ-Glutamyl-β-cyanoalanine** was determined on the amino acid analyzer with system A. In early experiments, elution volumes of natural and synthetic γ-Glu-β-CNAla tended to vary between 83 and 114 ml, which made it difficult with samples of natural origin to differentiate the peptide from taurine and urea emerging near 81 and 92 ml, respectively. Moreover, it was eluted sometimes as a single peak, other times as a broad, partly resolved peak with a trailing edge or as a double peak. Whereas the exact volume of pH 2.2 buffer and the manner in which the sample is applied to the chromatographic column appear to have little effect with common amino acids, these factors influence the elution pattern of γ-Glu-β-CNAla.

Standard Conditions. Samples were applied to the column in 1 ml of pH 2.2 buffer followed by an additional 1 ml of the buffer as a single rinse. γ-Glutamyl-β-cyanoalanine emerged consistently as a very sharp peak at effluent volume 114 ml immediately before β-CNAla. Its color yield constant was 20.9, compared to 22.1 for leucine. The absorbance of the ninhydrin product at 570 and 440 mμ had the ratio 4.6.

Quantitative determination of γ-Glu-β-CNAla in this manner was unreliable, however, when the chromatographic pattern of the amino acid analyzer was complicated in the region of this peptide, as in extracts of seeds of common vetch and of certain other plants. Instead, γ-Glu-β-CNAla was determined indirectly as total **bound β-CNAla.** The 30% ethanol extract corresponding to 200 mg of seed was concentrated to dryness in a 6-ml test tube, which was then surrounded by a cooling bath and set up for condensation of NH_3. Reduction with sodium–ammonia–

(16) C. Ressler, J. Nelson, and M. Pfeffer, *Biochem. Pharmacol.*, **16**, 2309 (1967).
(17) H. Scott, *Indian J. Med. Res.*, **18**, 51 (1930–1931).
(18) R. McCarrison, *ibid.*, **15**, 797 (1927–1928).
(19) M. Pfeffer and C. Ressler, *Biochem. Pharmacol.*, **16**, 2299 (1967).
(20) K. Sasaoka, C. Lauinger, S. N. Nigam, and C. Ressler, *Biochim. Biophys. Acta*, **156**, 128 (1968).
(21) E. A. Bell and A. S. L. Tirimanna, *Biochem. J.*, **97**, 104 (1965).
(22) M. M. Brysk, W. A. Corpe, and L. V. Hankes, *J. Bacteriol.*, **97**, 322 (1969).
(23) Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill. Melting points were determined in capillaries and are corrected. Many of them varied with the rate of heating. Samples were placed in a bath about 15° below the melting point and were heated at the rate of 2 or 3° per min. Optical rotations were measured in a 2-dm cell in a Rudolph polarimeter, Model 80. Concentrations were carried out below 30° under reduced pressure on a rotary evaporator. Hydrolyses were in twice distilled 6 *N* HCl under N_2 in sealed glass tubes at 110–120° for 14–16 hr. Amino acid analyses were performed on a Beckman-Spinco automatic amino acid analyzer, Model 120. Neutral and acidic substances were determined in system A as recommended for physiological fluids[12] with the 150-cm resin column with 0.2 *M* citrate buffer at pH 3.25 and 30° with a change to pH 4.25 and 50° at 11–13 hr. 2,4-Diaminobutyric acid and other basic substances were determined in system B with the 50-cm column at pH 4.26 and 50°.[5] Paper electrophoresis was on strips of Whatman No. 1 paper at room temperature at 9 V/cm for 3–6 hr unless indicated otherwise. Preparative electrophoresis was at 5°. System 1 was pyridinium acetate buffer, pH 3.5; system 2, pyridinium acetate, pH 5.7; system 3, triethylammonium acetate, pH 8.5; system 4, sodium barbital, pH 8.5. Paper chromatography was on the same paper in system 5, pyridine–water (65:35); system 6, *n*-butyl alcohol–acetic acid–water (5:1:5); or system 7, pyridine–water–acetic acid (10:7:3). The papers were developed by spraying with 0.15% ninhydrin in acetone and heating briefly at 105°. Paper strips were scanned for radioactivity as given in ref 4.

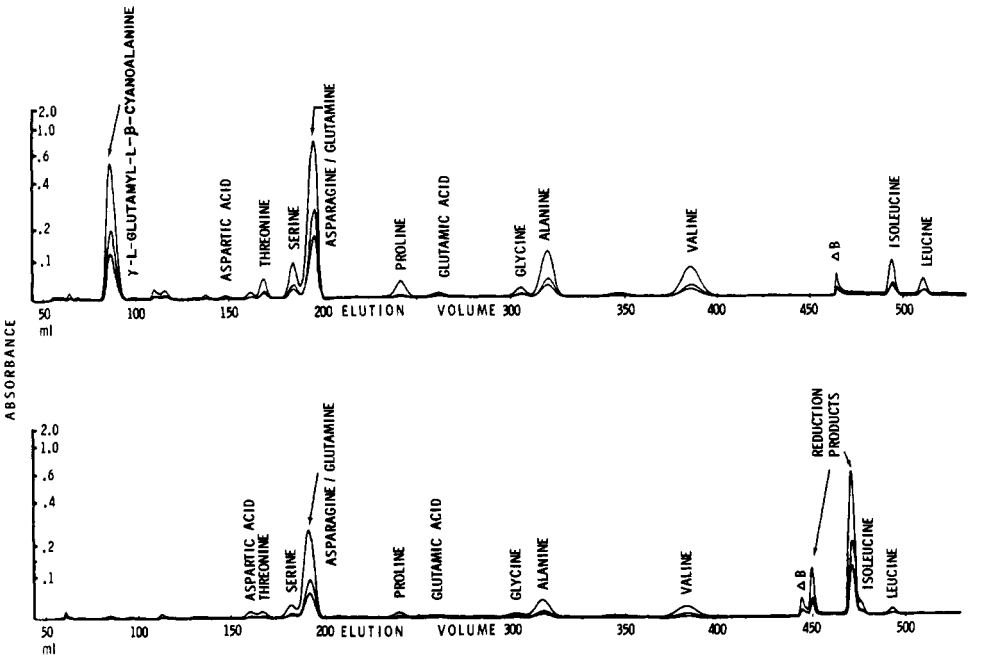


Figure 2. Amino acid analysis. The upper curve shows the presence of γ-glutamyl-β-cyanoalanine in a crude extract of tissues of 17-day-old seedlings of *V. sativa*. Material extracted from 4.75 mg of tissue by 30% ethanol was applied to the column in only 0.5 ml of pH 2.2 buffer, The lower curve shows the disappearance of γ-glutamyl-β-cyanoalanine and the products formed on reduction of the same extract with sodium–ammonia–methanol. [For chromatography and reduction of γ-glutamyl-β-cyanoalanine, see Determinations, Reduction of γ-Glutamyl-β-cyanoalanine, and Identification of N-γ-Glutamyl-β-cyanoalanine in Seeds and Seedlings, sections a and c.]

methanol, desalting, and hydrolysis were as described in a dehydration–reduction procedure,[5] except that 0.15 ml of methanol was used for reduction and the desalting column contained 8 ml of resin and was eluted with 6 *N* NH_3. Half of the hydrolysate was analyzed for 2,4-diaminobutyric acid (2,4-DAB) on the amino acid analyzer. The procedure allows detection of less than 0.01% free and bound 2,4-DAB *plus* free and bound β-CNAla. Extracts showing 2,4-DAB were checked for free and bound 2,4-DAB in the original extract by desalting, hydrolysis, and amino acid analysis. Corrections, which generally were minimal, were made for this except when noted. The 2,4-DAB so obtained represents the total free and bound β-CNAla in the extract. Deducting the free β-CNAla gave bound β-CNAla.[24]

Reduction of γ-Glutamyl-β-cyanoalanine. The procedure for determining bound β-CNAla was modified as follows. The sample containing 1.5–2.5 μmol of γ-Glu-β-CNAla, dissolved in 3 ml of liquid NH_3 and 50–100 μl of methanol, was treated with excess sodium. At the end of the reduction some crystals of ammonium chloride were added to the solution. The residue left after removal of NH_3 and methanol was cooled and dissolved in cold 0.1 *N* acetic acid. The solution was immediately adjusted to pH 5 and analyzed directly without hydrolysis on the amino acid analyzer with system A.

Experiments with Animals. Sherman strain, male albino rats and White Leghorn chicks of either sex were used. Their basal rations are given elsewhere.[10c,25] β-Cyanoalanine, γ-Glu-β-CNAla, and ground vetch seeds were incorporated into diets by mixing in the indicated proportions with the basal ration. The animals were maintained on the basal ration for 1–6 days before the experiments. Controls received the basal ration. Food and water were taken *ad libitum*.

Preliminary Indication of the Presence of Bound β-Cyanoalanine and Glutamyl-β-cyanoalanine. Nine-day-old common vetch seedlings growing in sterile culture had received 3.90 mg of L-β-cyanoalanine-4-^{14}C, 3.05 × 10^6 cpm, in nutrient medium.[3,4] Eight days later they were removed, rinsed, freed of seeds, and dried under vacuum; weight 366 mg. This was extracted at 5° and the soluble material was electrophoresed on paper in system 2. More than 88% of the recovered radioactivity was located in a ninhydrin-positive acidic area 6–12 cm from the origin and was eluted with water. Amino acid analysis showed two major unidentified peaks at effluent volumes 107 and 115 ml of 12 and 34 μmol, respectively, and minor amounts of other substances. Reduction and hydrolysis of a sample formed 4.1 μmol of 2,4-DAB. The material was reelectrophoresed in system 1 for 7 hr. The acidic area was cut into ninhydrin-positive fractions A (14–15 cm), B (10–11 cm), C (7 cm), and D (4.5 cm from the origin) which were eluted with water. Eluates were concentrated to dryness and analyzed for bound β-CNAla (see Determinations). Fraction A, containing over 87% of the recovered activity, was the only one to form 2,4-DAB. It was chromatographed on paper in 75% phenol (descending). Radioactive, ninhydrin-positive material, R_f 0.46, separated from highly radioactive, ninhydrin-negative materials, R_f 0.62 and 0.75. Area of R_f 0.46 was eluted with water and rechromatographed in system 5, R_f 0.57, and in system 7, R_f 0.26, with no separation of radioactivity from ninhydrin-reactive material in either system. Hydrolysis of the chromatographed material gave glutamic acid (Glu) and aspartic acid (Asp) in the molar ratio 1.00:1.04. Reduction and hydrolysis led to Glu, Asp, and 2,4-DAB in the molar ratio 1.00:0.84:0.3.[26] Another portion of 0.14 μmol in 0.55 ml of H_2O containing 4.1 mg of $NaHCO_3$ was treated with 5.8 mg of 2,4-dinitrofluorobenzene in 0.5 ml of ethanol for 16 hr. Processed in the usual way, it gave after hydrolysis Asp and Glu in the molar ratio 1.00:0.005. That the formed dipeptide is γ-L-glutamyl-L-β-cyanoalanine-^{14}C was established subsequently by chromatographic criteria and by cocrystallization with synthetic γ-Glu-β-CNAla-DCHA (LL) to constant specific activity.[3,4]

Forty-six-day-old common vetch seedlings, cultured in a similar manner, had been kept in a resting state until the 38th day, when they received nutrient medium only. Tissue extracts were electrophoresed in system 2, and the entire acidic fraction was eluted. One portion was hydrolyzed directly, and another was reduced and hydrolyzed, after which they showed Glu, Asp, and 2,4-DAB in the molar ratios of 1.00:0.88:0 and 1.00:0.29:0.73,[26] respectively. In

(24) For a direct, recently developed enzymatic method for determining β-cyanoalanine in simple peptides, see ref 20. The common vetch peptide yielded 1 mol each of β-cyanoalanine and glutamic acid.

(25) C. Ressler, J. Nelson, and M. Pfeffer, *Nature*, **203**, 1286 (1964).

(26) Reduction of bound β-cyanoalanine is expected to be quantitative (ref 5, Table I). The ratios could suggest the presence of GluAsp or GluAsn incompletely removed from Glu-β-CNAla. Although these may occur naturally, they may have arisen in preliminary experiments before reduction by solvolysis of the cyano group, perhaps on drying the paper chromatograms or electropherograms with heat.

addition to being formed by seedlings in response to administration of β-CNAla, bound β-CNAla thus occurs naturally.

Figure 2, upper curve, is a typical chromatogram of the neutral and acidic ninhydrin-positive substances present in a crude extract of tissues of 17-day-old common vetch seedlings. The simplicity of the physiological mixture is striking; it is apparent that γ-Glu-β-CNAla is a major constituent of the nonprotein nitrogen fraction of the plant at this stage; its concentration was 1.7%. Identification of the dipeptide in tissues of young seedlings is discussed further under Identification of N-(γ-Glutamyl)-β-cyanoalanine in Seeds and Seedlings, sections a and c.

Common vetch seeds also showed in 30% ethanol extracts material with the electrophoretic mobility in system D of glutamyl-β-cyanoalanine-^{14}C. Their total bound β-CNAla, expressed as γ-Glu-β-CNAla, was 0.58%.

Isolation of N-(γ-L-Glutamyl)-L-β-cyanoalanine from Seeds of *Vicia sativa*. a. By Preparative Electrophoresis. Pulverized *V. sativa* seeds, 200 g, were extracted repeatedly at room temperature with hexane until extracts were colorless. The powder was then extracted twice at 5° with a total of 2 l. of 30% ethanol. Extracts were concentrated, diluted to 100 ml with water, and clarified by centrifugation. The solution was adjusted to pH 2, again centrifuged, then applied to a 2.4 × 60-cm column of Amberlite CG-120 (H^+ cycle) (100–200 mesh) which was washed with water. Adsorbed material was eluted with 3 *N* NH_3 in 50-ml fractions which were rapidly concentrated to dryness under high vacuum. Residues were examined by paper electrophoresis in system 2. The first three basic fractions containing considerable amounts of ninhydrin-positive, acidic material were combined and dissolved in 200 ml of water. The solution was neutralized with 40% acetic acid, then warmed and treated with 6.2 g of activated charcoal for 20 min. The colorless filtrate was concentrated to dryness; yield 5.45 g. The latter, dissolved in 3.8 ml of water, was adjusted to pH 5.7 and electrophoresed on two blocks of Solka-floc (Brown Co., Concord, N. H., 12 × 38 × 1 cm) in system 2 for 48 hr. The acidic regions were examined by paper electrophoresis in systems 1 and 4. Fraction A, located 2.4–14 cm from the origin, contained Asp and Glu and material with the mobility of the desired peptide. Administered *per os* to 37-g chicks, it produced symptoms of β-CNAla toxicity[16] and was lethal at a dosage of 70 mg.

Fraction A was concentrated and reelectrophoresed on Solka-floc in system 1 at pH 3.5 for 76 hr. Consecutive segments in the acidic region were eluted and examined by electrophoresis in systems 1 and 3. Fraction 1 (0–3.8 cm from the origin toward the anode) contained chiefly Glu, and fraction 2 (3.8–7.5 cm), chiefly Asp. Material with the mobility of Glu-β-CNAla was present in fractions 3, 4, and 5 (7.5–16 cm), with a lesser amount in fraction 2. Except for a small amount of impurity in fraction 3, fractions 3–5 seemed free of ninhydrin-positive contaminants and were combined. However, because of indication of extensive heterogeneity when this material was chromatographed on the amino acid analyzer, it was set aside as a frozen aqueous solution. It was crystallized some months later after experience with synthetic γ-Glu-β-CNAla had suggested that heterogeneity could be a chromatographic artifact (see Determinations for Chromatography of γ-Glu-β-CNAla). The solution was concentrated to dryness. An aliquot of 28% was adjusted from pH 3.5 to 6.0 with a dilute solution of dicyclohexylamine in terahydrofuran. The solution was then reduced in volume, and warm tetrahydrofuran was added. Crystallization soon started. Cooling overnight and filtration gave 216 mg, mp 176–177° dec. Two recrystallizations from water–tetrahydrofuran left 174 mg, mp 183.5–185° dec. For analysis γ-glutamyl-β-cyanoalanine dicyclohexylammonium salt (γ-Glu-β-CNAla-DCHA) was recrystallized twice again, then air dried; mp 184–185° dec; $[\alpha]^{25}D$ +15.1° (*c* 0.6, 2.5% $KHCO_3$).

Anal. Calcd for $C_{21}H_{36}N_4O_5 \cdot 0.5H_2O$: C, 58.2; H, 8.60; N, 12.9; H_2O, 2.08. Found: C, 58.1; H, 8.46; N, 13.1; H_2O, 2.61.

Amino acid analysis gave Asp, Glu, and NH_3 in the molar ratios 1.00:1.02:1.00, with quantitative recovery. Analysis of 2.28 mg for bound β-CNAla gave Glu, Asp, and 2,4-DAB in the molar ratios 0.94:0.01:1.00. The average yield of Glu and 2,4-DAB was 89%; of Asp, less than 1%.

b. By Direct Column Chromatography of Crude Extracts of Seeds. Material extracted from 75 g of seeds was dissolved in 47 ml of water and applied to a 2.4 × 60-cm column of Dowex 1-X4 (acetate cycle) (Bio-Rad AG 1-X4), 100–200 mesh, followed by a 5-ml water rinse. The column was washed with 700 ml of deionized water, then eluted with pyridinium acetate buffer A, pH 4.1 (11 ml of acetic acid and approximately 3 ml of pyridine per l.). Fractions of 10 ml were collected at a flow rate of 0.8 ml/min until 900 ml had been collected. The pyridinium acetate was then increased five times in concentration, buffer B, pH 4.1. Analysis with ninhydrin[27] located material in fractions 1 (980–1100 ml), 2 (1240–1320 ml), and 3 (1400–1640 ml). Paper chromatography in system 5 showed fraction 1 to contain chiefly Glu; fraction 2, Asp and small amounts of other materials; and fraction 3, Glu-β-CNAla (R_f 0.55, ascending). The latter was concentrated to dryness, the residue was dissolved in 15 ml of water, and the solution was again concentrated. The process was repeated several times, and the colorless film was left overnight evacuated at 0.3 mm; yield 620 mg. The material was eluted on the amino acid analyzer at 112 ml. The film was dissolved in 2 ml of water and precipitated by excess ethanol as a gum that solidified on cooling; yield 380 mg. Crystallization was initiated from a small volume of water; 80% ethanol was then cautiously added. The mixture was allowed to stand first at room temperature, then in the cold. Recrystallization from water–ethanol in a similar manner yielded 146 mg of colorless needles, mp 182–186° dec. It had the expected elementary analysis but 92% homogeneity and 6% of a shoulder at 116 ml on amino acid analysis.

The extract of 540 g of seeds was chromatographed in two portions on a 4.5 × 55-cm column of Dowex-1 resin. Precipitation with ethanol afforded 3.41 g of crude solid which was crystallized from approximately 4 ml of water. After 2 days at 5° the mixture was diluted with 80% ethanol. The soft white solid was separated from the green mother liquor by centrifugal filtration. It was washed well on the filter once with 85% ethanol, then twice with 90% ethanol and dried under vacuum; yield 2.04 g, mp 182–184° dec. Recrystallization from water–ethanol yielded 1.7 g (55%), mp 183° dec. Recrystallization of 300 mg from 0.6 ml of hot water yielded after several days at 5° and filtration at the centrifuge 213 mg (38%), mp 186–187° dec; $[\alpha]^{26}D$ +14.5° (*c* 0.8, water).

Anal. Calcd for $C_9H_{13}N_3O_5$: C, 44.4; H, 5.39; N, 17.3. Found: C, 44.7; H, 5.49; N, 17.0.

The dipeptide was homogeneous when chromatographed on the amino acid analyzer, on paper in systems 5 and 6, and on electrophoresis in systems 1, 2, and 3. Chromatographic and electrophoretic constants and behavior on reduction are given under Determinations or Identification of N-(γ-Glutamyl-β-cyanoalanine in Seeds and Seedlings. Amino acid analysis of a hydrolysate gave Asp, Glu, and NH_3 in the molar ratios 1.01:1.00:1.00 with 98% recovery.

Synthesis of N-(γ-L-Glutamyl)-L-β-cyanolanine. α-Benzyl-N-carbobenzoxy-γ-L-glutamyl-L-β-cyanoalanine (I) Dicyclohexylammonium Salt. α-Benzyl-N-carbobenzoxy-L-glutamate was prepared by treating N-carbobenzoxyl-L-glutamic anhydride with benzyl alcohol[28] and was purified as described through its DCHA salt which had the reported properties.[29] α-Benzyl-N-carbobenzoxy-L-glutamate, obtained by treating 3.8 g of the DCHA salt with 8 g of moist Amberlite CG-120 H^+ resin in 80 ml of methanol–water (35:25), was recrystallized from carbon tetrachloride; mp 79.5–80.5°; lit. mp 94–96°[29,30] and 78–81°;[31] $[\alpha]^{23}D$ −11° (*c* 3.16 acetic acid), lit. $[\alpha]^{22}D$ −11.7° (*c* 3.15)[29] and $[\alpha]^{24}D$ −10.4° (1.7%).[30]

A solution of 5.92 g of α-benzyl-N-carbobenzoxy-L-glutamate and 2.24 ml of triethylamine in 56 ml of tetrahydrofuran was stirred magnetically and cooled to −12° with exclusion of moisture. Isobutyl chlorocarbonate, 2.04 ml, was added dropwise, and the mixture was maintained between −5 and −10° for 10 min. A suspension of 2.3 g of L-β-cyanoalanine[16,32] in 2.4 ml of water, 2.68 ml of triethylamine, and 4 ml of tetrahydrofuran, precooled to 5°, was added in several portions. Tetrahydrofuran, 2 ml, and water, 0.2 ml, were added as rinse. A white solid soon separated. The mixture was stirred at 0° for 15 min and then was allowed to warm to 20°. Water, 2 ml, was added, and the mixture was stirred at room temperature overnight. The solution was decanted, the solid was dissolved in a small volume of water, and both solutions were taken to dryness. The residues were diluted with water, and 20 ml of ethyl acetate was added. The mixture was acidified to pH 2 and extracted several times with fresh ethyl acetate. The organic extract was washed with water, then extracted three times into

(27) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).
(28) M. Bergmann and L. Zervas, *Chem. Ber.*, **65**, 1192 (1932).
(29) F. Weygand and K. Hunger, *ibid.*, **95**, 7 (1962).
(30) H. Sachs and E. Brand, *J. Am. Chem. Soc.*, **75**, 4610 (1953).
(31) W. J. LeQuesne and G. T. Young, *J. Chem. Soc.*, 1954 (1950).
(32) C. Ressler and H. Ratzkin, *J. Org. Chem.*, **26**, 3356 (1961).

excess 4% $KHCO_3$. Fresh ethyl acetate was added to the basic aqueous extract, which was then cooled, acidified, and extracted further with ethyl acetate. The extract was washed with water, dried ($MgSO_4$), and concentrated to dryness. The crystalline residue was triturated with 200 ml of ether for 10 min, then collected; yield 6.67 g. The crude product was dissolved in 5 ml of methanol, the solution was diluted with 40 ml of methylene chloride, and 3.34 ml of dicyclohexylamine were added. On cooling, crystallization started. Isopropyl ether, 75 ml, was added, and after 30 min the salt was collected on the filter and washed with methylene chloride; yield 7.62 g, mp 181–183° dec. This was dissolved in 115 ml of hot methanol and the solution was diluted with 100 ml of hot isopropyl ether; yield 7.05 g (68.5% of long felty needles), mp 186° dec. For analysis a sample was recrystallized twice from methanol–isopropyl ether; mp 186.5–188.5° dec, $[\alpha]^{26}D$ +10.9° (*c* 2, methanol); $[\alpha]^{26}D$ +14.5° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{36}H_{48}N_4O_7$: C, 66.6; H, 7.46; N, 8.64. Found: C, 66.5; H, 7.47; N, 8.41.

N-(γ-L-Glutamyl)-L-β-cyanoalanine (II). A suspension of 2 g of the DCHA salt of I in 75 ml of 65% methanol was stirred magnetically with 7.5 g of moist Amberlite CG-120 (H^+) resin for 2 hr at room temperature. The resin was filtered off and the solution was concentrated to dryness. Ethyl acetate was added, the solution was again taken to dryness, and the process was repeated. The crystalline residue was collected by filtration with the aid of a small amount of ether; yield 1.54 g, mp 92–95°. It was used without further purification.

α-Benzyl-N-carbobenzoxy-γ-glutamyl-β-cyanoalanine so obtained, 500 mg, was dissolved in 150 ml of methanol and treated with a stream of hydrogen in the presence of 70 mg of Pd black for 1.5 hr after evolution of CO_2 ceased. The catalyst was then filtered off and washed with hot water. The combined filtrates were concentrated to a syrupy residue. Paper electrophoresis in system 3 showed a major acidic component, approximately 25% of an impurity near the origin, and a minor basic component. The residue was reprecipitated twice from a small volume of water with excess ethanol and dried. The amorphous solid was dissolved in several drops of water and the thick mixture was allowed to stand at room temperature with occasional stirring and warming to 40° until crystallization was well under way, at which time it was diluted cautiously with 75% ethanol and after several hours placed in the cold. The needles were collected by filtration at the centrifuge; yield 133 mg (51%), mp 182–185° dec. The material was recrystallized twice from water–ethanol and dried under vacuum at 56°; mp 184–185° dec. Electrophoresis showed a trace of a neutral impurity.

Anal. Calcd for $C_9H_{13}N_3O_5$: C, 44.4; H, 5.39; N, 17.3. Found: C, 44.8; H, 5.78; N, 17.4.

Amino acid analysis of a hydrolysate gave Asp, Glu, and NH_3 in the molar ratios 1.00:1.00:0.86.

N-(γ-L-Glutamyl)-L-β-cyanoalanine Dicyclohexylammonium Salt (IIa). a. By Hydrogenolysis of the DCHA Salt of I. Hydrogen was bubbled through a solution of 1.0 g of the DCHA salt of I in 250 ml of methanol in the presence of 150 mg of Pd as described for the hydrogenolysis of I. Removal of catalyst and solvent left 0.52 g of a solid, mp 180–181° dec. This was dissolved in water and the solution was neutralized with dicyclohexylamine in tetrahydrofuran and concentrated to dryness. The residue was taken up in a minimum of warm water, and warm tetrahydrofuran was gradually added. The product separated slowly in 50% yield as sheaves of needles, mp 184–185° dec. The material was recrystallized three times from water–tetrahydrofuran and air dried with no change in melting point; $[\alpha]^{25}D$ +15.0° (*c* 0.5, 2.5% $KHCO_3$). Paper electrophoresis in system 3 showed a single, acidic substance.

Anal. Calcd for $C_{21}H_{36}N_4O_5$: C, 59.4; H, 8.5; N, 13.2. Calcd for $C_{21}H_{36}N_4O_5 \cdot 0.5H_2O$: C, 58.2; H, 8.60; N, 12.9; H_2O, 2.08. Found: C, 58.8; H, 8.70; N, 13.0; H_2O, 1.56.

This material when dried at 120° underwent 2.25% weight loss, then showed the following composition: C, 59.8; H, 8.81; N, 13.2.

Chromatographic and electrophoretic constants and behavior on reduction are given under Identification of N-(γ-Glutamyl)-β-cyanoalanine in Seeds and Seedlings, sections a and c.

b. By Hydrogenolysis of I. α-Benzyl-N-carbobenzoxy-γ-glutamyl-β-cyanoalanine, 275 mg, obtained from its DCHA salt as described under II, was dissolved in 90 ml of methanol and treated with hydrogen in the presence of 52 mg of Pd black. One hour after evolution of CO_2 ceased, the catalyst was filtered off, and the solution was titrated to pH 6.8 with dicyclohexylamine in tetrahydrofuran. The product was crystallized from water–tetrahydrofuran; yield 103 mg (50%), mp 183.5–185° dec. A second crop, obtained by adding tetrahydrofuran to the mother liquor, crystallized less readily and, unlike the first crop, showed some impurity on paper chromatography in system 5.

γ-Benzyl-N-carbobenzoxy-α-L-glutamyl-L-β-cyanoalanine (III) Dicyclohexylammonium Salt. γ-Benzyl-N-carbobenzoxy-L-glutamate-DCHA was obtained from the mother liquor of γ-benzyl-N-carbobenzoxy-L-glutamate-DCHA.[29] The salt was treated with Amberlite CG-120 H^+ resin as described for the α-benzyl compound. The liberated γ-benzyl-N-carbobenzoxy-L-glutamate was recrystallized from carbon tetrachloride, mp 73–74.5°. Large quantities were prepared more conveniently by treating γ-benzyl-L-glutamate[33] with carbobenzoxy chloride in the presence of potassium carbonate in ice-bath temperature as described for carbobenzoxylating α-benzyl-L-glutamate.[30] After two recrystallizations from carbon tetrachloride, the melting point was 73.5–75°; $[\alpha]^{26}D$ −23° (*c* 6.3, 1 *N* $KHCO_3$); lit. mp 76–78°[33] and 69–71°;[29] $[\alpha]^{22}D$ −23°.[33]

a. Mixed Anhydride Procedure. A solution of 1.48 g of γ-benzyl-N-carbobenzoxy-L-glutamate and 0.56 ml of triethylamine in 14 ml of tetrahydrofuran was treated with 0.51 ml of isobutyl chlorocarbonate at −14°. After 10 min, a mixture of 574 mg of L-β-cyanoalanine, 0.67 ml of triethylamine, 1 ml of tetrahydrofuran, and 1 ml of water was added. The reaction mixture was allowed to come to 0°, held there for 15 min, then allowed to warm to room temperature. After 45 min, 0.5 ml of water was added. The next day the mixture was concentrated to a syrup which was taken up in a small amount of ethyl acetate and water. The mixture was extracted several times with 5 ml of 4% $KHCO_3$, and the organic layer was removed. The basic extract and a third, oily layer that had separated were then acidified with 2 *N* HCl and extracted with fresh ethyl acetate. The organic extract was washed with water, dried ($MgSO_4$), and concentrated to a thin liquid that was taken up in ether. To four-fifths of this liquid was added 0.5 ml of dicyclohexylamine followed by isopropyl ether, at which time a fine white precipitate separated; yield 860 mg. This was crystallized from ethanol, then recrystallized; yield 428 mg (21%), mp 174–177° dec. For analysis the product was recrystallized twice in the form of rosettes, mp 180.5–183° dec; $[\alpha]^{26}D$ +10.7° (*c* 1, methanol).

Anal. Calcd for $C_{36}H_{48}N_4O_7$: C, 66.6; H, 7.46; N, 8.64. Found: C, 67.0; H, 7.75; N, 8.62.

A mixture melting point with the DCHA salt of I showed depression to 172–176°.

b. *p*-Nitrophenyl Ester Procedure. γ-Benzyl-N-carbobenzoxy-L-glutamate *p*-Nitrophenyl Ester. A solution of 5 g of γ-benzyl-N-carbobenzoxy-L-glutamate and 2.23 g of *p*-nitrophenol at 5° was treated with 2.57 g of N,N′-dicyclohexylcarbodiimide in a total of 57 ml of ethyl acetate. After 1.5 hr, 0.13 ml of acetic acid was added. The solid was filtered off after 5 min and washed with 40 ml of ethyl acetate, and the filtrates were concentrated to dryness. The residue was recrystallized from 30 ml of ethanol containing 0.04 ml of acetic acid, then recrystallized; yield 5.1 g, mp 110–112°; lit. mp 111–111.5°, prepared by a similar [34a] or alternate[34b] procedure.

To a mixture of 429 mg of L-β-cyanoalanine, 0.56 ml of triethylamine, and 0.96 ml of water was added with magnetic stirring a precooled solution of 1.48 g of γ-benzyl-N-carbobenzoxy-L-glutamate *p*-nitrophenyl ester in 6 ml of tetrahydrofuran. Some material soon separated. The mixture was stirred for 3.5 days at 5°, then filtered, and concentrated. The yellow oily residue was taken up in ethyl acetate and water, and the mixture was acidified with 2 *N* HCl and extracted four times with 10-ml portions of ethyl acetate. The organic extract was washed with water, then extracted three times with 18-ml portions of 4% $KHCO_3$. The basic extract was cooled, acidified, and extracted three times with ethyl acetate. The organic extract was washed with water and dried ($MgSO_4$). Solvent was removed and the light yellow syrup was dissolved in 7 ml of methylene chloride containing 0.5 ml of ethanol. To the cooled solution was added excess dicyclohexylamine followed by isopropyl ether. After further cooling, the crystalline solid was collected on the filter; yield 820 mg, mp 158–165°. This material, 483 mg, was triturated with 30 ml of boiling acetone. Most of it dissolved as a yellow solution from which the product crystallized almost immediately. After 1 hr at room temperature the soft white

(33) W. E. Hanby, S. G. Waley, and J. Watson, *J. Chem. Soc.*, 3239 (1950).

(34) (a) I. Photaki and V. duVigneaud, *J. Am. Chem. Soc.*, **87**, 908 (1965); (b) M. Goodman and K. C. Stueben, *ibid.*, **81**, 3980 (1959).

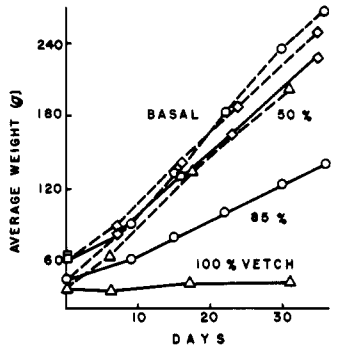


Figure 3. Growth of rats: on common vetch diets, solid line; on basal diet, broken line.

needles were collected on the filter and washed with hot acetone; yield 242 mg (21%), mp 180.5–182.5° dec. This product, 150 mg, was dissolved in tetrahydrofuran, and the solution was concentrated to half its volume. A small amount of gel that separated dissolved on gentle warming, and crystallization started. Recrystallization in the same way afforded 125 mg of needles, mp 181–182.5° dec; $[\alpha]^{26}D$ +11.8° (*c* 2, methanol).

Anal. Calcd for $C_{36}H_{48}N_4O_7$: C, 66.6; H, 7.46; N, 8.64. Found: C, 66.3; H, 7.37; N, 8.61.

A solution of 24 mg in 6 ml of methanol was treated with hydrogen in the presence of 10 mg of Pd black, as described for hydrogenolysis of I. Paper electrophoresis in system 2 showed a major acidic component and about 25% of a neutral impurity, presumably a by-product of the hydrogenolysis.

Identification of N-(γ-Glutamyl)-β-cyanoalanine in Seeds and Seedlings of *V. sativa*. a. Comparison with Synthetic N-(γ-L-Glutamyl)-L-β-cyanoalanine. Mixture melting points of the isolated material and synthetic γ-Glu-β-CNAla, or of their DCHA salts, showed no depression. Optical rotation and infrared spectra (KBr disk) of their DCHA salts agreed. The spectra showed weak cyano absorption at approximately 4.2 μ.

The isolated material and synthetic γ-Glu-β-CNAla were eluted as a single peak in the same position when chromatographed and cochromatographed (with 95% recovery) on the amino acid analyzer under the standard conditions. Similar results were obtained on Dowex 1-X4 resin with the natural peptide isolated as the DCHA salt and synthetic γ-Glu-β-CNAla. A crude 30% ethanol extract of tissues of seedlings of *V. sativa*, also chromatographed on the amino acid analyzer, then cochromatographed with synthetic γ-Glu-β-CNAla-DCHA (with 98% recovery), likewise afforded in each instance a single peak in the region of γ-Glu-β-CNAla. No difference in the mobility of the natural and synthetic dipeptides resulted on paper chromatography in system 5 (R_f 0.59) or in system 6 (R_f 0.13, descending), or on paper electrophoresis under three different conditions of pH, nor did separation result on electrophoresis of a mixture of the two in each system. Distances traveled from the origin in 5.5 hr at 9 V/cm in systems 1, 2, and 3 were 7.3, 11.9, and 7.1 cm, respectively.

b. Differentiation from N-(α-L-Glutamyl)-L-β-cyanoalanine. The isolated dipeptide and synthetic α-Glu-β-CNAla, as the major product of hydrogenolysis of III, were well separated when chromatographed on the amino acid analyzer under the standard conditions, elution volumes in system A being 114 and 216 ml, respectively. Their reduction products were likewise clearly differentiated on the analyzer; elution volumes are given under part c. Their mobilities on paper electrophoresis also differed. Distances traveled in 3 hr from the origin were: 3.8 and 2.1 cm in system 1, 6.3 and 4.7 cm in system 2, and 8 and 12 cm in system 4, respectively.

c. Reduction. Synthetic γ-Glu-β-CNAla-DCHA and various samples of the natural dipeptide were treated with sodium–ammonia–methanol as described under Reduction of γ-Glutamyl-β-cyanoalanine. Included were: (A) that isolated by preparative electrophoresis followed by recrystallization as the DCHA salt; (B) that isolated by chromatography of the crude seed extract on a column of Dowex-1 resin followed by recrystallization as the free peptide; and (C) crude 30% ethanol extract of tissues of 17-day-old common vetch seedlings. Amino acid analysis after reduction showed complete disappearance of synthetic γ-Glu-β-CNAla and of natural samples A and C and retention of 1.1% material in the region of γ-Glu-β-CNAla of sample B. All four reduced materials formed a major new peak eluting 26 ml after, and a minor peak 5 ml after ΔB, the indication of the pH change. Results with sample C are shown in Figure 2; the lower curve is the analysis of the reduced extract showing the characteristic reduction products and the disappearance of the peak corresponding to γ-Glu-β-CNAla seen before reduction in the upper curve.

If calculated with the ninhydrin-color yield constant of γ-Glu-β-CNAla, recovery of reduction products would be 171 and 200%. Since natural γ-Glu-β-CNAla-DCHA, when reduced, desalted, and hydrolyzed, gave the expected amount of Glu and 2,4-DAB, the chief reduction product, presumably N^2-γ-glutamyl-2,4-diaminobutyric acid, appears to yield about twice the color with ninhydrin as most α-amino acids, including dibasic α-amino acids. The major peak in each of the four reduced samples constituted 80–87% of the product and the minor one 13–20%. During reduction or on subsequent handling or chromatography some transformation probably occurs. Since the chief reduction product of γ-benzyl-N-carbobenzoxy-α-glutamyl-β-cyanoalanine (III) is eluted 610 ml after ΔB, the by-product probably is not N^2-α-glutamyl-2,4-diaminobutyric acid.[35]

Survey for β-Cyanoalanine in Seeds of Some Vetches and Other Legumes. Commercial *V. sativa* seeds from which β-CNAla and γ-Glu-β-CNAla were isolated contained 0.15% free and 0.27% bound β-CNAla (0.58% expressed as γ-Glu-β-CNAla). *V. sativa* var. *chlorosperma* Alef. and var. *leucosperma* Moench had 0.1 and 0.14% β-CNAla and 0.1 and 0.22% γ-Glu-β-CNAla, respectively. The following had 0.1% β-CNAla or less: *V. sativa* L. and the varieties, fuligenosa Alef., cordata (Wulf.) Arc., frisea Alef., hirsutissima (Cyr.) Alef., ludoviciana Alef., marmorata Alef., segitalis (Thuill.) Ser., "Moravská krajová," "Taborská krajová," "Slovenská krajová," and "Prerovská astrá." They were not investigated for bound β-CNAla.

V. angustifolia had 0.15% free and 0.19% bound β-CNAla; *V. grandiflora* and *V. lathyroides*, 0.07 and 0.15% free β-CNAla and 0.60 and 0.36% γ-Glu-β-CNAla, respectively. No free β-CNAla was detected in *V. faba*, *V. pisiformis*, L., *V. ervilia* (L.) Willd., *V. dasycarpa*, *V. pannonica*, *V. cracca*, *V. monantha*, *V. villosa* Roth, and *V. bengalensis*. The last two species had <0.004% bound β-CNAla. The findings with the vetch species agree with those of Bell and Tirimanna[21] except for *V. monantha* and for concentrations of free β-CNAla which were about ten times those we obtained.

Free β-CNAla was not detected in *Lathyrus hirsutus*, *L. sphaericus*, *L. tingitanus*, and *L. latifolius*, or in *L. sativus*, which had <0.003% bound β-CNAla, or *L. sylvestris*, which had 0.01% γ-Glu-β-CNAla, or *L. odoratus*, which had 0.01% γ-Glu-β-CNAla, identified by elution volume only, or *Lotus corniculatus*, *Astragalus wootonii*, and commercial samples of *Lupinus*, *Medicago* (alfalfa) *Lens esculenta* (lentil), *Soja max* (soybean bansei), and *Pisum sativum* (garden pea), which had <0.004% bound β-CNAla.

Species Difference of Rats and Chicks. a. Toward Toxicity of Common Vetch Seeds in the Diet. Groups of four rats (32–62 g) received diets incorporating 50, 85, or 100% common vetch seed. As seen in Figure 3, growth rate was unaffected on the 50% vetch ration, but was retarded at the two higher levels. Mortality was 25% with the diet of 100% vetch seed, but none resulted at the other levels.

In seven groups of chicks (43–75 g) on a 50% vetch ration all died within 4.8 to 8.8 days. Vetch seeds extracted with 30% ethanol before incorporation as 50% of the diet produced no deaths within 15 days but some depression of growth. Mortality was 50% within 21 days (average survival, 18 days) on an 18% vetch seed ration. An occasional survivor after 3 weeks on the 50% vetch diet showed "curled toe" or enlarged hock. None of the controls died.

b. Toward Toxicity of β-Cyanoalanine in the Diet. In groups of rats (37–53 g) fed diets containing 1.5, 1.0, and 0.75% β-CNAla, all died within 13 hr, 2.8, and 4.5 days, respectively; mortality was 25% on a diet containing 0.5% β-CNAla. Survivors were hyper-

(35) α ↔ ω acyl migration apparently is a general property of acyl (including aminoacyl) derivatives of 2,4-diaminobutyric acid and 2,3-diaminopropionic acid. It has been observed to occur under the alkaline conditions of work-up of N^2-glycyl-2,4-diaminobutyric acid and N^4-glycyl-2,4-diaminobutyric acid liberated from their tosyl derivatives with sodium in liquid ammonia [K. Poduska, G. S. Katrukha, A. B. Silaev, and J. Rudinger, *Collection Czech. Chem. Commun.*, **30**, 2410 (1965), and references cited therein]. If the result of such rearrangement, the by-product in the reduced natural and synthetic γ-Glu-β-CNAla could then be N^4-γ-glutamyl-2,4-diaminobutyric acid.

Table I. Toxicity of Common Vetch Seeds Compared to γ-Glutamyl-β-cyanoalanine and β-Cyanoalanine in Diets of Chicks[a]

Diet	Total β-CNAla content,[b] wt %	Duration of expt, days	Average survival, days	Mortality, %[c]
50% common vetch	0.211	9	6.5	100
0.075% β-CNAla[c] + 0.29% γ-Glu-β-CNAla[d]	0.075 + 0.136	6	6	100
0.45% γ-Glu-β-CNAla	0.211	6	5.5	100
0.61% γ-Glu-β-CNAla[e]	0.286	4	3.5	100
0.075% β-CNAla[d]	0.075	11	10.8	100
0.05% β-CNAla	0.050	30[f]	30	0
Basal ration		30	30	0

[a] Average starting body weight 36–43 g. [b] Free, bound, or both. [c] Treated chicks frequently died in the opisthotonic convulsive state characteristic of β-CNAla toxicity.[16] [d] Concentration in the 50% vetch seed ration. [e] 16.5 g of diet consumed per chick. [f] Average final body weight 249 g compared to 309 g of controls.

irritable, and growth was retarded. In chicks (39–52 g) fed diets incorporating 0.25, 0.15, and 0.1% β-CNAla, all died within 4, 7.5, and 15 days, respectively, with no deaths within 30 days on 0.05% β-CNAla or on the basal ration.

The low toxicity to the rat on diets of 50–100% common vetch seed is thus consistent with their total content of β-CNAla, 0.211–0.422%, which is below the level of β-CNAla that is toxic to the rat. Likewise, the high toxicity to the chick of diets of 18–50% vetch seed is in accord with their total content of β-CNAla, 0.08–0.211%, which is a range that is toxic to this species.

Comparison of Toxicity in the Chick on Diets Containing Common Vetch Seeds with Those Reconstituted with β-Cyanoalanine and γ-Glutamyl-β-cyanoalanine.[36] The 50% vetch diet contains 0.075% free and 0.136% bound β-CNAla, or 0.211 total β-CNAla. If expressed as γ-Glu-β-CNAla, bound β-CNAla is 0.29% and total β-CNAla 0.45%. That the toxicity of the vetch seeds cannot be accounted for by their content of free β-CNAla alone can be seen in Table I by comparing the average survival times on 50% vetch and on 0.075% β-CNAla (6.5 and 10.8 days, respectively). However, rations with the same total β-CNAla content as 50% vetch, either all as γ-Glu-β-CNAla (0.45%) or in the form present in the vetch ration (0.075% free and 0.29% γ-Glu-β-CNAla), resulted in survival times similar to those on the vetch ration. The ration containing a higher content of β-CNAla (0.286%), all in the form of γ-Glu-β-CNAla (0.61%), was more toxic (average survival, 3.5 days).

(36) Divicine, 2,4-diamino-4,6-diketopyrimidine, thought to be formed from its glycoside vicin by enzymatic hydrolysis, had previously been suggested to be a toxic factor in vetch.[9] When administered as 1% of the diet, divicine had no effect on mortality of chicks [G. H. Arscott and J. A. Harper, *J. Nutr.*, **80**, 251 (1963)].

Acknowledgment. The authors thank Miss H. Malodeczky, Mrs. J. Nelson, and Mrs. H. R. Levie for their capable assistance. For help in obtaining seed specimens, we are indebted to Dr. G. Kalmbacher, Brooklyn Botanic Garden; H. O. Jacobsen, Craver-Dickinson Seed Co., J. D. Powell, Experiment Station of the University of Georgia College of Agriculture; and Drs. P. R. Henson and D. D. Dolan, U. S. Department of Agriculture.