

seemed somewhat low, based on the toxicity of β -cyanoalanine to the rat,⁶ to warrant serious consideration of this amino acid as an active factor in lathyrism. We now wish to report that common vetch contains, as well, significant quantities of β -cyano-L-alanine in a bound form which can also be neurotoxic. Moreover, observations with a second species indicate that both the seed of common vetch and its active principles can be far more toxic than experience with the rat had suggested.

Preliminary analytical procedures allowed characterization of the bound β -cyanoalanine as the acidic dipeptide glutamyl- β -cyanoalanine (R_f 0.59, pyridine-water 65:35). The β -cyanoalanine peptide was then isolated from common vetch by chromatography of material in 30% ethanol seed extracts directly on Dowex 1-X4 with pyridinium acetate buffer, pH 4.1, as well as by preparative electrophoresis on blocks of Solka-floc at pH 5.7 and pH 3.5. The purified peptide was then crystallized from water-ethanol, yield 59%, m.p. 185.5–186° dec. (*Anal.* Calcd. for $C_9H_{13}N_3O_5$: C, 44.4; H, 5.39; N, 17.3. Found: C, 44.4; H, 5.52; N, 17.0). Its *dicyclohexylammonium* (DCHA) salt melts at 184–185° dec.; $[\alpha]^{23}_D +15.1^\circ$ (c 0.6, 2.5% $KHCO_3$). *Anal.* Calcd. for $C_{21}H_{36}N_4O_5 \cdot 0.5 H_2O$: 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. Hydrolysis in 6 *N* HCl for 10 hr. at 110° followed by analysis on the Beckman automatic amino acid analyzer⁸ gave a quantitative yield of aspartic acid, glutamic acid, and ammonia, all in a molar ratio to each other of 1:1. On treatment⁶ of the DCHA salt with sodium in ammonia containing methanol followed by desalting, hydrolysis, and analysis, less than 1% of aspartic acid was found. Glutamic acid and 2,4-diaminobutyric acid, which were present in molar ratio of 1:1, were obtained in 89% yield.

The isolated peptide was established to be N-(γ -L-glutamyl)- β -cyano-L-alanine through synthesis of the latter, accomplished by hydrogenolysis in the presence of a palladium catalyst of the intermediate carbobenzoxy- γ -L-glutamyl- β -cyano-L-alanine α -benzyl ester which was prepared in 69% yield by coupling carbobenzoxy-L-glutamic acid α -benzyl ester and β -cyano-L-alanine by the mixed anhydride procedure with isobutyl chlorocarbonate. Synthetic γ -L-glutamyl- β -cyano-L-alanine, which possessed the expected elementary composition, agreed with the isolated peptide in melting point, and admixture of them, as well as of their DCHA salts, caused no depression in the respective melting points. Optical rotations and infrared spectra of the DCHA salts were the same for both substances. The two materials showed identical behavior on chromatography on paper in two systems as well as on Dowex 1-X4 (acetate) and Amberlite CG-120 (H^+) ion-exchange resin columns, and had the same electrophoretic mobility on paper at pH 5.7 or 8.6. Moreover, the natural and synthetic peptides were readily distinguishable chromatographically and electrophoretically from the product of hydrogenolysis of carbobenzoxy- α -L-glutamyl- β -cyano-L-alanine γ -benzyl ester prepared in a similar way from carbobenzoxy-L-glutamic acid γ -benzyl ester. The natural and synthetic γ -L-glutamyl- β -cyano-L-alanines showed similar toxicities when injected subcutaneously into young rats.

Isolated γ -L-glutamyl- β -cyano-L-alanine is similar in potency on a molar basis to β -cyano-L-alanine when administered to male weanling Sherman rats subcutaneously, or as a single dose by stomach tube. In White Leghorn chicks the dipeptide, when administered

subcutaneously or *per os*, is approximately half as toxic as β -cyano-L-alanine. The concentration of γ -glutamyl- β -cyanoalanine is 0.58% in the seed of common vetch and rises to 1.67 to 2.6% (dry wt.) in the young seedling, suggesting the latter stage of development is potentially more toxic than the seed. A mixture of γ -L-glutamyl- β -cyano-L-alanine and β -cyano-L-alanine, incorporated into a basal ration at half the concentration (0.29 and 0.075%) at which these occur in the seed, has an effect in young chicks similar to a 50% common vetch seed ration, the two diets producing within 6 and 6.5 days (average), respectively, terminal convulsive states with a characteristic opisthotonus.⁹

There has been some question of the neurotoxicity of common vetch seed,⁷ and in view of seeming conflicting reports in the literature^{10–12} we wish to note that toxicity varies strikingly in different species. In general confirmation of recently reported studies with the chick,¹³ diets incorporating 20 to 50% of common vetch seed have been found highly neurotoxic to this species, whereas, in our earlier experiments with the rat, similar concentrations of vetch had appeared nontoxic. As in other experiments,¹¹ even diets very high in vetch (85 and 100%), although tending to retard growth, produced no obvious neurotoxicity in the rat. Considerable species difference holds also for β -cyano-L-alanine, which, in the diets employed, has been found neurotoxic to the chick near the 0.075% level (average survival 10.5 days) in contrast to the rat which tolerates more than 9 times this concentration.⁶ On the basis of the concentrations of γ -L-glutamyl- β -cyano-L-alanine and β -cyano-L-alanine in the seed of common vetch and the now established levels of toxicity of these two principals in the two species, one should indeed expect diets which contain 20 to 100% of common vetch seed to be highly neurotoxic to the chick while they could be nontoxic to the rat. The advisability of using diverse species when testing suspect foods or chemicals for neurotoxicity is evident.

These studies constitute the first report of the natural occurrence of β -cyanoalanine in peptide or bound form and also suggest that γ -glutamyl- β -cyanoalanine and β -cyanoalanine are probably the chief neurotoxic principals of common vetch seed.

(9) The basal ration was commercial Wayne High Fiber Pullet Developer obtained from Allied Mills, Inc., Chicago, Illinois. It contained minimum 14% protein, 2.5% fat, and 9% fiber. The test diets used for the vetch and the isolated principles may not be comparable nutritionally. The isolations were followed chiefly by chemical means due to the lack of a sensitive bioassay at the start of this work.

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Isolation and Reactions of a Stable Enol Phosphonium Salt

Sir:

Enol phosphonium salts I have been postulated^{1–3} as intermediates in the Perkow⁴ and halogen migration¹

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