The Isolation and Characterization of β-*N*-Oxalyl-L-α,β-Diaminopropionic Acid: A Neurotoxin from the Seeds of *Lathyrus sativus**

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A neurotoxic compound has been isolated from the seeds of *Lathyrus sativus* in 0.5% yield and characterized as β-*N*-oxalyl-L-α,β-diaminopropionic acid. The compound is highly acidic in character and forms oxalic acid and diaminopropionic acid on acid hydrolysis. The compound has a specific rotation of −36.9° and has apparent *pK* values in the order of 1.95, 2.95, and 9.25, corresponding to the two carboxyl and one amino functions, respectively. The compound has been synthesized by reacting an aqueous methanolic solution of the copper complex of L-α,β-diaminopropionic acid prepared at *p*H 4.5–5.0 with dimethyl oxalate under controlled *p*H conditions and isolating the compound by chromatography on a Dowex 50-H^+ column after precipitating the copper. The compound induced severe neurological symptoms in day-old chicks at the level of 20 mg/chick, but not in rats or mice. It also inhibited the growth of several microorganisms and of the insect larva *Corcyra cephalonica* Staint. L-Homoarginine had no neural action in chicks. It is suggested that the neurotoxic compound is species specific in its action and may be related to "neurolathyrism" associated with the human consumption of *L. sativus* seeds.

The clinical syndrome "neurolathyrism" is attributed to the prolonged consumption of the seeds of several species of *Lathyrus* and notably *Lathyrus sativus* (Ganapathy and Dwivedi, 1961). The latter is grown and consumed under famine conditions in certain parts of central India. Patients suffering from this disease are afflicted by symptoms which are primarily of a neurological character. Though many explanations have been advanced and much research work has been carried out to characterize the causative factor(s), the etiology of this crippling disease is still unknown (Strong, 1956; *Nutrition Reviews*, 1963). Ressler *et al.* (1961) have reported the presence of L-α,γ-diaminobutyric acid, a neurotoxic compound in the seeds of *L. latifolius* and the same has also been detected in eleven other species of *Lathyrus* by Bell (1962a). However, this neurotoxic compound has not been detected in the seeds of *L. sativus* (Bell, 1962b). Recently Ressler (1962) reported the presence of β-cyano-L-alanine in the seeds of *Vicia sativa*, a common vetch which is reported as a contaminant of *L. sativus* seeds (Anderson *et al.*, 1924) and suggested that the toxicity of the seeds might be due to this contaminant.

Earlier reports from this laboratory on the analysis of the *L. sativus* seeds for unusual constituents which may have potent biological activity dealt with the isolation and characterization of L-homoarginine (Rao *et al.*, 1963) as well as the isolation and properties of a toxic compound (Adiga *et al.*, 1962, 1963). This compound has now been characterized as β-*N*-oxalyl-L-α,β-diaminopropionic acid and in this paper a detailed account of its isolation characterization and toxic action is presented. β-Cyano-L-alanine could not be detected in the seed samples used in the present investigation.

Experimental and Results

Chromatography.—One-dimensional ascending chromatograms were developed using Whatman No. 1 filter paper. The solvents used were generally those recommended by Smith (1958) and Block *et al.* (1955).

Electrophoresis.—Electrophoresis was carried out on Whatman No. 1 filter paper strips using a horizontal open-strip paper electrophoretic apparatus for 3 hours at 400 v (8–10°). The following buffer systems were employed: citrate-phosphate buffer (*p*H 3–5), sodium phosphate buffer (*p*H 6–8), barbital buffer (*p*H 9) (Gomori, 1957).

Infrared Spectra.—Infrared spectra of the compounds were recorded in a Perkin-Elmer infracord spectrophotometer Model 137 using Nujol.

pK Determination.—A 0.025 M solution of the isolated compound was titrated against 0.5 M HCl and 0.5 M NaOH at 20°. Measurements of *p*H were made with a Coleman Metrion *p*H meter Model 28. Corrections for the 20 ml of water used as solvent were similarly determined. L-Aspartic acid was also titrated for comparison and the following apparent dissociation constants were obtained: pK_1', 2.0; pK_2', 3.6; pK_3', 9.6. (lit. pK_1', 1.9; pK_2', 3.7; pK_3', 9.6 at 25°).

Reagents and Chemicals.—Dimethyl oxalate was prepared according to the method of Bowden (1943). L-α,β-Diaminopropionic acid HCl was prepared by Hoffman degradation of carbobenzoxy-L-asparagine (Sigma) following the procedure of Schneider (1937).

Isolation and Properties of the Compound.—The toxic compound was isolated essentially as described in our earlier report (Adiga *et al.*, 1962). However, a modification of the extraction procedure as described below secured better yields of the compound.

The seed powder (400 g) was extracted for 15 hours with 2 liters of water at 60–65° with occasional stirring. The residue from the filtered slurry was further extracted with 1 liter of water. The combined water extracts were treated with alcohol to 75% concentration, stirred, and filtered after 0.5 hour. The filtrate was concentrated under reduced pressure to about 1.5 liters. The concentrate was extracted first with ether (1 liter) and subsequently with chloroform (1 liter). The aqueous phase, after being freed of solvents, was percolated through a 1 × 7.5-in. column of Dowex 50-X-8 (H^+), (200–400) and washed with water. Fractions (100 ml) were collected at a flow rate of 25–30 ml/hour, the elution being followed by periodic testing of the effluent by circular paper chromatographic technique of Giri and Rao (1952) using the solvent system, 1-butanol–acetic acid–water, 4:1:1 (v/v), and locating

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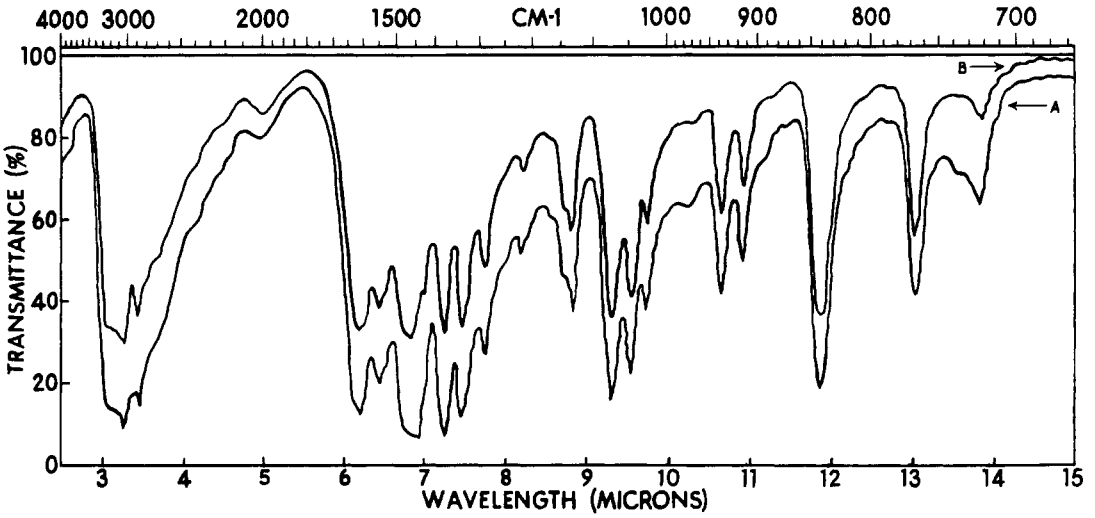

FIG. 1.—Infrared spectra of L-α,β-diaminopropionic acid HCl. A, authentic sample; B, isolated sample.

the compound by spraying with ninhydrin. The compound emerged after about 200 ml of the eluate was collected, and the next 400 ml of the eluate secured complete elution of the compound. The pooled extracts containing the compound were then concent ated to about 100 ml at 40–50° in a rotary flash evaporator and treated with excess of acetone (3 liters) and stirred. The precipitated compound was collected and reprecipitated from hot water by the addition of acetone. Yield, 2–2.1 g. The compound was recrystallized from hot water; mp 206° decomp, $[\alpha]_D^{25} = -36.9°$ (*c*, 0.66; 4 N HCl).

Anal. Calcd. for $C_5H_8O_5N_2$: C, 34.09; H, 4.57; N, 15.90. Found: C, 33.32; H, 4.56; N, 15.57.

The compound in solution (2 mg/ml) was highly acidic (*p*H 2.5) and was negatively charged below *p*H 9.0 and moved faster than aspartic and glutamic acids. The compound when tested with ninhydrin according to Rosen (1957) gave a quantitative color yield, and failed to react with ninhydrin after spraying with copper nitrate reagent of Larsen and Kjaer (1960) or with pyridoxal reagent of Kalyankar and Snell (1957), thus suggesting the presence of a free α-NH_2 group. The compound gave the apparent *pK* values 1.95, 2.95, and 9.25 for the ionizable functional groups.

Hydrolysis of the Compound.—The compound (200 mg) was hydrolyzed with 5 ml of 4 N HCl for 2 hours at 100° in a sealed tube. The hydrolysate was concentrated to a thick syrup *in vacuo* over NaOH pellets and triturated with portions of ether. The crystalline residue (170 mg) left after ether extraction was recrystallized from aqueous methanol when 155 mg of white needles was obtained (compound A). The ether extracts were evaporated to dryness *in vacuo* when 130 mg of colorless crystals was left (compound B).

Identification of Compound A as L-α,β-Diaminopropionic Acid HCl.—Compound A was ninhydrin positive and gave a 33% color yield (Gallop *et al.*, 1960) with ninhydrin when tested according to the method of Rosen (1957). A 2 mg/ml solution, when tested separately on paper with copper nitrate and pyridoxal reagents of Larsen and Kjaer (1960) and Kalyankar and Snell (1957), respectively, followed by ninhydrin, gave a positive reaction. The compound proved to be more basic than lysine and ornithine upon electrophoresis and testing in a Dowex 50-H^+ column. A three-times-crystallized compound had mp 236–237° (lit. 236–237°), $[\alpha]_D^{25} = +20.5°$[1] (*c*, 1.0; 4 N HCl); (Schneider, 1937, $[\alpha]_D^{25} = +25.25°$).

[1] This lowered value of specific rotation has been ascribed by Kjaer and Larsen (1959) as due to acid-induced racemization.

TABLE I

R_F VALUES OF NATURALLY OCCURRING AND SYNTHETIC β-*N*-OXALYL-L-α,β-DIAMINOPROPIONIC ACID AND ISOLATED AND AUTHENTIC L-α,β-DIAMINOPROPIONIC ACID

Solvent[a]	OX-Dapro[b] (Natural)	OX-Dapro[b] (Synthetic)	Dapro[c] (Isolated)	Dapro[c] (Authentic)
A	0.09	0.09	0.17	0.17
B	0.08	0.08	0.41	0.41
C	0.2	0.2	0.37	0.37
D	0.09	0.09	0.34	0.34

[a] Solvents: A, isopropanol-water-ammonia (sp gr, 0.88), 80:20:2, v/v; B, phenol-ethanol-water-ammonia (sp gr, 0.88), 150:40:10:1, v/v; C, 1-butanol–1-propanol–0.1 N HCl, 1:1:1, v/v; D, collidine-water, 125:44, v/v (NH_3 atmosphere). [b] OX-Dapro = β-*N*-oxalyl-L-α,β-diaminopropionic acid. [c] Dapro = L-α,β-diaminopropionic acid.

Anal. Calcd. for $C_3H_8O_2N_2 \cdot HCl$: C, 25.64; H, 6.45; N, 19.90; Cl, 25.23. Found: C, 25.5; H, 6.44; N, 20.05; Cl, 25.24 (ionic).

A comparison of the R_F values of compound with that of authentic L-α,β-diaminopropionic acid in various solvents (Table I) established its identity. The infrared spectrum of compound A was indistinguishable from that of authentic L-α,β-diaminopropionic acid HCl (Fig. 1). Compound A was further characterized as its dipicrate. Compound A (50 mg) was dissolved in 0.5 ml water and treated with excess of hot saturated solution of picric acid, heated on a steam bath for 15 minutes, and cooled. The excess of picric acid was removed by extraction with ether and the aqueous solution was concentrated to dryness *in vacuo*. The residue was collected and washed with ethanol and ether and dried. Yield, 140 mg; mp 235° decomp.

Anal. Calcd. for $C_3H_8O_2N_2$ $(C_6H_3O_7N_3)_2$: N, 19.92. Found: N, 19.38.

Identification of Compound B as Oxalic Acid.—Since the elemental analysis of L-α,β-diaminopropionic acid differed from that of the original compound by a C_2 fragment and the infrared spectrum of the original compound was suggestive of a —CO—NH— stretching, the nature of the ether-soluble component (compound B) was investigated. Compound B decolorized acidified permanganate, but did not reduce Tollens' reagent, formed an insoluble calcium salt, and produced a red color on heating with indole and sulfuric acid (Bergerman and Elliot, 1955). A few mg of the compound, when melted with diphenylamine over a

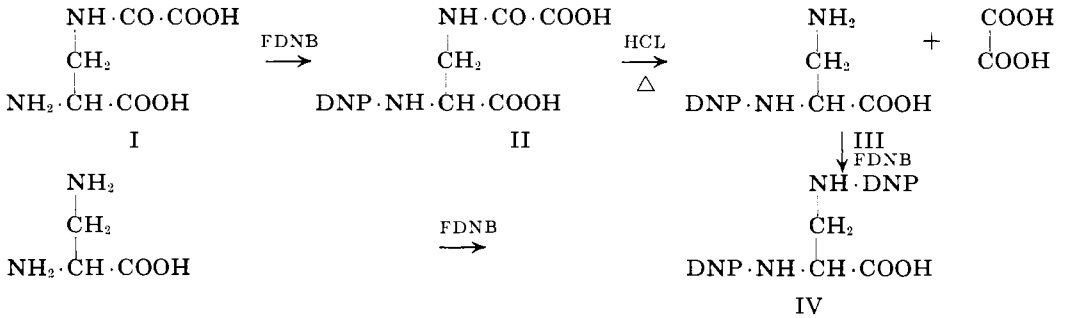

FIG. 2.—Schematic representation of the dinitrophenylation reactions.

free flame, cooled, and dissolved in alcohol, gave a blue color, which is a specific test for oxalic acid (Huntress and Mulliken, 1941). Compound B was recrystallized from a small quantity of water and it analyzed exactly for oxalic acid. Mp 101° (lit. 101–102°).

Anal. Calcd. for $C_2H_2O_4 \cdot 2\ H_2O$: C, 19.06; H, 4.8. Found: C, 18.64; H, 5.01.

Compound B was further characterized as oxalic acid by preparation of its ethanolamine complex according to Keisser (1940). Mp 199° (rep: 199–200°). Again the R_F values of compound B were the same as that of an authentic sample of oxalic acid in the solvent systems recommended by Smith (1958).

Proof for the Structure of the Compound.—The yields of L-α,β-diaminopropionic acid and oxalic acid obtained by hydrolysis of the original compound suggest them to be present in equimolar ratios, and this was further confirmed by estimating the oxalic acid with indole (Bergerman and Elliot, 1955) and L-α,β-diaminopropionic acid with ninhydrin (Rosen, 1957). Since the original compound did not give positive reaction with ninhydrin after treatment with copper nitrate and pyridoxal reagents, the β-amino group of L-α,β-diaminopropionic acid is probably linked with the carboxyl group of oxalic acid in an amide linkage. This is conclusively proved by the following reactions represented in Figure 2. About 20 mg of the original compound (I) was dinitrophenylated with excess of FDNB[2] according to Sanger (1945). The resulting DNP compound (II) which was ninhydrin negative was hydrolyzed with 4 N HCl for 2 hours at 100° in a sealed tube. After removal of HCl the DNP compound (III) was found to be ninhydrin positive both before and after spraying with cupric nitrate reagent showing thereby the formation of a free β-NH_2 group during hydrolysis. Compound III was further treated with FDNB. The resulting di-DNP compound (IV) had the same R_F values as that of an authentic di-DNP-L-α,β-diaminopropionic acid in various solvents. The R_F values of these compounds are listed in Table II (also the R_F values of these DNP compounds obtained from synthetic β-*N*-oxalyl-L-α,β-diaminopropionic acid are included for comparison).

Synthesis of β-N-Oxalyl-L-α,β-diaminopropionic Acid.—Since Kjaer and Larsen (1959) have successfully used the copper complex of L-α,β-diaminopropionic acid prepared at *p*H 4.5–5.0 for the introduction of a β-ureido group in synthesizing albizzine, the same method of temporary protection of the α-NH_2 group of L-α,β-diaminopropionic acid has been used in this synthesis. Since reaction of the copper complex prepared in this fashion with oxalyl chloride led to diminished yields of the expected compound, dimethyl oxalate was used and found to result in better yields of the compound.

[2] Abbreviations used in this work: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl.

TABLE II
R_F VALUES OF DNP COMPOUNDS (FIG. 3)[a]

Solvent[b]	II_a	II_b	III_a	III_b	IV_a	IV_b	IV_c
A	0	0	0.11	0.11	0.63	0.63	0.63
B	0.05	0.05	0.23	0.23	0.08	0.08	0.08
C	0.43	0.43	0.59	0.59	0.87	0.87	0.87
D	0.03	0.03	0.22	0.22	0.68	0.68	0.68

[a] II, III, and IV refer to the compounds shown in Fig. 3. Subscript letters refer to DNP compounds obtained from: (a) naturally occurring and (b) synthetic β-*N*-oxalyl-L-α,β-diaminopropionic acid, and (c) from authentic L-α,β-diaminopropionic acid. [b] Solvents: A, toluene-chloroethanol-pyridine-ammonia (sp gr, 0.88), 5:3:1:3, v/v; B, chloroform–acetic acid (1.5 N)–1-propanol, 10:6:10, v/v; C, 1-butanol–acetic acid–water, 3:1:1, v/v; D, pyridine–isoamyl alcohol–ammonia (1.6 N), 6:14:20, v/v.

A solution of L-α,β-diaminopropionic acid HCl (140 mg) in 2 ml of water, brought to *p*H 4.5–5.0 by the addition of 1 N NaOH, was gently boiled with excess of cupric oxide for 4–5 minutes and the excess of cupric oxide was subsequently removed by centrifugation The aqueous solution containing the copper complex was treated with 1–1.5 ml of methanol. Dimethyl oxalate (170 mg) dissolved in 3 ml of methanol was added to the solution during the course of 40 minutes with rapid stirring, the *p*H of the solution being maintained between 4.5 and 5.0 by the periodic addition of 1 N NaOH. The stirring was continued for a further 20 minutes at this *p*H. The *p*H of the solution was then increased to 8.2–8.5 and after 30 minutes the volume was increased to about 80 ml with hot water. After the *p*H of the solution was adjusted to 4.0 with 1 N HCl the solution was treated with hydrogen sulfide and filtered. The filtrate was freed of hydrogen sulfide and put through a 1 × 6-cm column of Dowex 50-H^+ and eluted with water. The ninhydrin-positive fractions were pooled and concentrated to a small bulk *in vacuo*, treated with excess of acetone, and stirred. The precipitated compound was collected and reprecipitated from an aqueous solution with acetone. Yield, 70–80 mg. The compound recrystallized with hot water had mp 206° decomp; $[\alpha]_D^{27} = -35.1°$ (c = 0.66, 4 N HCl).

Anal. Calcd. for $C_5H_8O_5N_2$: C, 34.09; H, 4.57; N, 15.90. Found: C, 34.32; H, 5.206; N, 15.3.

Admixture of the synthetic compound with the naturally occurring compound did not show any depression in melting point.

The identity of the naturally occurring compound with that of synthetic β-N-oxalyl-L-α,β-diaminopropionic acid was established on the basis of (1) their R_F values (Table I), (2) their DNP compounds (Table

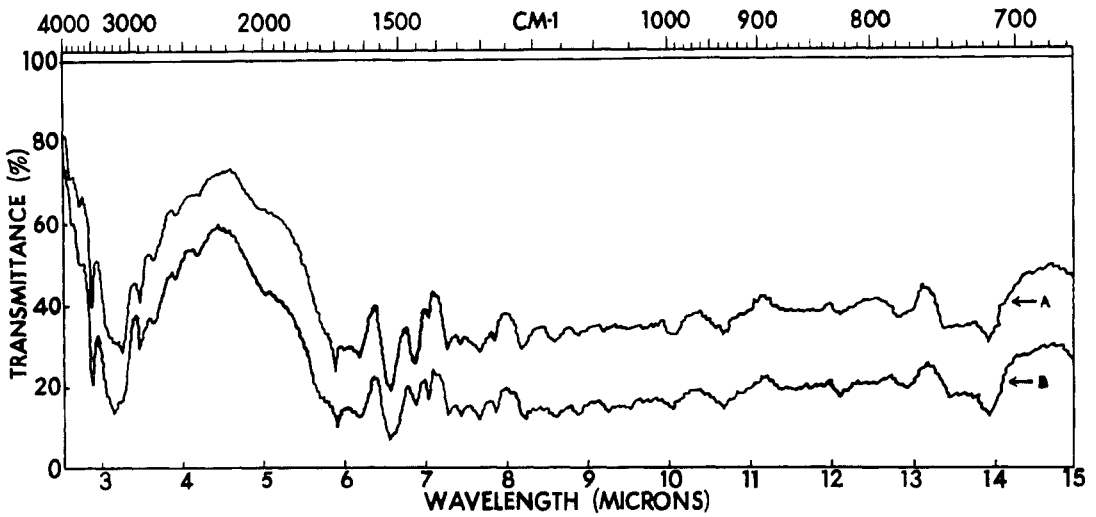

FIG. 3.—Infrared spectra of β-*N*-oxalyl-L-α,β-diaminopropionic acid. A, natural material; B, synthetic material.

II), and (3) their electrophoretic mobilities. The infrared-absorption spectra (Fig. 3) of the two compounds were also found to be similar.

Toxicology.—In preliminary experiments the compound caused growth inhibition of *Staphylococcus aureus* N-15, *Escherichia coli* N-52, and *Candida albicans* Z-242 by 50% at 7 μg/ml and completely at 15 μg/ml in a 24-hour culture. When the mold *Neurospora crassa* (wild Em 5297a) was grown on the minimal medium in test tubes in shake culture experiments as described by Sivarama Sastry *et al.* (1963), the compound caused 50% and complete growth inhibition at levels of 4 μg/ml and 8 μg/ml, respectively.

In view of the toxicity of the compound to the above microorganisms the effect of the compound on the growth of the larvae of the rice moth *Corcyra cephalonica* Staint was next investigated following the experimental procedure of Sivarama Sastry *et al.* (1958). The results presented in Table III show the marked growth-inhibitory effect of the compound on the larvae, while oxalic acid or L-α,β-diaminopropionic acid at comparable levels had no effect.

Since the report by Roy *et al.* (1963) of induction of neurological symptoms in day-old chicks receiving *L. sativus* concentrates, the effect of the isolated compound was studied on chicks, rats, and mice. Day-old white leghorn chicks (35–40 g each, obtained from Central Poultry Farm, Hesserghatta, Bangalore) were divided into groups of four and maintained on stock diet in electrically heated brooders. The chicks in the control group were administered sterile distilled water (0.5 ml). One-half hour after a single intraperitoneal administration of the compound at the level of 20 mg/chick (0.5 ml, *p*H 6.8–7.0) the birds developed typical neurological symptoms such as head retraction, bending and stiffening of the neck, inability to stand and walk, and extensor paralysis of the limbs. The symptoms persisted for 8–12 hours while at higher dosages (30–60 mg/chick) it persisted even after 24 hours and in some cases resulted eventually in death. Neither oxalic acid nor L-α,β-diaminopropionic acid at comparable levels produced any visible symptoms. The compound failed to produce any visible symptoms when administered either to rats or mice. L-Homoarginine, which has been shown to be present in *L. sativus* seeds (Bell, 1962c; Rao *et al.*, 1963), had no visible effect in chicks.

TABLE III
EFFECT OF β-*N*-OXALYL-L-α,β-DIAMINOPROPIONIC ACID, L-α,β-DIAMINOPROPIONIC ACID, AND OXALIC ACID ON RICE-MOTH LARVAE

Supplement to Basal Diet of Wheat Flour (10 g)	Weight of Ten Larvae (mg)				
	At Transfer	1 Week	2 Weeks	3 Weeks	4 Weeks
None (control)	2.8	19.2	69.2	130.0	218.0
OX-Dapro[a] (0.5%)	2.7	10.6	30.8	56.2	90.2
Dapro[b] (0.3%)	2.8	15.4	51.6	109.6	190.0
Oxalic acid (0.26%)	2.8	18.2	56.8	113.2	195.0

[a] β-*N*-Oxalyl-L-α,β-diaminopropionic acid. [b] L,α,β-Diaminopropionic acid.

DISCUSSION

The characterization of oxalic acid and L-α,β-diaminopropionic acid (partially racemized) as the products of hydrolysis of the original compound, together with its properties and dinitrophenylation studies, shows the compound to be β-*N*-oxalyl-L-α,β-diaminopropionic acid (or β-*N*-oxalyl-L-β-aminoalanine). This structure is confirmed by the synthesis of the compound from L-α,β-diaminopropionic acid. Both the naturally occurring compound as well as the synthetic compound had the same specific rotation and chromatographic and electrophoretic mobilities, and possessed a similar infrared-absorption spectrum.

The occurrence of this compound in relatively high concentrations (0.5%) in the seeds of *L. sativus* is interesting, first because of its pronounced neurotoxic action in chicks, which may have a relation to the syndrome caused in human beings on consumption of these seeds; and second because it is a β-acyl derivative of a rather uncommon amino acid. L-α,β-Diaminopropionic acid has hitherto been shown to be present in the hydrolysates of the antibiotic viomycin sulfate (Haskell *et al.*, 1952) and in the free state, and as its β-ureido derivative (albizzine) in the seeds of some plants of the family *Mimosaceae* by Gmelin *et al.* (1958, 1959). It would be of interest in this connection to examine whether other forms of L-α,β-diaminopropionic acid exist in plants.

The same constant yield of the compound (0.5%) was always realized from the different batches of seeds used. Further, when the cold 30% ethanolic extracts of the hexane-extracted seed meal from different batches was concentrated and chromatographed in the differ-

ent solvent systems and sprayed with ninhydrin, they showed no trace of any greenish band characteristic of β-cyanoalanine. This extraction and identification procedure has been employed by Ressler (1962) in the identification of β-cyano-L-alanine in the seeds of *Vicia sativa*. It is thus held that the toxic compound isolated is not derived from any contaminant of *L. sativus* seeds, and as such it is unlikely that a contaminant like *Vicia sativa* (Anderson *et al.*, 1924; Ressler, 1962) is present in the sample of seeds used in this investigation.

Though L-α,β-diaminopropionic acid has been shown to inhibit the growth of several microorganisms (Kjerulf and Schmidt, 1945) and also to inhibit the transamination of aspartate (Hernandez and Kun, 1957), it had no visible effect in the organisms tested by us. As L-α,β-diaminopropionic acid and oxalic acid had no visible neural action in chicks, the neurotoxic effect of β-*N*-oxalyl-L-α,β-diaminopropionic acid in chicks and growth inhibitory action in the other organisms, may depend on its structural integrity. It is possible that the toxicity is associated with the interesting structure of the compound, which can be considered as a N-substituted oxamic acid (HOOC—CO—NH—). Investigations are at present in progress to elucidate this aspect of its structure and activity using β-*N*-oxalyl-L-α,β-diaminopropionic acid as a model compound.

The behavior of this compound in inducing neural symptoms in chicks but not in rats can be attributed to its species-specific action. In this connection it may be mentioned that prolonged feeding of *L. sativus* seeds to rats fails to induce any lathyritic symptoms (McCarrison and Krishnan, 1934).

Acknowledgments

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