β-Cyanoalanine, Product of Cyanide Fixation and Intermediate in Asparagine Biosynthesis in Certain Species of *Lathyrus* and *Vicia*[1]

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

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

Abstract: β-Cyanoalanine, in the form of γ-glutamyl-β-cyanoalanine, has been established as a major product of fixation of inorganic cyanide in *Vicia sativa* seedlings. In *Lathyrus sylvestris* W., in which asparagine is the chief product of cyanide fixation, β-cyanoalanine serves as an intermediate. It probably serves in this role also in *L. odoratus* and *V. villosa* judged by demonstrated conversions of cyanide and β-cyanoalanine into asparagine. Double labeling of nitrogen and carbon has shown cyanide to be incorporated into β-cyanoalanine as a unit with its carbon–nitrogen bond intact. In *V. sativa*, the β-cyanoalanine route to asparagine is not active. β-Cyanoalanine, like cyanide, accumulates as γ-glutamyl-β-cyanoalanine, and synthesis of asparagine from free aspartic acid is now enhanced. A number of other compounds, including asparagine, γ-glutamyl-β-cyanoalanine, formate, serine, valine, phenylalanine, tyrosine, and cystine, have been tested as precursors of β-cyanoalanine or asparagine.

In an earlier study we reported the isolation and identification from *Vicia sativa* (common vetch) of the neurotoxic amino acid, β-cyanoalanine.[2] Its structural similarity to asparagine prompted us to investigate the existence of a biological relationship between these two amino acids. Sparse information was available about the metabolism and biosynthesis of either of them in plants and animals. In addition to *V. sativa*, we had in the laboratory certain other legumes known or recently found to contain lathyrogenic or neurotoxic principles, including *L. odoratus* (γ-glutamyl-β-aminopropionitrile),[3] *L. sylvestris* (2,4-diaminobutyric acid),[4] and *V. angustifolia* (β-cyanoalanine).[2] Since young seedlings of these plants all accumulate large amounts of asparagine, they were employed, in addition to *V. sativa*, as systems to study the possible relation of β-cyanoalanine to asparagine. *V. villosa*, a less toxic,[5] β-cyanoalanine-free vetch, also was used.

Radiochemical syntheses of L-β-cyanoalanine-4-^{14}C, L-asparagine-4-^{14}C, and L-aspartic acid 4-^{14}C were developed for this study and are described elsewhere.[6] When these labeled amino acids were administered to seedlings of *L. sylvestris* under sterile conditions, it soon became clear that β-cyanoalanine was not derived from asparagine, as had been conjectured earlier,[4] but that the reverse was true. β-Cyanoalanine-4-^{14}C served as an excellent precursor of asparagine in all the plants examined except *V. sativa*. Here the administered radioactivity accumulated in the form of an unknown substance.

The insect, *Sitophilus granarius*, had been reported to incorporate labeled HCN into a bound form of aspartic acid,[7] and while our study was in progress, observations were made of the labeling of asparagine, in the amide carbon, by H^{14}CN in a variety of plants.[8] K^{14}CN then was administered to the *Lathyrus* and vetch seedlings under investigation. Asparagine indeed became labeled extensively in *L. sylvestris*, *L. odoratus*, and *V. villosa*. In *V. sativa*, however, an unknown labeled substance accumulated that appeared to be electrophoretically identical with the unknown formed from β-cyanoalanine-4-^{14}C. The unknown substance was found to be present also in untreated seeds of *V. sativa*. Its isolation from this source, its identification as γ-glutamyl-β-cyanoalanine, and its synthesis, outlined earlier,[9] are given in detail in the accompanying paper.[10] With the use of synthetic γ-glutamyl-β-cyanoalanine dicyclohexylammonium salt as carrier, the product of cyanide and β-cyanoalanine incorporation has been established in each case to be γ-glutamyl-β-cyanoalanine.

Incorporation of cyanide into the cyano group of γ-glutamyl-β-cyanoalanine in *V. sativa* has been traced with K^{14}CN and K^{14}C^{15}N. Distribution of activity in asparagine in *L. sylvestris* upon incorporation of β-cyanoalanine-4-^{14}C also has been determined. In the latter plant, specific activities of tissue γ-glutamyl-β-cyanoalanine and asparagine resulting after administration of K^{14}CN are consistent with the role, suggested by the labeling patterns, that β-cyanoalanine or its dipeptide is an intermediate in the incorporation of cyanide into asparagine. Efficiency of asparagine synthesis from β-cyanoalanine has been compared with that from aspartic acid in this plant, as well as in *V. sativa*, which is unable to convert β-cyanoalanine to asparagine. Also administered to *L. sylvestris* as asparagine precursors were L-serine-3-^{14}C, DL-tyrosine-2-^{14}C, and DL-cystine-1-^{14}C.

In a preliminary attempt to assess the physiological significance of the β-cyanoalanine pathway for binding inorganic cyanide, a natural source or precursor of cyanide in some of the seedlings under study was sought. Cyanogenetic glycosides found in some plants are capable of releasing HCN upon enzymatic hydrolysis,[11] and in several instances the aglycon moiety is derived from that

(1) Aided by Grant NB 04316 from the U. S. Public Health Service and by the Muscular Dystrophy Associations of America.
(2) C. Ressler, *J. Biol. Chem.*, **237**, 733 (1962).
(3) E. D. Schilling and F. M. Strong, *J. Am. Chem. Soc.*, **77**, 2843 (1955).
(4) C. Ressler, P. A. Redstone, and R. H. Erenberg, *Science*, **134**, 188 (1961).
(5) J. A. Harper and G. H. Arscott, *Poultry Sci.*, **41**, 1968 (1962).
(6) Y.-H. Giza and C. Ressler, *J. Labelled Compounds*, in press.
(7) E. J. Bond, *Can. J. Biochem. Physiol.*, 39, 1793 (1961).
(8) (a) S. Blumenthal-Goldschmidt, G. W. Butler, and E. E. Conn, *Nature*, **197**, 718 (1963). We thank Drs. Butler and Conn for informing us of these findings before publication; (b) B. Tschiersch, *Flora*, **153**, 115 (1963).
(9) C. Ressler, S. N. Nigam, Y.-H. Giza, and J. Nelson, *J. Am. Chem. Soc.*, **85**, 3311 (1963).
(10) C. Ressler, S. N. Nigam, and Y.-H. Giza, *ibid.*, **91**, 2758 (1969).

amino acid corresponding most closely to it in structure[12,13] Cyanogenetic material and HCN were not detected in seeds or young seedlings of *L. odoratus*, *L. sylvestris*, or *V. villosa*. However, vicianin (mandelonitrile vicianoside) is present in seeds of *V. angustifolia*,[14] a β-cyanoalanine-accumulating species closely related to *V. sativa*, and its presence and varying amounts of HCN have been noted also in seeds of the latter.[11,15,16] That vicianin and β-cyanoalanine may be related biogenetically had been pointed out earlier.[2] Phenylalanine-2-^{14}C, suggested by the structure of the mandelonitrile aglycon of vicianin, accordingly was tested as a precursor of cyanide in these two vetches. This was accomplished by examining incorporation of activity into the cyano carbon of γ-glutamyl-β-cyanoalanine since *V. angustifolia* seedlings, like *V. sativa*, readily fix cyanide into this dipeptide. Conversion of phenylalanine-2-^{14}C into cyanide, also observed by Tschiersch,[17] took place in *V. angustifolia* only, but to a low degree. Also tested as a γ-glutamyl-β-cyanoalanine precursor in *V. angustifolia* was L-valine-U-^{14}C, and in *V. sativa*, sodium formate-^{14}C and L-serine-3-^{14}C. Results are discussed. Preliminary reports of some of this work have appeared.[18-20]

Experimental Section

Labeled Materials. L-β-Cyanoalanine-4-^{14}C, L-asparagine-4-^{14}C, and L-aspartic acid-4-^{14}C were synthesized as described.[6] L-Aspartic-U-^{14}C acid, K^{14}CN (crystalline, alkali free), L-serine-3-^{14}C, and DL-cystine-1-^{14}C were purchased from New England Nuclear Corp., Boston, Mass.; DL-phenylalanine-2-^{14}C, DL-tyrosine-2-^{14}C, and sodium formate-^{14}C from Nuclear-Chicago Corp., Des Plaines, Ill.; L-valine-U-^{14}C from Schwarz Bioresearch Inc., Orangeburg, N. Y.; KC^{15}N from Isomet Corp., Palisades Park, N. J. γ-Glutamyl-β-cyanoalanine-4-^{14}C was the biosynthetic product of K^{14}CN in expt 38. Specific activities are given in Tables II and III.

Other Materials. Sources of *V. sativa*, *V. angustifolia*, and *V. villosa* seeds are indicated elsewhere.[10] Seeds of *L. sylvestris* were obtained from New York State Agricultural Experiment Station, Geneva, N. Y.; seeds of *L. odoratus* (sweet pea) were from Burnett-Seedsmen, Inc., N. Y. *Clostridium welchii* NCTC 6784 and almond emulsin were obtained from Worthington Biochemical Corporation, Freehold, N. J.

Cultural Conditions and Administration of Labeled Materials. Seeds of *V. sativa* were immersed for 7 min in 0.2% mercuric chloride solution containing 5 ml of concentrated HCl per liter. Seeds of *L. odoratus*, *L. sylvestris*, and *V. angustifolia* were immersed for 10 min in 0.7% calcium hypochlorite (1% HTH, product of Olin Mathieson Chemical Corp., New York, N. Y.). Seeds of *V. villosa* were coated for 90 min with 96% chloranil (Spergon seed protectant, product of U. S. Rubber Co., Naugatuck, Conn.), covered with sterile water for 5 min, rinsed, and treated with mercuric chloride solution for 5 min. The seeds then were rinsed with sterile water and allowed to germinate in the dark under aseptic conditions as given elsewhere.[21] After 6–12 days they were cultured as also described. Labeled substances were dissolved in 3 ml of nutrient medium and administered to the roots through a sterile Swinny filter. At the end of the experiment, the medium was retested for sterility with Bacto Fluid Thioglycollate Medium and Bacto AC Medium.[22] In some of the short-term experiments, seedlings were grown in soil or gravel and the labeled substance was administered to cut stems. The seedlings were removed, rinsed, dried, pulverized, and extracted with hexane followed by 30% ethanol.[21]

Analyses. β-Cyanoalanine (β-CNAla), other amino acids, and γ-glutamyl-β-cyanoalanine (γ-Glu-β-CNAla) in extracts were determined by the chromatographic–ninhydrin procedure[23] with the Beckman-Spinco Model 120 automatic amino acid analyzer. Figure 2 of the accompanying paper[10] shows a representative analysis of extracts of *V. sativa* seedlings. Asparagine was determined indirectly by treating an aliquot of the extract containing 2 μmol of asparagine plus glutamine with 0.05 ml of guinea pig asparaginase in 100 μl of 0.01 *M* sodium borate buffer, pH 8.5, at 37° for 1 hr[24] and was calculated from the resultant increase in aspartic acid. To detect HCN and cyanogenetic material susceptible to emulsin, macerated tissue (0.025–1 g) was incubated at pH 5 with 3 mg of the enzyme preparation in a closed test tube;[25] HCN was detected by the alkaline picrate paper test[26] with a sensitivity of 0.05 μmol. Hydrolysis of vicianin by emulsin has been reported.[14,27]

Chromatographic Isolation of γ-Glutamyl-β-cyanoalanine on Dowex 1-X4 Resin. The procedure is based on the large-scale chromatographic isolation of this dipeptide described in the accompanying paper.[10] Extracts or other samples containing 1–10 μmol of γ-Glu-β-CNAla in 0.5 ml of buffer A were applied to a 0.8 × 60 cm resin column, previously equilibrated with 0.5 *N* acetic acid, and rinsed with 0.5 ml of the buffer. The column was washed with 50 ml of water, and the material was eluted with 50 ml of buffer A followed by buffer B. Fractions of 2 ml were collected at a flow rate of approximately 0.2 ml/min. Samples from alternate tubes were analyzed with ninhydrin,[28] dried, and counted. In metabolic experiments with ^{14}C precursors in which less than 0.5 μmol of γ-Glu-β-CNAla was chromatographed, 1–2 μmol of synthetic γ-L-glutamyl-L-β-cyanoalanine dicyclohexylammonium salt (γ-Glu-β-CNAla-DCHA)[9,10] was added before chromatography. Figures 1 and 2 show the chromatographic pattern on Dowex 1-X4 of crude extracts of *V. sativa* containing γ-Glu-β-CNAla.

In expt 27 and 36 the chromatographed peptide was determined, diluted with carrier synthetic γ-Glu-β-CNAla-DCHA, and crystallized from water–tetrahydrofuran to constant specific activity. The dipeptide isolated from *L. sylvestris* seedlings contained another ninhydrin-positive substance that could be removed by paper chromatography in pyridine–water (65:35). The isolated peptide was chromatographed further on paper in this system in expt 19, 20, 37, 38, and 40, and in *n*-butyl alcohol–pyridine–water (1:1:1) in expt 39 and 41. The isolated peptide was diluted 20-fold with carrier synthetic γ-Glu-β-CNAla-DCHA and crystallized in expt 20. The dipeptide was isolated directly by Dowex 1-X4 chromatography in all other experiments.

Isolation of Aspartic Acid, Asparagine, and β-Cyanoalanine. Aspartic acid was isolated from extracts by chromatography on Dowex 1-X4 acetate at the same time as γ-Glu-β-CNAla (Figure 2). It was purified further by paper chromatography in *n*-butyl alcohol–acetic acid–water (4:1:5). Sometimes it was isolated by paper

(11) J. M. Kingsbury, Ed., "Poisonous Plants of the United States and Canada," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1964, pp 23–26.

(12) Tyrosine, phenylalanine, isoleucine, and valine provide the *p*-hydroxymandelonitrile, mandelonitrile, α-hydroxy-α-methylbutyronitrile, and α-hydroxyisobutyronitrile aglycons of dhurrin, prunasin, lotaustralin, and linamarin, respectively (see ref 13).

(13) For a review of these and other recent developments in this field, see J. P. Ferris in "Chemistry of the Cyano Group," Z. Rappoport, Ed., Interscience Publishers, New York, N. Y., in press.

(14) G. Bertrand, *Compt. Rend.*, **143**, 832 (1906).

(15) A. K. Kupche, *Chem. Abstr.*, **35**, 5537 (1941).

(16) C. M. F. Porley and L. Echenique, *ibid.*, **37**, 1739 (1943).

(17) B. Tschiersch, *Flora*, **157**, 43 (1966).

(18) (a) Y.-H. Giza, H. Ratzkin, and C. Ressler, *Federation Proc.*, **22**, 651 (1963); (b) C. Ressler, Y.-H. Giza, and S. N. Nigam, *J. Am. Chem. Soc.*, **85**, 2874 (1963), and ref 4 cited therein.

(19) C. Ressler, *Federation Proc.*, **23**, 1350 (1964).

(20) After our preliminary publications on this subject had appeared,[18] some essentially similar conclusions were reported with *Linum usitatissimum* and *V. sativa* [(a) B. Tschiersch, *Flora*, 154, 445 (1964)]. The role of β-cyanoalanine in asparagine synthesis from cyanide has since been extended to include several bacteria and a variety of plants. Accumulation of β-cyanoalanine has recently been observed in *Chromobacterium violaceum*, and its synthesis has been confirmed *in vitro* with certain plant and bacterial enzyme preparations (see ref 13).

(21) S. N. Nigam and C. Ressler, *Biochim. Biophys. Acta*, 93, 339. (1964).

(22) Difco Manual, Difco Laboratories, Inc., Detroit, Mich., 1953, pp 195, 201.

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

(24) A. Meister, *Methods Enzymol.*, **2**, 383 (1955).

(25) T. Robinson, Ed., "The Organic Constituents of Higher Plants: Their Chemistry and Interrelationships," Burgess, Minneapolis, Minn., 1963, p 289.

(26) G. Gutzeit, *Helv. Chim. Acta*, **12**, 713 (1929).

(27) J. E. Courtois, F. Percheron, and H. N. Quang, *Bull. Soc. Chim. Biol.*, **46**, 984 (1964).

(28) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

Table I. Concentrations of γ-Glutamyl-β-cyanoalanine and Related Amino Acids in *Vicia sativa* and *Vicia angustifolia* during Development and Administered β-Cyanoalanine Precursors

Expt	Stage	Age, days	Conditions of growth or source	Chromatographic ninhydrin values, expressed as wt %: γ-Glu-β-CNAla	Bound β-CNAla[a]	Free β-CNAla[b]	Asn + Gln[c]	Asp	Glu
			V. sativa						
1	Seed			(0.58)[d]	0.27[e]	0.15	0.09	0.03	0.10
2	Seedling	5	Sterile, H_2O, dark	2.43	1.29	Trace	5.87	0.04	0.17
3	5–7 in.	8	Nonsterile, gravel, nutrient medium[f]	1.82	(0.85)		7.66	0.14	0.16
4	3–4 in.[g]	23	Sterile, nutrient medium, dark	2.31	(1.08)	Trace	10.0	0.05	0.16
5	5–7 in.[g]	27	Sterile, nutrient medium	1.58	(0.74)		5.61	0.06	0.07
6		28	Sterile, nutrient medium + NaCN, 0.5 mg daily for 9 days	3.68	(1.73)		8.68	0.04	0.12
7		30	Sterile, nutrient medium + NaCN, 0.5 mg, + L-serine, 1 mg daily for 8 days	3.60	(1.69)		12.2	0.03	0.11
8	Young plant[h]	35	Nonsterile, soil	1.03	(0.48)		10.4	0.02	0.07
9	Mature plant[h]	80	Nonsterile, soil	0.31	(0.15)		5.02	0.04	0.12
10	Hay	*i*	Nonsterile, soil	0.05	0.02[j]		1.81	0.06	0.07
			V. angustifolia						
11	Germ		*k*	0.70	(0.33)		0.15	0.01	0.21
12	Seed			(0.40)[d]	0.19[e]	0.15	0.05	0.03	0.05
	Seedling	21	Nonsterile, soil						
13	Roots			0.95	(0.45)		9.53	0.11	0.13
14	Greens			1.61	(0.76)		12.1	0.11	0.05

[a] Values for bound β-CNAla within parentheses were calculated from γ-Glu-β-CNAla content and may be minimal since this dipeptide is assumed to be the sole form of bound β-CNAla. [b] In expt 5, 10, 11, 13, and 14, β-CNAla was not detected when 20 mg of material was examined by paper electrophoresis at pH 8.6, or at a concentration of <0.01%. β-CNAla was not detected on the amino acid analyzer in expt 3, 8, and 9. In expt 6 and 7, small amounts of β-CNAla could have been obscured by the large γ-Glu-β-CNAla peak preceding it by 6 ml. [c] Composite peak calculated as asparagine. In 17- and 18-day-old common vetch seedlings of expt 27 and 28 in Table II, 94 and 87% of the composite peak was asparagine. [d] Although the presence of γ-Glu-β-CNAla was indicated chromatographically, direct determination of it in the seed extract was considered unreliable because of the complicated chromatographic pattern in this region. γ-Glu-β-CNAla was calculated from the content of bound β-CNAla and may be high since this dipeptide is assumed to be the sole form of bound β-CNAla. [e] Obtained as the difference in micromoles per 100 g and calculated as the percentage of β-CNAla, between total reducible β-CNAla, determined as 2,4-DAB, and free β-CNAla, determined chromatographically (see ref 10). Free and combined 2,4-DAB were <0.005%; free 2,4-DAB, <0.001% in *V. sativa* seeds. [f] Received light and nutrient medium 2 days before end of expt; in expt 4–7 received nutrient medium 8–9 days before end. [g] Shoots and roots. [h] Green parts. [i] Grown indoors, plants had browned and dried at end of season without producing seed. [j] Uncorrected for 2,4-DAB content (see footnote *e*). [k] Obtained by dissecting seeds soaked in water for 36 hr.

electrophoresis of the extract in pyridinium acetate buffer, pH 5.7; the acidic fraction so obtained was reelectrophoresed in this buffer at pH 3.5, and the aspartic acid region was eluted with water.

Asparagine and β-CNAla were isolated either directly from extracts of seedlings, or from the fraction of basic and neutral substances obtained on washing the Dowex 1-X4 acetate columns with water. The material was electrophoresed on paper in pyridinium acetate buffer, pH 5.7, for 7 hr. The neutral material was eluted and reelectrophoresed in sodium barbital buffer, pH 8.5, for 5 hr at 9 V/cm. Areas were eluted that showed yellow (asparagine) or green[2] (β-CNAla) color when sprayed with 0.15% ninhydrin in acetone. In some cases, asparagine was then chromatographed on paper in *n*-butyl alcohol–pyridine–water (1:1:1). The isolated amino acids were determined on the amino acid analyzer, mixed with carrier, and crystallized from water–ethanol to constant specific activity.

Isotope Determinations. Carbon-14-containing compounds were converted to CO_2 by wet combustion[29] and counted as $BaCO_3$ with a Nuclear-Chicago C-115 low-background system with a Micromil window and gas flow detector, except in the $K^{14}C^{15}N$ experiment, when all compounds were counted in a Nuclear-Chicago 722 liquid scintillation system in Bray's mixture.[30] Corrections were made for self-absorption or quenching.[31] Specific activity of $K^{14}CN$ of other experiments was based on the supplier's value in microcuries. Distribution of radioactivity in column effluents was determined by plating samples in planchets and counting them as solids. Paper strips were cut into 1-cm² segments that were scanned in planchets as solids. Nitrogen-15 was determined by mass spectrometry by Analytica Corporation, Westbury, N. Y.

(29) D. D. van Slyke and J. Folch, *ibid.*, **136**, 509 (1940).
(30) G. A. Bray, *Anal. Biochem.*, **1**, 279 (1960).
(31) G. E. Francis, W. Mulligan, and A. Wormall, "Isotopic Tracers," Athlone Press, London, 1959, p 238.

Degradation of γ-Glutamyl-β-cyanoalanine and Asparagine. In expt 32 the isolated γ-Glu-β-CNAla (2.064 μmol, 1.05×10^6 cpm) was diluted with 3.12 mg of nonisotopic γ-Glu-β-CNAla and hydrolyzed for 6 hr in 6 *N* HCl at 110°. The hydrolysate was chromatographed on a 0.8 × 50 cm column of Dowex 1-X4 (acetate) and eluted with 0.5 *N* acetic acid.[32] Fractions of 2 ml were collected at a flow rate of 0.2 ml/min, and samples were analyzed with ninhydrin and counted. Figure 3 shows a typical distribution of activity in the formed glutamic and aspartic acids. Part of the isolated aspartic acid, 9.46 μmol, was diluted with 18.7 mg of L-aspartic acid and recrystallized three times from aqueous ethanol to constant specific activity, 4.79×10^3 cpm/μmol. The material, 3.67 mg, was further diluted with 20.2 mg of aspartic acid, then subjected to β decarboxylation[33] with 128 mg of *Clostridium welchii* in the presence of 1.06 mg of sodium pyruvate in 15 ml of acetate buffer, pH 4.9, for 24 hr at 37° with magnetic stirring. The mixture was centrifuged and the solution was taken to dryness. The residue was decolorized with activated carbon and crystallized twice from aqueous ethanol. The alanine, 5 mg, then was chromatographed on paper in pyridine–water (65:35) and the alanine band eluted with water. Three recrystallizations yielded alanine, 80% pure on amino acid analysis, 0.6 cpm/μmol.[34]

In expt 38 the isolated γ-Glu-β-CNAla (5.3 μmol, 7.05×10^3 cpm) was degraded in a similar manner to aspartic acid, then to

(32) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Am. Chem. Soc.*, **76**, 6063 (1954).
(33) A. Meister, H. A. Sober, and S. V. Tice, *J. Biol. Chem.*, **189**, 577 (1951).
(34) The validity of this procedure was confirmed by degrading commercial L-aspartic acid-$U^{14}C$, sp act 1.69×10^4 cpm/μmol, which yielded alanine, 1.05×10^4 cpm/μmol. Moreover, in a subsequent study in which serine-3-^{14}C was administered with nonisotopic KCN to *V. sativa* seedlings,[21] alanine obtained on degradation contained 92% of the activity of the β-CNAla residue of γ-Glu-β-CNAla.

Table II. Asparagine Biosynthesis from L-β-Cyanoalanine-4-^{14}C, N-γ-L-Glutamyl-L-β-cyanoalanine-4-^{14}C, K^{14}CN, and Other Substances in *Lathyrus* and *Vicia* Species[a]

		Precursor			Asparagine[b]				
Expt	Compound	Amt, μmol (*A*)	Sp act., cpm/μmol × 10^{-5} (*B*)	Total act., cpm × 10^{-6} (*C*)	Tissue content, μmol (*D*)	Sp act., cpm/μmol × 10^{-3} (*E*)	Total act., cpm × 10^{-4} (*D* × *E*)	Sp act. ratio[c]	% incorpd[d]
				L. sylvestris W.					
15	L-β-Cyanoalanine-4-^{14}C	26.2	0.89	2.33	53.3	14.7	78.4	100	33.6
16	L-Asparagine-4-^{14}C	27.4	0.35	0.96	49.8	4.40	21.9	76	24.1
17	L-Aspartic acid-4-^{14}C	34.4	0.48	1.65	50.7	0.74	3.75	7.2	2.27
18	γ-L-Glutamyl-L-β-cyanoalanine-4-^{14}C (6.5 hr, cut seedlings)	15.5	5.25	8.14	33.2	12.1	40.2	10.6	6.10
19	K^{14}CN (3 days)	0.29	96.6	2.80	25.5	15.9[e]	40.6	0.9	14.5
20	K^{14}CN (11 hr)	0.98	106.8	10.5	61	35.3[e]	215	5.3	20.5
21	L-Serine-3-^{14}C	0.79	16.0	1.26	34.3	1.11	3.81	0.4	3.02
22	DL-Tyrosine-2-^{14}C	0.59	27.0	1.59	62.4	0.062	0.39	0.02	0.25
23	DL-Cystine-1-^{14}C	0.51	33.4	1.70	74.9	0.229	1.72	0.03	1.01
				L. odoratus					
24	K^{14}CN	1.28	26.9	3.44	30.4	3.51	10.7	1.3	3.16
25	L-β-Cyanoalanine-4-^{14}C[f]	36.3	0.89	3.23	51.5	6.25	32.2	73	10.2
26	γ-L-Glutamyl-L-β-cyanoalanine-4-^{14}C (23 hr)	10.4	5.25	5.46	41.5	1.85	7.68	1.6	4.69
				V. sativa					
27	K^{14}CN	1.28	26.9	3.44	55.5	0.537[e]	2.98	0.3	0.87
28	L-β-Cyanoalanine-4-^{14}C	43.8	0.89	3.90	67.8	0.187	1.27	1.6	0.35
29	L-Aspartic acid-4-^{14}C (4 days)	19.7	0.48	0.95	62.7	0.355	2.23	3.1	3.45
				V. villosa					
30	K^{14}CN	2.72	26.9	7.32	28.8	6.57	18.9	2.0	2.58
31	L-β-Cyanoalanine-4-^{14}C	32.4	0.89	2.88	63.8	5.54	35.3	59	12.3

[a] Age of *L. sylvestris* plants, 24–60 days; *L. odoratus*, 23–29 days; *Vicia*, 16–32 days. Except where indicated, the experimental period was 7 or 8 days. [b] Amount isolated was 4–20 μmol; concentration in plant was 1.7–6.4%; lowest values in each group were in the KCN experiments. [c] Ratio of specific activity of isolated asparagine to that of precursor, compared to that ratio in expt 15 taken as 100 after adjusting for difference in seedling weight from that of expt 15. Ratios can be compared only in experiments in which similar quantities of precursor were used. [d] Uptakes are not significant for KCN experiments because of possible loss by volatilization. In expt 16, 18, 26, 28, and 29, uptake was 95, 81, 30, 93, and 68%; in all other experiments, 98% or more. Percentage incorporated into asparagine is $[(D \times E)/C]100$ after correction for uptake, except for KCN, where it is based on administered activity, *C*. Percentage of uptake incorporated into seedlings was 45–66% except for 23, 8, and 35% in expt 17, 29, and 30. [e] Specific activity of tissue γ-Glu-β-CNAla is given in Table III. [f] A similar experiment with cut seedlings resulted in 17% β-CNAla incorporated into asparagine after 6 hr.

alanine. Decarboxylation of 2.4 mg of aspartic acid was carried out in a stoppered micro-Warburg cell. Carbon dioxide liberated from C-4 was absorbed in CO_2-free 1 *N* NaOH in the center well and precipitated as $BaCO_3$, which also was counted.

In expt 15 the isolated asparagine was diluted with carrier and crystallized, then hydrolyzed for 3.5 hr in 1 *N* HCl at 110°. Formed aspartic acid was crystallized to constant activity, then diluted and decarboxylated to alanine in the manner described. Results corrected for dilution are given in Table IV.

Incorporation of Doubly Labeled Cyanide (K^{14}C^{15}N) into γ-Glutamyl-β-cyanoalanine by *Vicia sativa* Seedlings. K^{14}C^{15}N was prepared by mixing 18.5 mg of KC^{15}N (80.2% assay, 98.1% ^{15}N) and 25 μCi of K^{14}CN (17.1 μCi/μmol) in 25 ml of water. The solution was distributed immediately into sealed ampoules and refrigerated. On 4 alternate days, six flasks of 23-day-old seedlings in sterile culture each received from a newly opened ampoule 0.43 mg of the K^{14}C^{15}N; this represents a total administration of 10.2 mg of K^{14}C^{15}N and 17.23 μCi. At the end of 9 days the sterile seedlings were rinsed and dried; combined weight 2.86 g. The content of γ-Glu-β-CNAla was 1.72%; of asparagine, 5.42%. The extract of 2.07 g of seedlings that had 146 μmol of dipeptide was chromatographed on a 1 × 70 cm column of Dowex 1-X4 resin (see Chromatographic Isolation of γ-Glutamyl-β-cyanoalanine). Tubes 84–98 constituted the only radioactive peak and yielded 127 μmol (87%) of γ-Glu-β-CNAla. Of this, 124.3 μmol was diluted 2.367 times with 41.3 mg of carrier γ-Glu-β-CNAla[9,10] and crystallized twice to give 51.5 mg (91.6%) of γ-Glu-β-CNAla of specific activity 1.86 × 10^4 cpm/μmol.

Acid hydrolysates (1.33 mg in 1.5 ml of 2 *N* HCl for 6 hr at 115°) were diluted 8.367 times with 2 ml of 0.01 *M* (40 μmol) reagent ammonium sulfate in a Kjeldahl apparatus, made alkaline with 12 *N* potassium hydroxide, and steam distilled into 8 ml of 0.01 *N* H_2SO_4. The ammonia in the distillates contained 1.025 atom % excess ^{15}N.

Another acid hydrolysate of the labeled recrystallized γ-Glu-β-CNAla (14.52 mg in 2 ml of 6 *N* HCl for 8 hr at 100°) was taken to dryness and chromatographed on a 0.6 × 50 cm column of Dowex 1-X4 (acetate). Glutamic and aspartic acids were eluted separately with 0.5 *N* acetic acid, as described under Degradation of γ-Glutamyl-β-cyanoalanine and Asparagine. Glutamic acid was diluted 1.79 times with 4.0 mg of carrier and crystallized; sp act. 29.2 cpm/μmol. Aspartic acid was diluted 2.049 times with 6.2 mg of carrier and crystallized four times to the constant activity 9.57 × 10^3 cpm/μmol. Table V gives findings corrected for dilution.

Results and Discussion

β-Cyanoalanine and γ-Glutamyl-β-cyanoalanine Concentrations in *Vicia sativa* and *V. angustifolia* during Development

Both free and bound β-CNAla are present in seeds of *V. sativa* and *V. angustifolia* (Table I, expt 1, 12).[10] In young seedlings of both species, however, essentially all the β-CNAla is bound. Only in seedlings cultured in the dark were traces of free β-CNAla detected. Concentration of γ-Glu-β-CNAla in young *V. sativa* seedlings was three- to fourfold higher than in seeds. However, total content of β-CNAla in the 5-day-old seedling was the same as that of the ungerminated seed (the excised seedling, 200 mg, had 1.29%; the remaining seed portion, 1.55 g, had 0.32%; the ungerminated seed, 0.42%). This could

suggest that during this period, under the existing conditions, no net synthesis, and perhaps no synthesis at all, of β-CNAla is taking place. β-Cyanoalanine can certainly be synthesized by the young seedling if cyanide is supplied, as is evidenced by the extensive incorporation of $K^{14}CN$ into γ-Glu-β-CNAla (22–63%; Table III, expt 27, 32, 40). Moreover, repeated administration of sodium cyanide raises the concentration of γ-Glu-β-CNAla from 1.6 to 3.7% (Table I, expt 5–7). The limiting factor in the synthesis of β-CNAla in the young seedling could be its ability to form cyanide. Possibly γ-Glu-β-CNAla in the shoot of the young seedling is derived by the process of translocation from the seed's free and bound β-CNAla.

L. sylvestris W. had only 0.01% γ-Glu-β-CNAla in seeds but 0.19% in untreated 41-day-old seedlings. Increases in concentration of other toxic amino acids and derivatives on germination have been noted elsewhere.[19] However, it apparently is not a general occurrence with γ-glutamyl peptides since Virtanen noted that the many γ-glutamyl peptides in onion and garlic disappear rapidly as the green leaves appear.[35]

In *V. sativa* concentration of γ-Glu-β-CNAla was at a maximum in the young seedling. In the first few weeks after germination, it decreased only slowly in resting seedlings, being 1.8 and 1.6% in 8- and 27-day-old seedlings of comparable size (expt 3, 5). Concentration fell off as the plant grew and matured, being 1 and 0.3% in green parts of the 35- and 80-day-old-plant (expt 8–10). It would seem that β-CNAla derived from the seed or formed during germination is rather inert metabolically during the next few weeks and gradually is diluted in the growing plant with other substances that have been newly formed, or is formed more slowly in the older plant or utilized more rapidly than it is formed, compared to the younger seedling.

The dipeptide seemed to accumulate more in the dark than when the seedlings received light daily (expt 4, 5). However, this may reflect only an increase in the concentration of the dipeptide owing to the significantly reduced size of the plants grown in the dark. Asparagine and glutamine, determined together, also increased in concentration, to 10%; this finding agrees with the well-recognized accumulation of asparagine in young vetches and certain other legumes, especially in the dark.[36]

Biosynthesis of Asparagine. That asparagine is metabolically rather inert in young *L. sylvestris* seedlings was indicated by the large amount of radioactivity remaining incorporated in tissue asparagine 7 days after administering asparagine-4-^{14}C (Table II, expt 16). Periods ranged from 6.5 hr to 8 days in experiments on the biosynthesis of asparagine.

a. From β-Cyanoalanine. β-Cyanoalanine served as an excellent precursor of asparagine in *L. sylvestris*, *L. odoratus*, and *V. villosa* seedlings, as judged both by the percentage of radioactivity incorporated and the degree of dilution of specific activity on incorporation into asparagine. Percentages of β-cyanoalanine-4-^{14}C incorporated into asparagine were 34, 10, and 12%, respectively (expt 15, 25, 31). With *L. sylvestris*, 99.6% of the administered β-CNAla was taken up and 54% was incorporated into the seedlings; of this, 62% was present as free asparagine. Incorporation of β-CNAla into asparagine compared favorably even with that of asparagine itself (expt 15, 16). Asparagine biosynthesized from β-cyanoalanine-4-^{14}C was hydrolyzed to aspartic acid, and this was decarboxylated at the β position. The alanine thus obtained, representing carbons 1, 2, and 3, had only 0.7% of the activity of the aspartic acid, which indicated that β-CNAla was converted into asparagine without randomization of C-4 (Table IV, expt 15).

b. From KCN. In *L. sylvestris*, $K^{14}CN$ was 15% incorporated in 3 days (expt 19) and 21% in 11 hr (expt 20). Extent of cyanide incorporated was thus similar to that with *Sorghum vulgare*.[8a] With *L. odoratus* and *V. villosa* for longer periods, $K^{14}CN$ was 3.2 and 2.6% incorporated into asparagine (expt 24, 30).

c. From γ-Glutamyl-β-cyanoalanine. Asparagine also formed readily from γ-Glu-β-CNAla. Whole seedlings of *L. odoratus* incorporated 4.7% of this dipeptide into asparagine in 23 hr (expt 26). Cut seedlings of *L. sylvestris* incorporated 6.1% γ-Glu-β-CNAla in 6.5 hr (expt 18). Moreover, if adjusted for the finding that 57% of the dipeptide taken up was still present in the seedling at the end of the latter experiment, incorporation of γ-Glu-β-CNAla into asparagine was 14%. It was of interest also to find some highly labeled β-CNAla, 1.53×10^5 cpm/μmol, in the free state at the end of the experiment; perhaps γ-Glu-β-CNAla is hydrolyzed to the constituent amino acids before being converted to asparagine. Since specific activity of the reisolated peptide (in cpm/μmol) was 4.45×10^5, aspartic acid 4.06×10^4, and asparagine 1.21×10^4, it is possible that, before forming asparagine, γ-Glu-β-CNAla or β-CNAla is converted into aspartic acid.

d. From Aspartic acid. L-Aspartic-4-^{14}C acid was a much less effective precursor of asparagine than either β-cyanoalanine-4-^{14}C or cyanide in *L. sylvestris* (2.3% incorporated, Table II, expt 17; *cf.* expt 15, 19). Aspartic acid was metabolized more extensively than was β-CNAla (23% *vs.* 54% of the activity taken up was incorporated into the seedlings). Even when adjusted for this factor, aspartic acid was 5.4% incorporated into asparagine, or only one-sixth as much as was β-CNAla. Moreover, aspartic acid underwent 14-fold more dilution than did β-CNAla on forming asparagine. In *L. sylvestris*, the β-CNAla pathway would seem a feasible one for the synthesis of asparagine. If, in accord with the results of expt 18, aspartic acid is an intermediate in the conversion of β-CNAla into asparagine,[37] presumably it could be formed close to the site at which it is converted into asparagine. Exogenous aspartic acid, in contrast, would be distributed more widely, and, participating in a variety of other reactions, probably less available for asparagine synthesis than aspartic acid generated from β-CNAla *in situ*. However, the data do not rule out the existence of separate, perhaps simultaneous, pathways to asparagine, *i.e.*, direct conversion of β-CNAla into asparagine, conversion of β-CNAla into aspartic acid that is converted into asparagine, and amidation of aspartic acid derived from sources other than β-CNAla.

e. From Aspartic Acid and Other Substances in *V.*

(35) A. I. Virtanen, *Angew. Chem. Intern. Ed. Engl.*, **1**, 299 (1962).

(36) J. P. Greenstein and M. Winitz, Ed., "Chemistry of the Amino Acids," Vol. 3, John Wiley & Sons, Inc., New York, N. Y., 1961, pp 1858–1859.

(37) Enzymes capable of hydrolyzing β-CNAla to aspartic acid have been observed, *e.g.*, guinea pig serum asparaginase [Y.-H. Giza, H. Ratzkin, and C. Ressler, *Federation Proc.*, **22**, 651 (1963)], *Pseudomonas* nitrilase [R. H. Hook and W. G. Robinson, *J. Biol. Chem.*, **239**, 4263 (1964)].

Table III. N-γ-Glutamyl-β-cyanoalanine Biosynthesis in *Vicia sativa*, *V. angustifolia*, and *Lathyrus sylvestris* Seedlings[a]

Expt	Compound	Precursor: Amt, μmol (*A*)	Precursor: Sp act., cpm/μmol × 10^{-5} (*B*)	Precursor: Total act., cpm × 10^{-6} (*C*)	γ-Glutamyl-β-cyanoalanine[b]: Tissue content, μmol (*D*)	γ-Glutamyl-β-cyanoalanine[b]: Sp act., cpm/μmol × 10^{-2} (*E*)	γ-Glutamyl-β-cyanoalanine[b]: Total act., cpm × 10^{-4} (*D* × *E*)	% incorpd[c]
				Whole Seedlings				
				V. sativa				
27	$K^{14}CN$	1.28	26.9	3.44	39.4	234	92.2	26.8
32	$K^{14}CN$	5.85	96.6	56.5	67.6	5250	3549	62.8[d]
33	Sodium formate-^{14}C	0.82	12.9	1.06	26.8	8.81	2.36	2.23
34	L-Serine-3-^{14}C[e]	1.58	16.2	2.56	27.1	4.46	1.21	0.47
35	L-Asparagine-4-^{14}C	27.1	0.53	1.44	23.7	1.41[f]	0.33	0.23
36	L-β-Cyanoalanine-4-^{14}C	34.2	0.89	3.04	37.4	240	89.8	29.5
29	L-Aspartic-4-^{14}C acid (4 days)	19.7	0.48	0.95	6.78	8.36	0.57	0.9
				L. sylvestris				
19	$K^{14}CN$ (3 days)	0.29	96.6	2.80	3.24	232[g]	7.52	2.69
20	$K^{14}CN$ (11 hr)	0.98	107	10.5	4.94	483[g]	23.9	2.28
				Cut Seedlings, 10–11 hr				
				V. angustifolia				
37	$K^{14}CN$	1.2	96.6	11.6	17.4	1130	197	17.0
38	DL-Phenylalanine-2-^{14}C	2.2	18.9	4.16	20.6	13.3	2.74	0.7
39	L-Valine-U-^{14}C	0.05	414	2.07	15.8	0.55	0.09	0.04
				V. sativa				
40	$K^{14}CN$	1.2	96.6	11.6	27.2	930	253	21.8
41	DL-Phenylalanine-2-^{14}C	2.2	18.9	4.16	43.8	0.38	0.17	0.04

[a] Age of plants 12–21 days except for 25 and 37 days in expt 19 and 20. Except where indicated, the experimental period was 7 or 8 days. Carried out simultaneously were expt 37 and 40, 38 and 41. [b] Amount isolated was 0.6–52 μmol; concentration in dried plant, 1.4–1.9%, except for 2.4% in expt 27 and 36 and 0.47 and 0.25% in expt 19 and 20. [c] $(D \times E)/C$. Uptake was 97–100%, except for 68% in expt 29 and in experiments with KCN (see Table II, footnote *d*). Percentage incorporated into γ-glutamyl-β-cyanoalanine is based on administered activity, *C*, except for expt 29. Percentage incorporated into seedlings was 55–60% except for 40, 79, 26, and 48% in expt 27, 36, 37, and 40. In expt 29, percentage of uptake incorporated into seedlings was 8%. [d] Seedling weight and amount of cyanide administered were 2.6–2.9 times those in expt 27 and 40. [e] Data of ref 21. [f] Aspartic acid, also isolated, had sp act. 2.06 × 10^3 cpm/μmol. [g] Specific activity of asparagine is given in Table II.

sativa. Of the plants investigated in Table II, β-CNAla and cyanide were poor precursors of asparagine for *V. sativa*, which is the only one of this group that has a high content of γ-Glu-β-CNAla. β-Cyanoalanine and cyanide were only 0.4 and 0.9% incorporated (expt 28, 27). Ratios of specific activity were low as well. In *V. sativa*, β-CNAla underwent 37–62 times more dilution in forming asparagine than it did in *V. villosa*, *L. odoratus*, and *L. sylvestris*; KCN underwent 3–18 times more dilution in *V. sativa* than in the three other plants. Interestingly, however, in *V. sativa*, aspartic acid was now superior to β-CNAla and cyanide as a precursor of asparagine (expt 29). If adjusted for the fact that only 8% of the activity taken up was incorporated into the seedlings compared to 62% for β-CNAla in this plant, incorporation of aspartic acid into asparagine was as much as 27%. Furthermore, aspartic acid underwent less dilution than did β-CNAla on forming asparagine. In *V. sativa*, in which conversion of β-CNAla into asparagine is limited, it appears that a route involving free aspartic acid now is enhanced in asparagine biosynthesis.

Biosynthesis of β-Cyanoalanine (γ-Glutamyl-β-cyanoalanine). a. Conversion of β-Cyanoalanine into γ-Glutamyl-β-cyanoalanine. It is clear from the results of expt 35 of Table III that β-CNAla is not synthesized in *V. sativa* by dehydration of asparagine. The reverse reaction, whereby β-CNAla is converted to asparagine, can take place in *L. sylvestris*, *L. odoratus*, and *V. villosa*, as discussed under Biosynthesis of Asparagine. In *V. sativa*, β-CNAla seems to be converted mainly into γ-Glu-β-CNAla. In expt 36 identity of the main labeled soluble product as γ-Glu-β-CNAla after β-cyanoalanine-4-^{14}C administration was established by isolation by Dowex 1-X4 chromatography, dilution 91.3-fold with synthetic γ-Glu-β-CNAla-DCHA,[9,10] and repeated crystallization.

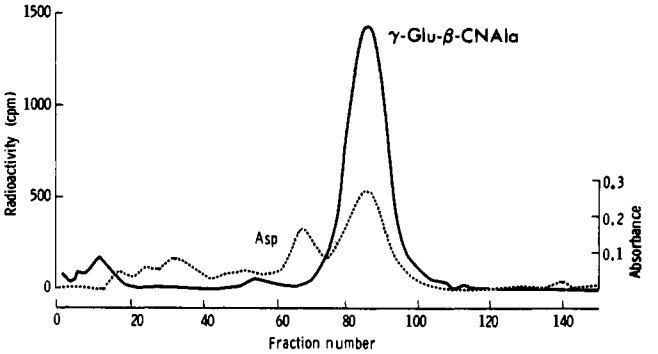


Figure 1. Conversion of β-cyanoalanine into γ-glutamyl-β-cyanoalanine by *V. sativa* seedlings. Seedlings, 366 mg, were fed L-β-cyanoalanine-4-^{14}C (3.04 × 10^6 cpm, Table III, expt 36). Extract of 24 mg contained 0.03 μmol of aspartic acid, 0.05 μmol of glutamic acid, and 2.38 μmol of γ-glutamyl-β-cyanoalanine. Authentic L-aspartic acid, 1.14 μmol, and synthetic γ-glutamyl-β-cyanoalanine dicyclohexylammonium salt, 1.21 μmol, were added. The material was chromatographed on a column of Dowex 1-X4 resin as in Figure 2 except that the buffer was changed at 30 ml. Samples of 0.3 ml were analyzed with ninhydrin (- - -) and counted for radioactivity (—).

Table IV. Distribution of Radioactivity in Asparagine Formed from L-β-Cyanoalanine-4-^{14}C in *L. sylvestris W.* and in γ-Glutamyl-β-cyanoalanine Formed from K^{14}CN in *V. sativa* and from DL-Phenylalanine-2-^{14}C in *V. angustifolia*

Precursor (expt)	Isolated compd[a] (sp act., cpm/μmol)	Sp act., cpm/μmol, of[b]: Aspartic acid	α-Alanine (C_{1-3}) (% of act. of Asp)	CO_2 (C_4)
β-Cyanoalanine (15)	Asparagine	1.47×10^4	103 (0.7)	
K^{14}CN (32)	γ-Glutamyl-β-cyanoalanine (5.25×10^5)	5.49×10^5	440 (0.08)	
DL-Phenylalanine-2-^{14}C (38)	γ-Glutamyl-β-cyanoalanine (1.33×10^3)	1.08×10^3	42 (3.9)	838 (78; 95[c])

[a] Isolated chromatographically. [b] Procedures are given under Degradation of γ-Glutamyl-β-cyanoalanine and Asparagine. Distribution of activity between glutamic and aspartic acids in expt 27 and 38 is seen in Figures 3 and 4. [c] Calculated on the basis of radioactivity recovered on decarboxylation in CO_2 and alanine.

Table V. Incorporation of K^{14}C^{15}N into γ-Glutamyl-β-cyanoalanine in Seedlings of *V. sativa*[a]

Compd	^{15}N, atom % excess	^{14}C, cpm/μmol × 10^{-4}	^{15}N/^{14}C × 10^4 (R)	R(β-cyanoalanine)/R(KCN)
K^{14}C^{15}N	97.47	23.8	4.10	
γ-Glu-β-CNAla		4.40		
Cyano nitrogen[b]	19.75			
Aspartic acid[c]	0.027	4.64	4.26[d]	1.04
Glutamic acid[c]	0.007	0.01		

[a] Percentage of K^{14}C^{15}N incorporated into γ-Glu-β-CNAla = [(total μmol × sp act. of γ-Glu-β-CNAla)/(total μmol × sp act. of K^{14}C^{15}N)]100 = [(202.1 × 4.40 × 10^4)/(155.7 × 2.38 × 10^5)]100 = 24%. This is a minimal value uncorrected for uptake of K^{14}C^{15}N from the medium and loss of H^{14}C^{15}N by volatilization. [b] As ammonia liberated by acid hydrolysis. [c] Amino nitrogen. [d] ^{15}N concentration of cyano nitrogen/specific activity of aspartic acid.

Uncorrected for dilution specific activities after the first, second, third, and fourth crystallizations were 395, 580, 636, and 606 cpm/mg.

Of the β-CNAla administered in this experiment, 98% was taken up and 79% incorporated into the seedlings, 38% of which was γ-Glu-β-CNAla. Over 88% of the radioactivity of the soluble material was acidic, of which γ-Glu-β-CNAla was the major labeled product as indicated by electrophoresis at pH 5.7 as well as by chromatography of the crude extract on Dowex-4 resin (Figure 1). β-Cyanoalanine-4-^{14}C was 30% incorporated into γ-Glu-β-CNAla. β-Cyanoalanine would appear to be rather stable metabolically in *V. sativa* since these results were obtained 7 days after administering β-CNAla.

b. From Cyanide. The same dipeptide, γ-Glu-β-CNAla, accounts for the major product of incorporation of inorganic cyanide in *V. sativa*. Presence of γ-Glu-β-CNAla in the crude plant extract after K^{14}CN administration was confirmed in expt 27 by cochromatography with synthetic material on the amino acid analyzer and by reduction to γ-glutamyl-2,4-diaminobutyric acid.[10] Identity of the major labeled soluble product as γ-Glu-β-CNAla was established by dilution with synthetic γ-Glu-β-CNAla-DCHA, isolation by Dowex 1-X4 chromatography, further dilution with the carrier, and repeated crystallization. Uncorrected for 92.3-fold dilution, specific activities after the first, second, third, and fourth crystallizations were 415, 597, 570, and 585 cpm/mg.

Virtually all the radioactivity in the soluble fraction of the plant was associated with the acidic, ninhydrin-reactive region on paper electrophoresis. The chromatographic pattern in Figure 2 shows the predominance of the labeling as γ-Glu-β-CNAla among the acidic soluble substances. Of the cyanide administered in this experiment, 40% was bound into the seedlings, 67% of which was present as γ-Glu-β-CNAla. Incorporations of K^{14}CN into γ-Glu-β-CNAla were 27 and 63% in 8 days with whole seedlings (expt 27, 32). In 11 hr as much as 22% K^{14}CN was incorporated into the β-CNAla dipeptide by cut seedlings of *V. sativa* (expt 40); similar results were obtained with cut seedlings of *V. angustifolia* (expt 37). Specific activity of γ-Glu-β-CNAla in the latter was even somewhat higher than in the former; however, the smaller content of dipeptide (1.49 *vs.* 1.83%) results in somewhat lower calculated incorporation. Biosynthesis of β-CNAla and of γ-Glu-β-CNAla from inorganic cyanide apparently takes place rapidly as well as efficiently and does not require root systems. These incorporations resulted with administration of 3–6 μmol of cyanide per gram (dry weight) of tissue. Even when, as in the doubly labeled K^{14}C^{15}N experiment of Table V, as much as 55 μmol of cyanide, *i.e.*, 3.6 mg of KCN, in several milliliters of medium were supplied per gram to the *V. sativa* plant, at least 24% of the cyanide was incorporated into γ-Glu-β-CNAla. As these experiments, moreover, were conducted in erlenmeyer flasks closed only with porous cotton to preserve sterility, or in open vessels, the avidity of both vetches to incorporate volatile cyanide into γ-Glu-β-CNAla is certainly impressive. It is not known whether this ability to bind inorganic cyanide is the sole factor determining their resistance to such large amounts of cyanide.

γ-Glutamyl-β-cyanoalanine, rather than β-CNAla, is present in young *V. sativa* seedlings (Table I), and direct

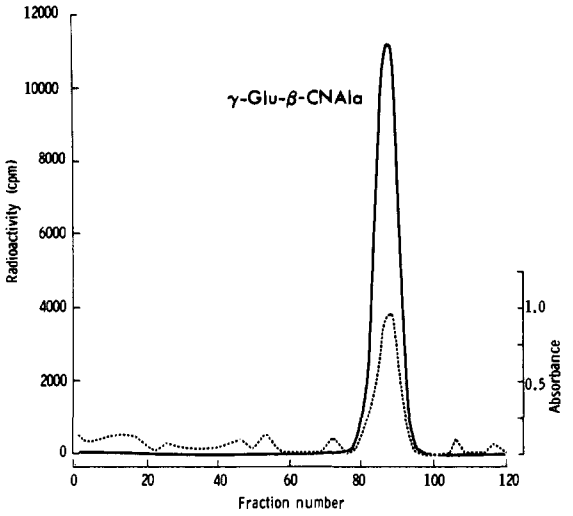


Figure 2. Incorporation of cyanide into γ-glutamyl-β-cyanoalanine by *V. sativa* seedlings. Whole seedlings were fed $K^{14}CN$. The extract containing 1.27×10^6 cpm, prepared from 120 mg of green parts and stems only, was chromatographed on a column of Dowex 1-X4 as described under Chromatographic Isolation of γ-Glutamyl-β-cyanoalanine. Samples of 0.5 ml were analyzed with ninhydrin (- - -) and counted (—).

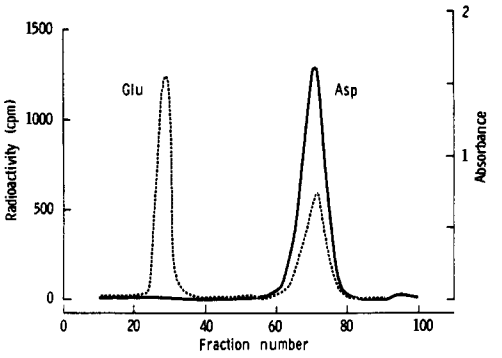


Figure 3. Incorporation of cyanide into the β-cyanoalanine residue of γ-glutamyl-β-cyanoalanine by *V. sativa* seedlings. Seedlings were fed $K^{14}CN$ (Table III, expt 27). γ-Glutamyl-β-cyanoalanine was isolated, diluted, hydrolyzed, and chromatographed on Dowex 1-X4, as described under Degradation of γ-Glutamyl-β-cyanoalanine and Asparagine. Samples were analyzed with ninhydrin (- - -) and counted (—).

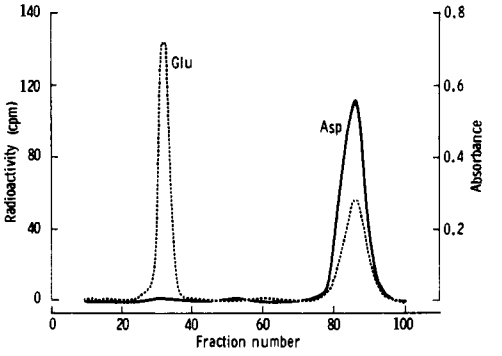


Figure 4. Incorporation of C-2 of phenylalanine into the β-cyanoalanine residue of γ-glutamyl-β-cyanoalanine by *V. angustifolia* seedlings. Cut stems were fed DL-phenylalanine-2-^{14}C (Table III, expt 38). γ-Glutamyl-β-cyanoalanine was isolated, hydrolyzed, and chromatographed on Dowex 1-X4 as in Figure 3 except that the size of the column was 0.8 × 60 cm; ninhydrin (- - -), radioactivity (—).

evidence that inorganic cyanide was forming β-CNAla was desired. In an inverse isotope dilution experiment, 4.2 mg of nonisotopic β-CNAla was administered together with 100 μCi of $K^{14}CN$. After 3 days, free β-CNAla, 3.6 μmol, was reisolated from the plant tissues and found to be labeled significantly with sp act. 1.31×10^5 cpm/μmol. It therefore seemed justifiable to study the biosynthesis of β-CNAla in the form of γ-Glu-β-CNAla.[38]

The position in γ-Glu-β-CNAla that became labeled by the administered cyanide was determined by degrading the dipeptide to glutamic and aspartic acids and separating these chromatographically (Figure 3). β-Decarboxylation of the aspartic acid placed over 99% of the radioactivity in C-4, corresponding to the cyano carbon of the β-CNAla moiety (Table IV, expt 32). That cyanide is incorporated into β-CNAla as a cyano unit with the carbon–nitrogen bond intact was established after administering doubly labeled $K^{14}C^{15}N$ likewise by isolating and degrading tissue γ-Glu-β-CNAla. As seen in Table V, specific activities of this dipeptide and the aspartic acid derived from it agreed within experimental error. Of the dipeptide's activity, aspartic acid had 99.7%; glutamic acid, 0.3%. The ratio in the isolated γ-Glu-β-CNAla of the ^{15}N concentration of the cyano group to the ^{14}C specific activity of the derived aspartic acid[39] agreed within 4% with the ratio of ^{15}N to ^{14}C in the starting $K^{14}C^{15}N$. Only very small amounts of ^{15}N were present in the amino groups of the β-CNAla and glutamic acid moieties, which supports the deduction concerning the metabolic stability of β-CNAla in this plant.

c. From Aspartic Acid and Formate. In *V. sativa*, aspartic-4-^{14}C acid was a much less effective precursor of γ-Glu-β-CNAla than either KCN or β-CNAla (Table III, expt 27, 29, 32, 36). However, if the more extensive metabolism of aspartic acid is considered, its incorporation becomes significant, increasing from 0.9 to 8.7% (*cf.* 30% for β-CNAla). Incorporation of formate was low (2.23%, expt 33) but likewise significant. Over 96% of the dipeptide's activity was in the β-CNAla moiety, as determined after hydrolysis. In addition to direct fixation of cyanide, perhaps alternative, less important routes to γ-Glu-β-CNAla exist, in which formate or aspartic acid can participate, or these could provide precursors of cyanide or of the molecule condensing with cyanide.

d. From Serine. Incorporation of L-serine-3-^{14}C into γ-Glu-β-CNAla in *V. sativa* was low (0.5%, expt 34). Subsequent studies of ours[21] and others[20a] have shown, however, that incorporation of serine into the β-CNAla dipeptide depends upon the presence of cyanide. In two experiments addition of cyanide increased incorporation of serine 18-fold to 25% and 92-fold to 46%. Serine was diverted from other reactions to provide without rearrangement a three-carbon unit that combined with cyanide to form β-CNAla. This suggested that β-CNAla

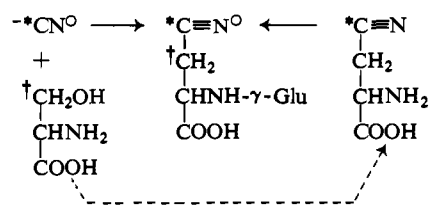
(38) It seems likely that β-CNAla is formed from cyanide, then placed into peptide linkage with glutamic acid. This sequence has been observed in time-course experiments with *Chlorella pyrenoidosa* [L. Fowden and E. A. Bell, *Nature*, **206**, 110 (1965)].

(39) Assignment of the specific activity of aspartic acid to the cyano carbon of β-CNAla is based upon results of degradations in expt 32, which showed over 99% of the activity of the β-CNAla moiety synthesized from $K^{14}CN$ to be located in C-4 of the derived aspartic acid.

synthesis is a detoxication process controlled chiefly by the formation of cyanide.[21] Incorporation of serine into asparagine in *L. sylvestris* was low but significant (3% incorporated, sp act. 1.11×10^3 cpm/μmol; Table II, expt 21). It perhaps is under similar control, as it is in linseed seedlings.[20a]

The metabolism of cyanide, β-CNAla, and serine in the presence of cyanide in *V. sativa* may be represented as in Scheme I.

Scheme I



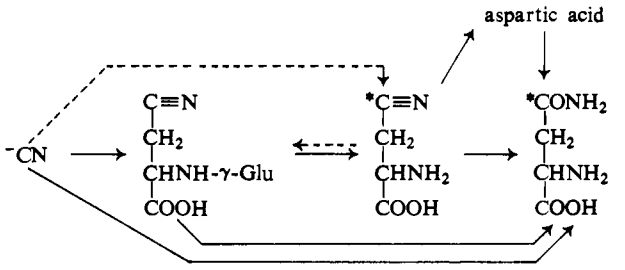
e. From Phenylalanine. Conversion of DL-phenylalanine-2-^{14}C into γ-Glu-β-CNAla in *V. angustifolia* was low but significant (0.7% incorporated, sp act. 1.33×10^3 cpm/μmol; Table III, expt 38). As found after hydrolysis (Figure 4), the dipeptide's activity resided in the β-CNAla moiety. Of this, most was in C-4 (Table IV, expt 38). The reaction of phenylalanine to yield cyanide has some structural specificity in this plant, since under the same conditions valine, which furnishes the α-hydroxybutyronitrile aglycon of linamarin in white clover,[40] was not a significant precursor of γ-Glu-β-CNAla (expt 39). The low degree of incorporation of C-2 of phenylalanine into β-CNAla despite the efficiency of the incorporation of KCN (17%, expt 37) suggests that, under the conditions studied, cyanide formation is only a minor route of the metabolism of phenylalanine.

Administration of DL-phenylalanine-2-^{14}C to cut seedlings of *V. sativa* did not lead to appreciable labeling of γ-Glu-β-CNAla (specific activity 38 cpm/μmol; Table III, expt 41). The *V. sativa* seeds contained about one-twentieth as much material capable of liberating HCN on treatment with emulsin as did *V. angustifolia* and furnished only 0.1 μmol of HCN/g. Such material could not be detected in 6-day-old seedlings of either species. In agreement with observations of Tschiersch,[17] the ability of a plant to convert an amino acid into cyanide is not necessarily associated with accumulation of cyanogenic material. In *V. sativa*, one or more steps in the conversion of phenylalanine into cyanogenetic material, or of the latter to cyanide, perhaps is absent or limited at the stage of development selected in the present study, *i.e.*, in the young seedling. However, the data do not preclude the existence of other metabolic routes or precursors for the synthesis of cyanide. The factors regulating the production of cyanide in *V. sativa* and in many other plants capable of assimilating cyanide by the β-cyanoalanine pathway remain to be clarified.

β-Cyanoalanine as an Intermediate between Inorganic Cyanide and Asparagine. In *L. sylvestris*, K^{14}CN was 2.7 and 2.3% incorporated into γ-Glu-β-CNAla (Table III, expt 19, 20). This was much less than the amount of K^{14}CN incorporated into asparagine in the same experiments (15 and 21%; Table II), and into γ-Glu-β-CNAla by *V. sativa* and *V. angustifolia* in various other experiments (17–63%). In these two vetches, the high content (1.4–1.9%) of γ-Glu-β-CNAla in young seedlings reflects the metabolic stability of β-CNAla as well as the efficiency of cyanide fixation. In contrast, *L. sylvestris* seedlings metabolize cyanide, β-CNAla, and γ-Glu-β-CNAla extensively into asparagine, and their low content of γ-Glu-β-CNAla accordingly may reflect the role of γ-Glu-β-CNAla or β-CNAla as an unstable intermediate in the conversion of cyanide into asparagine. The low incorporation of KCN into γ-Glu-β-CNAla in *L. sylvestris* may reflect the low content of this dipeptide, or the possibility that β-CNAla synthesized from cyanide is not all converted into the peptide before being converted into asparagine. That formation of β-CNAla or γ-Glu-β-CNAla from cyanide in expt 19 and 20 is more significant than would be suggested by the low degree of incorporation of K^{14}CN into this dipeptide is revealed by the high specific activity of γ-Glu-β-CNAla in each case.

Tissue γ-Glu-β-CNAla and asparagine were compared in specific activity after K^{14}CN administration. In expt 20 after 11 hr, the dipeptide had sp act. (in cpm/μmol) 4.83×10^4; asparagine 3.53×10^4. In expt 19 after 3 days, γ-Glu-β-CNAla had sp act. (in cpm/μmol) 2.32×10^4; asparagine 1.59×10^4; aspartic acid 2.06×10^4. When γ-Glu-β-CNAla had deen given to *L. sylvestris* seedlings for 6.5 hr, as discussed for expt 18 under Biosynthesis of Asparagine from γ-Glutamyl-β-cyanoalanine, tissue asparagine likewise had a lower specific activity than β-CNAla and the γ-Glu-β-CNAla that remained. These and preceding data summarized in the tables are consistent with Scheme II for the biosynthesis of asparagine and aspartic acid from cyanide in *L. sylvestris*. Only reactions established directly by experimentation in this study are indicated by heavy lines.

Scheme II



As with *V. sativa*, it would seem likely in *L. sylvestris* that β-CNAla is formed from cyanide before γ-Glu-β-CNAla. The data do not indicate whether incorporation of β-CNAla into the dipeptide is an obligatory step for asparagine synthesis in this plant. Probably at least some β-CNAla formed initially from cyanide is converted into asparagine without forming the dipeptide.

β-Cyanoalanine and γ-Glu-β-CNAla normally are not found in young *L. odoratus* and *V. villosa* seedlings. Like *L. sylvestris*, these plants incorporate both KCN and β-CNAla into asparagine, the latter to a greater degree than the former, as could be expected of a closer precursor. It seems likely that the same general pathway exists in these plants as in *L. sylvestris* for converting cyanide to asparagine in which β-CNAla serves as an unstable intermediate. The increasing number of species belonging to various higher plants, an insect, bacteria, and fungi that are reported[13] to incorporate cyanide into asparagine or

(40) G. W. Butler and B. G. Butler, *Nature*, **187**, 780 (1960).

aspartic acid without noticeably accumulating free or bound β-CNAla may be of this type.

Acknowledgment. We thank Mrs. Harriet R. Levie for skillful help in culturing seedlings, Mrs. Jeanne A. Nelson for amino acid analyses and assistance, Dr. Julian R. Rachele for advice concerning isotope determinations, and Dr. Leslie Fowden for suggesting the use of short-term experiments.

Superoxocobalamin, the First Intermediate in the Autoxidation of Vitamin B_{12r}

J. H. Bayston, N. Kelso King, F. D. Looney, and M. E. Winfield

Contribution from the Division of Applied Chemistry, Commonwealth Scientific and Industrial Research Organization, Fishermen's Bend, Victoria, Australia.
Received October 7, 1968

Abstract: Crystalline cob(II)alamin (vitamin B_{12r}) and its solutions in various liquids react reversibly with O_2 to yield a complex which is shown by epr spectroscopy to be mononuclear. In accord with the known shielding of the cobalt atom of the cobalamin against collision with large molecules, the mononuclear product of oxygenation does not combine with a second cob(II)alamin molecule to give a dicobalt complex. Eight hyperfine lines are found in the spectrum of methanolic solutions of oxygenated cob(II)alamin at temperatures close to the melting point. The coupling constant due to the cobalt nucleus is 12 G. At about 159°K there is a transition attributed to cessation of tumbling to give a spectrum different in over-all shape and in having an additional set of eight hyperfine lines. Superhyperfine structure due to the coordinated nitrogen atom of dimethylbenzimidazole is lost on oxygenation. Comparison with the hyperfine coupling constants of related binuclear complexes indicates that oxygenated cob(II)-alamin may be regarded as superoxocobalamin, most of the unpaired spin density being concentrated at the coordinated $O_2 \cdot^-$ group. Detection of a reversibly formed mononuclear product of oxygenation establishes the first reaction step in the autoxidation of B_{12r} and indicates the likelihood of transient occurrence of mononuclear superoxo complexes during autoxidation of hemoproteins. When cob(II)inamides are oxygenated the epr signals obtained resemble those from superoxocobalamin at temperatures below 159°K. The hyperfine coupling constants may be larger or smaller than for superoxocobalamin, depending upon whether the strength of bonding of the fifth ligand is less or greater than that for the nucleotide in the cobalamin.

In many catalyzed autoxidations, both enzymatic and nonbiological, the participation of O_2 begins with its activation, *i.e.*, a weakening of the bond between the two oxygen atoms. Sometimes the activation is effected by an organic free radical (mono or bi), sometimes by a metal atom of an inorganic compound or of a coordination complex. It is the latter class with which we have been principally concerned. Elsewhere we have listed eight conceivable ways in which activation by the metal may commence[1] and have, together with other groups of investigators (see, for example, references 2–4), provided some experimental evidence for the reality of four of them.[1,5-8]

Pauling[9] and many later investigators have studied the reversible complexing of O_2 to give a diamagnetic mononuclear product such as oxymyoglobin. Neither partner behaves as a free radical. The present report is concerned with the combination of O_2 with a free radical-like metal ion to yield a paramagnetic mononuclear product.

$$M\cdot + O_2 \rightarrow MO_2\cdot$$

A complex of this type had previously been detected[6] during the autoxidation of $[Co^{II}(CN)_5]^{3-}$, but we were unable to prove that it could be formed in the initial reaction step, apparently because it reacted swiftly with a second pentacyanocobaltate(II) ion to give a binuclear diamagnetic complex.

$$MO_2\cdot + M\cdot \rightarrow M\text{-}O\text{-}O\text{-}M$$

To avoid the difficulty we have chosen for study the oxygenation of cob(II)alamin (henceforth referred to as B_{12r}), a complex in which the cobaltous ion is shielded against close approach of similarly protected metal atoms.[10]

Some of the later steps in the autoxidation of B_{12r} to aquocobalamin have been elucidated by King, *et al.*[8]

Experimental Section

Materials. Glaxo aquocobalamin was recrystallized from acetone–water before use. Cyanoaquocobinamide was prepared

(1) M. E. Winfield in "Oxidases and Related Redox Systems," T. E. King, H. S., Mason, and M. Morrison, Ed., John Wiley & Sons, New York, N. Y., 1965, p 115.
(2) A. Haim and W. K. Wilmarth, *J. Am. Chem. Soc.*, **83**, 509 (1961).
(3) E. A. V. Ebsworth and J. A. Weil, *J. Phys. Chem.*, **63**, 1890 (1959).
(4) W. P. Schaefer and R. E. Marsh, *Acta Cryst.*, **21**, 735 (1966).
(5) J. H. Bayston, R. N. Beale, N. K. King, and M. E. Winfield, *Australian J. Chem.*, **16**, 954 (1963).
(6) J. H. Bayston, F. D. Looney, and M. E. Winfield, *ibid.*, **16**, 557 (1963).
(7) J. H. Bayston and M. E. Winfield, *J. Catal.*, **3**, 123 (1964).
(8) N. K. King, F. D. Looney, and M. E. Winfield, unpublished.
(9) L. Pauling in "Haemoglobin," F. J. Roughtan and J. C. Kendrew, Ed., Butterworth and Co. (Publishers), Ltd., London, 1949, p 57.
(10) D. C. Hodgkin, quoted by E. L. Smith, L. Mervyn, P. W. Muggleton, A. W. Johnson, and N. Shaw, *Ann. N. Y. Acad. Sci.*, **112**, 565 (1964).