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Isolation and Identification from Common Vetch of the Neurotoxin β-Cyano-L-alanine, a Possible Factor in Neurolathyrism*

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Neurological effects have for centuries been observed to accompany in many instances the consumption of excessive amounts of lathyrus meal as food, particularly in parts of India and in the Mediterranean area in times of food scarcity. This condition, which is known as spontaneous clinical neurolathyrism, occurs chiefly in the male and is characterized principally by reflex irritability, weakness, spasticity, and rigidity of the leg muscles, followed sometimes by death (1). The etiology of lathyrism is unknown. However, the condition has been variously ascribed to a nutritional deficiency and to the presence of a neurotoxin in the lathyrus plant. Many studies in experimental lathyrism were made possible by the isolation of a lathyrus factor from *Lathyrus pusillus* (singletary pea) (2) and *Lathyrus odoratus* (sweet pea) (3–5), the identification of this factor as β-*N*-(γ-L-glutamyl)-aminopropionitrile, and the recognition of β-aminopropionitrile as the active principle of the factor.[1] However, the experimental condition produced with either the *odoratus* factor or with a number of synthetic compounds related to β-aminopropionitrile has resulted chiefly in abnormalities of bone and mesenchymal tissue in contradistinction to human lathyrism which is neurologic in character.[1]

Recently, in an investigation of a new reaction encountered in peptide synthesis, which involves conversion of asparagine to α,γ-diaminobutyric acid (9), the amino acid nitrile, β-cyano-L-alanine, was synthesized (10). The latter was shown to be a likely intermediate in the conversion, arising through dehydration of the amide group of asparagine, and forming α,γ-diaminobutyric acid through reduction of its cyano group (10). Structurally, β-cyano-L-alanine also represents the parent amino acid which by biological decarboxylation could form the lathyrogen β-aminopropionitrile. In view of the latter relationship and the prevalence and importance of biological decarboxylation reactions, β-cyano-L-alanine was tested for possible neurological activity. Fed at the 1% level, this amino acid nitrile is neurotoxic to the rat, and within 3 to 5 days it results in hyperirritability, tremors, convulsions, and death (11). The D isomer at approximately 3 times the dose is also neurotoxic.[2] In contrast, γ-cyano-L-α-aminobutyric acid, the amino acid nitrile which represents the dehydration product of L-glutamine (10), is tolerated with no obvious toxicity.[2]

These results led us to consider the possibility that β-cyanoalanine may be the neurolathyrogen which occurs naturally. Since Lewis *et al.* (12, 13) and Schulert and Lewis (14) had shown the seeds of two species of lathyrus, *i.e. Lathyrus latifolius* (perennial sweet pea) and *Lathyrus sylvestris Wagneri* (flat pea), to be neurotoxic to the rat and the mouse, we examined these seeds for the presence of β-cyanoalanine. These studies led recently to the isolation of the chief neurotoxic principle of *L. latifolius* and its identification as L-α,γ-diaminobutyric acid (11). This same amino acid occurs in very high concentration in the toxic seed of *L. sylvestris Wagneri* (11). It is perhaps more than fortuitous that although the isolated neurotoxin was not the sought-for β-cyanoalanine, it is, nevertheless, chemically related to the latter, L-α,γ-diaminobutyric acid being a reduction product of β-cyano-L-alanine. For this reason, and in analogy with the chemical reaction found with peptide asparagine, a biosynthetic pathway in lathyrus was considered likely in which L-asparagine is dehydrated to β-cyano-L-alanine. In *L. odoratus* this intermediate amino acid nitrile would be decarboxylated to yield β-aminopropionitrile, whereas in *L. latifolius* and *L. sylvestris Wagneri* it would be reduced to L-α,γ-diaminobutyric acid (11).

It appears that, in human neurolathyrism, one of the chief species of lathyrus involved has been *Lathyrus sativus* (15, 16). Yet, it has been found that consumption of the latter by experimental animals has not produced toxic effects (12, 17, 18).[1] A botanical examination carried out some years ago with a number of samples of *L. sativus* collected from various localities in India, where lathyrism was common, showed that in all the samples a leguminous weed, *Vicia sativa*,[3] was present (18). Moreover, ducks and monkeys fed diets containing *V. sativa*[3] developed a syndrome suggestive of deleterious effects on the nervous system. It was pointed out that the seeds of *L. sativus* itself are harmless, but that the danger of the disease lay probably in the contamination of the latter with other peas known

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[1] For reviews of the subject of experimental and clinical lathyrism, see references (6–8).

[2] C. Ressler, unpublished experiments.

[3] Identified as *Vicia sativa* L. var. *angustifolia* (18). Because *Vicia angustifolia*, which is regarded as the progenitor of *Vicia sativa*, was not available to us at the start of this study, we have investigated chiefly the latter which is considered to differ scarcely from the former except in dimensions (19).

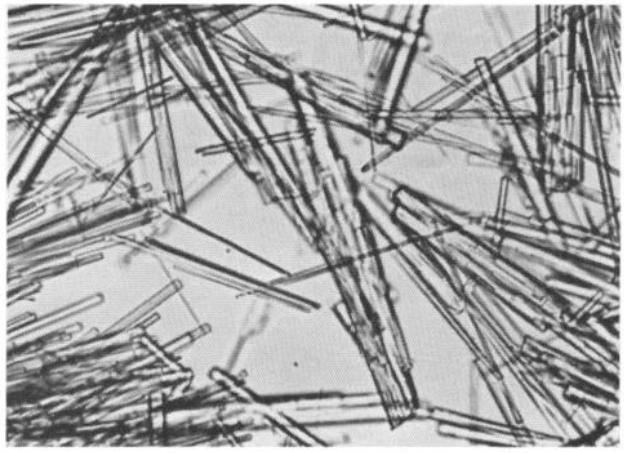

FIG. 1. Photomicrograph of crystals of β-cyano-L-alanine isolated from *Vicia sativa* seed.

to be toxic (18).[4] A later report has described an outbreak in an Indian village of lathyrism which appeared to be associated with the consumption of wheat, a sample of which was examined and found to contain seeds of *V. sativa* (22).

We have now examined *V. sativa* (common vetch) for the presence of L-α,γ-diaminobutyric acid and β-cyano-L-alanine as possible neurolathyrogens. In this paper we report for the first time the natural occurrence of β-cyano-L-alanine, and describe the isolation and identification of this neurotoxic amino acid from *V. sativa* seed. β-Cyanoalanine was identified also in the seed of *Vicia angustifolia* (narrow leaf vetch), which is of interest also from the biogenetic standpoint, since it is in this seed that the cyanogenetic glycoside vicianin (mandelonitrile vicianoside) occurs (23, 24). *L. sativus*, which was examined as well, showed no detectable amount in the free state of either amino adid.

The isolation of the neurotoxic amino acid from the seed was based upon the observation that synthetic β-cyanoalanine, like β-aminopropionitrile (25), produces, when allowed to react with ninhydrin, a rather unusual bright green color (10); the color reaction was used in conjunction with chromatographic and electrophoretic procedures. Identification of the isolated material rested on a comparison of physical, chemical, and biological properties with the recently synthesized β-cyano-L-alanine (10).

Pulverized, hexane-extracted *V. sativa* seeds,[5] 460 g, were extracted twice with 2.3 liters of 30% ethanol overnight in the cold room. After removal of the solvent, the residue in 230 ml of water was applied at pH 2 to two 2.2 × 55 cm columns of Amberlite CG-120 (H^+) resin. The columns were washed with water, and the adsorbed material was eluted with 3 N ammonia. The alkaline effluent yielded 16 g of material which was passed through a 2.2 × 50 cm column of Amberlite IR-45 resin. The effluent was analyzed qualitatively on paper by spraying with 0.2% ninhydrin in acetone, and the fractions which gave evidence of a green color reaction were collected and combined. After concentration of the solution, 832 mg of ninhydrin-negative, nontoxic crystals separated, m.p.[6] 240°, with decomposition. The residue of 767 mg from the mother liquor was subjected to preparative electrophoresis on a block of Solka-Floc[7] in pyridinium acetate buffer, pH 5.7. The neutral fraction was eluted and electrophorized in barbital buffer, pH 8.6, for 1½ days at 9 volts per cm. A contact print of the block with the use of the green ninhydrin color reaction located the material 6.5 to 13 cm from the origin toward the anode. Buffer components were removed from the eluate with Amberlite CG-120 (H^+) resin, and, after concentration and adjustment of the solution to pH 4.9, 125 mg of rectangular needles separated, m.p. 207°, with decomposition. Three recrystallizations from aqueous dioxane left 75 mg, m.p. 214.5°, with decomposition; $[\alpha]_D^{23}$ −0.2° (*c* 0.74, 1 N acetic acid); *analysis*:[8]

$C_4H_6N_2O_2$

Calculated: C 42.1, H 5.30, N 24.6

Found: C 42.4, H 5.40, N 24.5

reported for β-cyano-L-alanine, m.p. 218–218.5°, with decomposition; $[\alpha]_D^{26}$ −2.9° (*c* 1.4) (10). A mixed melting point with synthetic β-cyano-L-alanine showed no depression. The recrystallized isolated material is shown in Fig. 1.

A sample of 0.5 mg was hydrolyzed in 6 N hydrochloric acid for 12 hours at 110°, and the hydrolysate was analyzed by the quantitative chromatographic-ninhydrin procedure (26) on the Beckman model 120 automatic amino acid analyzer. Only aspartic acid and ammonia were found, in the ratio 1:1.04. The recovery of material was 104%. Another sample was dissolved in 2 ml of liquid ammonia and 200 μl of methanol, and the solution was treated briefly with sodium (10). After evaporation of the liquids, quantitative amino acid analysis of the residue on the 15-cm resin column of the amino acid analyzer, pH 5.28, 50°, indicated the presence of α,γ-diaminobutyric acid, effluent volume, 60 ml, in a yield of 102%. The isolated material and synthetic β-cyano-L-alanine were chromatographed and co-chromatographed on the 50-cm resin column of the amino acid analyzer at pH 3.25 and 30°. A single band was obtained in each case, effluent volume, 111 ml; color yield constant, 5.48. The electrophoretic behavior on paper of both substances at pH 5.7, as well as at pH 8.6, was identical. In each instance a single green spot was obtained when the strip was developed with ninhydrin in acetone. The absorption spectra of the reaction products formed with ninhydrin in *n*-butyl alcohol at 100° for 8 minutes were identical for both substances (λ_{max} 320, 410, 645 mμ and high general absorption in the 520- to 620-mμ region). The natural material and synthetic β-cyano-L-alanine possessed infrared spectra which were essentially identical and which exhibited characteristic nitrile absorption at 2250 cm^{-1}. That the isolated β-cyanoalanine possessed the L configuration was established through preparation of the carbobenzoxy derivative, m.p. 136°, $[\alpha]_D^{23}$ −46.0° (*c* 0.47, dimethylformamide), reported for carbobenzoxy-β-cyano-L-alanine, m.p. 134–135° (27), 132–133° (10), $[\alpha]_D^{22}$ −45.2° (*c* 0.93) (10). There was no depression in melting point on admixture with carbobenzoxy-β-cyano-L-alanine prepared through dehydration of carbobenzoxy-

[4] In later feeding experiments with the pony and the rat no neurotoxic effects could be demonstrated with *Vicia sativa* as well as with *Lathyrus sativis* (20, 21).

[5] Purchased from Craver-Dickinson Seed Company, Buffalo, New York.

[6] Melting points were determined in capillaries and are corrected.

[7] Purchased from Brown Company, Berlin, New Hampshire.

[8] Microanalyses were performed by Schwarzkopf Microanalytical Laboratories, Woodside, New York.

L-asparagine with dicyclohexylcarbodiimide (10). The infrared spectrum of the carbobenzoxy derivative of the isolated material was indistinguishable from that of synthetic carbobenzoxy-β-cyano-L-alanine. Like the synthetic amino acid, natural β-cyano-L-alanine, administered to a weanling male rat by stomach tube in dosage of 15 mg per 100 g, caused hyperactivity, followed by tremors, convulsions, and rigidity, from which the rat recovered after 4 hours. A dose of 20 mg per 100 g, injected subcutaneously, caused convulsions, rigidity, prostration, and death.

In any attempt to evaluate the natural occurrence in *V. sativa* of β-cyano-L-alanine as a causative factor of human neurolathyrism, it should be noted that the presence in this legume of additional neurotoxins, or even the presence of additional β-cyano-L-alanine in bound form, has not been excluded, since we have not followed the isolation with biological studies on the distribution of toxicity. There may also be some uncertainty about the similarity of the human condition and the biological effects of the neurotoxin in the rat. It is recognized, however, that the rat may not be the most suitable experimental animal for a definitive comparison with man with respect to the nature of neurological lesions and behavior. It may be noted also that, as has been found with the rat and the mouse,[2] there may be considerable species difference in the degree of susceptibility to β-cyano-L-alanine. Ultimately, the question remains whether, in the absence of additional toxins, β-cyanoalanine, present to the extent of approximately 0.1%[9] in *Vicia* seed, which itself occurs as a mixture with nontoxic seeds, such as *L. sativus*, in a diet of unknown total composition, is present in high enough concentration to be toxic to man. In feeding experiments with the rat, toxicity with a level of 0.75% of synthetic β-cyano-L-alanine was evident within several days,[2] and it may not be unexpected that the consumption of β-cyano-L-alanine even at very low levels, in conjunction with a nutritionally poor diet of long duration, could lead to neurotoxicity in man. If β-cyano-L-alanine should indeed prove to be a factor in human neurolathyrism, a striking example of a nutritional disease is provided which has its origin in the presence of a toxic substance rather than, as has been more usually the case, in the deficiency of an essential factor.

SUMMARY

Specimens of the seeds of *Vicia angustifolia* and *Vicia sativa*, which are closely allied botanically to the seeds that have been reported as contaminants in the *Lathyrus sativus* seeds and in the wheat consumed during several outbreaks of human lathyrism, have been examined electrophoretically and chromatographically for their amino acid content. The neurotoxic amino acid β-cyanoalanine has been found to be present in the seeds of both vetches. β-Cyano-L-alanine has been isolated from *V. sativa* seed in crystalline form and has been identified through comparisons of its physical, chemical, and biological properties with synthetic material. The possibility that β-cyano-L-alanine is a factor in causing human neurolathyrism is discussed.

[9] Quantitative amino acid analysis (26) of 30% ethanol extracts showed a concentration of β-cyanoalanine of 0.15% in seeds of *Vicia sativa* and of 0.09 and 0.12% in seeds of *Vicia angustifolia*, the latter of Turkish and Iranian origin, respectively.

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