Reports

Isolation and Identification of a Neuroactive Factor from Lathyrus latifolius

Abstract. A neuroactive principle of *Lathyrus latifolius* has been isolated in crystalline form as a monohydrochloride and has been identified as L-α,γ-diaminobutyric acid. This amino acid has also been found in very high concentration in the toxic seed of *Lathyrus sylvestris Wagneri*. A structural relationship between this factor, the lathyrus factor from *Lathyrus odoratus*, and β-cyano-L-alanine, a new, synthetic neuroactive amino acid nitrile, is pointed out, and a possible biosynthetic pathway relating these is indicated.

Recently the synthesis of β-cyano-L-alanine, the amino acid nitrile derived from L-asparagine through dehydration of its β-carboxamide, was reported, and some of its physical and chemical properties were described (*1*). It may be noted that β-cyano-L-alanine represents structurally the parent amino acid which by biological decarboxylation could form β-aminopropionitrile, the active moiety (*2*) of the lathrogen isolated from *Lathyrus odoratus* (flowering sweet pea) and characterized as β-N-(γ-L-glutamyl) aminopropionitrile (*3*). We therefore became interested in examining the biological properties of the amino acid nitrile. It was found that β-cyano-L-alanine at a 1-percent level in a standard laboratory diet produces in the young male white rat within 3 to 5 days severe nervous symptoms—that is, hyperirritability, tremors, and convulsions, followed by death (*4*). Lewis and co-workers, in their studies on experimental lathyrism, have described these same symptoms in the rat after ingestion of the lathyrus species *L. latifolius* (perennial sweet pea) and *L. sylvestris Wagneri* (flat pea) (*5*). From their work, the presence of a second lathyrus principle, a powerful neurotoxin, was suggested (*6*), and efforts to isolate the principle from *L. sylvestris Wagneri* were described (*7*). The neurological effects of these plants and of β-cyano-L-alanine are of particular interest in connection with the occurrence of spontaneous clinical neurolathyrism, a condition which has been known for centuries and which has been associated in many instances with the consumption of excessive amounts of lathyrus meal in times of food scarcity (*8*).

A number of synthetic aminonitriles structurally related to β-aminopropionitrile have been administered before to experimental animals, and several of these were found to possess the osteolathrogenic activity of β-aminopropionitrile (*2*). β-Cyano-L-alanine, however, is so far unique in reproducing the neurological symptoms associated with some of the plants. We have therefore examined *L. latifolius* and *L. sylvestris Wagneri* for the presence of β-cyano-L-alanine as the possible natural neurolathrogen in those seeds (*9*). In this report (*10*) we describe the isolation of the chief neurotoxic principle of *L. latifolius*, and its identification as L-α,γ-diaminobutyric acid. The same substance is also present in *L. sylvestris Wagneri*.

In the course of the purification and isolation of the *latifolius* factor, the distribution of activity was followed by a bioassay which consisted of the administration of aqueous extracts by stomach tube to weanling male Sherman rats (*11*). Within 48 hr weakness in the hind legs was apparent, and tremors in the upper extremities suddenly started, followed after several hours by convulsions and death. Subcutaneous administration was less satisfactory since neurological symptoms were not as obvious although toxicity developed. Hexane-extracted ground *L. latifolius* seed (*12*), 370 g, was extracted three times with a total of 5.4 liters of 30 percent ethanol (*13*). The extract yielded 57 g of an active brown semisolid; toxic dose 2.3 g. This was treated in 10 percent aqueous solution with 5 g of activated charcoal, and ethanol was added to 75 percent. The resulting semisolid, 29 g, toxic dose 1.0 g, was dissolved in water and was subjected in 2.7-g aliquots to preparative electrophoresis in pyridine acetate, *p*H 5.58, on Solka-floc (Brown Company, New Hampshire) for 36 hr at +5°C at 8 volt/cm. Assay of the block in segments showed activity only in one basic region 10 to 17 cm from the origin. The combined active eluate of three electrophoretic runs after evaporation yielded 0.96 g; toxic dose 120 mg. This material, 0.8 g, was again subjected to electrophoresis, over a longer distance. Active material was eluted from fraction 1 at 23.8 to 27 cm, 179 mg, and from fraction 2 at 19.5 to 23.7 cm, 157 mg. Each fraction was lyophilized from 30 ml of 0.1*N* hydrochloric acid. The residues were dissolved in water and the solutions were adjusted to *p*H 5. On addition of ethanol, fraction 1 crystallized as prisms; wt. 125 mg; mp 229.5–230° dec.; $(\alpha)^{22}D + 23.3°$ (*c*, 2.1; 5*N* HCl); anal. C, 31.2; H, 7.18; N, 17.7; Cl, 22.8; picrate mp, 181–181.5°; toxic dose 68 mg. Fraction 2 yielded 104 mg, mp 228.5° dec.; toxic dose 68 mg. The active crystalline material represents a recovery of approximately 59 percent of the activity and 1.7 percent of the weight in the initial aqueous ethanol extracts, and approximately 0.25 percent of the weight of the seed.

In the electrophoretic isolation, activity was closely associated with basic, ninhydrin-positive material. The latter was identified as α,γ-diaminobutyric acid by comparison with an authentic sample in the following systems: electrophoresis in pyridine acetate, *p*H 5.7; electrophoresis in barbital buffer, *p*H 8.6; chromatography on Dowex-50 with the Beckman-Spinco automatic amino acid analyzer (*14*) with the 50-cm column and 0.38 *N* sodium citrate buffer, *p*H 4.26, at 30°C, and

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Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to contributors" [*Science* **125**, 16 (1957)].

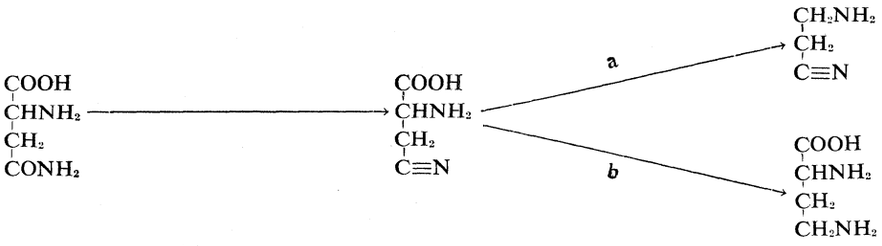

Fig. 1. Hypothetical biosynthetic pathway in *Lathyrus*. (*a*) *L. odoratus;* (*b*) *L. latifolius* and *L. sylvestris Wagneri*.

also at 50°C. The latter system at 50°C, like electrophoresis at *p*H 8.6, allows differentiation of α,γ-diaminobutyric acid (191 ml) from ornithine (182 ml). That the crystalline *latifolius* factor was indeed L-α,γ-diaminobutyric acid monochloride was confirmed by comparison of the isolated active material with an authentic sample (*15, 16*) with the following properties: anal. (calc.) C, 31.1; H, 7.17; N, 18.1; Cl, 22.9; mp 230–230.5° dec., reported 225° (*17*), picrate mp 180.5–181.5°, reported 180° (*18*); $(\alpha)^{23}D$ +23.9°, (*c* 2.1, 5*N* HCl), reported $(\alpha)^{25}D$ + 24.2° (*c* 2, 5*N* HCl) (*16*). There were no differences in infrared spectra (KBr disk). Finally, synthetic L-α,γ-diaminobutyric acid monohydrochloride led at a level of 68 mg within 2 days to weakness in the hind legs, tremors, convulsive behavior, and death.

Quantitative determination (*14*) of α,γ-diaminobutyric acid in 30 percent ethanol extracts of several samples indicated concentrations in *L. latifolius* seed of 0.51 to 0.67 percent, expressed as the monohydrochloride. Similar analysis of *L. sylvetris Wagneri* (*19*) showed a 1.4-percent level. High concentrations of arginine (0.3 to 0.4 percent) were also present in both seeds. The concentration of L-α,γ-diaminobutyric acid in *L. latifolius*, together with the knowledge of the toxic level of this amino acid in the rat, allows one to account fully for the toxicity of the seed (*13*). Since, in addition, activity was found only in fractions containing the diaminoacid, it is evident that L-α,γ-diaminobutyric acid is the chief neurotoxic principle in *L. latifolius*. The extremely high level of free α,γ-diaminobutyric acid found in the seed of *L. sylvestris Wagneri* now explains why ingestion of as little as several grams of this seed has proved so highly toxic to the rat (*5–7*), and this amino acid probably also accounts for the toxicity to some classes of livestock encountered during trials of the immature flat pea plant as a forage (*20*). It may be noted that the relative toxicities of the two seeds are roughly in accord with their concentrations of α,γ-diaminobutyric acid, *L. sylvestris Wagneri* being reportedly three to four times more toxic to the rat than *L. latifolius* (*7*).

It is of interest that although synthetic L-α,γ-diaminobutyric acid has long been available, its neurotoxic action, although briefly mentioned in the literature (*21*), has remained virtually unnoticed in neurochemistry. Although present in peptide linkage in the naturally occurring polymyxin, circulin and colistin families of antibiotics, α,γ-diaminobutyric acid has been encountered before this study in plants in only trace amounts (*22*), and this appears to be the first report in which free L-α,γ-diaminobutyric acid has been recognized as a naturally occurring neurotoxin. Noteworthy in view of the pharmacological activity of α,γ-diaminobutyric acid is its recent identification in mammalian liver (*23*) and in the road snail (*24*). It will be of interest to ascertain its presence in common foodstuffs as well as in those lathyrus species implicated in clinical lathyrism (*25*). Whether it is a natural factor of significance to man, perhaps, in connection with the action of the structurally related γ-aminobutyric acid in cerebral metabolism, remains to be seen.

It is interesting that in attempting to determine the presence of β-cyano-L-alanine as the active factor in *L. latifolius* we have instead established the active factor to be its reduction product, L-α,γ-diaminobutyric acid. Although our evidence for a biological relationship between these two substances is only circumstantial, chemical analogy (*26*) could suggest the metabolic relationship illustrated in Fig. 1. β-Cyano-L-alanine, derived possibly from asparagine by dehydration in analogy with its laboratory preparation, could serve as the biological intermediate which in *L. odoratus* is decarboxylated to β-aminopropionitrile, whereas in *L. latifolius* and in *L. sylvestris Wagneri* it is reduced to α,γ-diaminobutyric acid. These reactions involving the formation and reduction of a nitrile would presumably be catalyzed by enzymes of types—that is, "amide dehydrase" and "nitrile reductase"—which are as yet unknown.

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9. We should like to thank Quentin Jones, U.S. Department of Agriculture, and Richard M. Klein, New York Botanical Gardens, for assistance in locating the seeds and Gilbert N. Schnirman for capable assistance with the preparative experiments.
10. This work was supported by a grant from the Muscular Dystrophy Associations of America, Inc. One of us (P.A.R.) was the recipient of a MDAA student summer scholarship (1960).
11. We are indebted to Maurice B. Feinstein for suggesting this procedure.
12. The seed was purchased from Herbst Brothers Seedsmen, Inc., New York, N.Y.
13. It had been shown previously in experiments with mice that the toxic principle is removed with 30 percent ethanol (*5*). Estimation here of the toxicity of the seed has been based on that of the ethanol extracts. This toxicity, 15 g, was somewhat greater and much less variable than that which resulted when the powdered seed was administered in a 50 percent diet mixed with laboratory chow, average 22.5 g, perhaps due to the more gradual assimilation through the latter route. Toxic dose in the stomach tube assay is weight per 100 g of rat, which consistently caused death and was usually not lethal at a 20 percent lower level.
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Puyallup, Wash., to whom we express appreciation.
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25. There appears to be some uncertainty concerning the identity of the species involved (*8*).
26. Chemical analogy exists, for example in a reaction encountered in the synthesis of an asparagine-containing pentapeptide, whereby the asparagine moiety was converted in part by a peptide-coupling agent, that is, dehydrating agent, followed by reduction, to a residue of α,γ-diaminobutyric ac → [C. Ressler, *J. Am. Chem. Soc.* **78**, 5956 (1956)]. A β-cyanoalanine derivative was shown to be the likely intermediate in this conversion (*1*).

11 March 1961

Sulfate-Reducing Bacteria and Pyritic Sediments in Antarctica

Abstract. Black lacustrine and marine sediments occur in the McMurdo Sound region of Antarctica. The black color is due to the presence of iron sulfide, precipitated by sulfate-reducing bacteria *(Desulfovibrio)* in the presence of decaying organic matter of algal origin. Viability of sulfate-reducing bacteria in the sediments was demonstrated in the laboratory by culturing in anaerobic liquid media. It is probable that sulfate-reducing bacteria are widely distributed in Antarctica.

The significance of sulfate-reducing bacteria *(Desulfovibrio)* as biological and geologic agents has been widely recognized since the classic work of Beijerinck (*1*) and van Delden (*2*). Numerous investigators have confirmed the significance of these organisms in the geochemical cycle of sulfur, although their quantitative role is not clearly established in either recent or ancient sediments. The ubiquitous presence of sulfate reducers in a wide range of sedimentary environments has been demonstrated in studies of bottom muds from fresh-water, marine, and saline basins. Their temperature tolerance under natural conditions, however, has not been delimited. The presence of sulfate-reducing bacteria as active agents in sedimentary processes under the extreme conditions of the antarctic environment is therefore of interest both with respect to their ecological tolerance and geographic distribution.

Kettle holes are common on the south side of the Wright Dry Valley in the McMurdo Sound region of Antarctica. These holes are located in an area extending westward from the western terminus of the Lower Wright Glacier for about 2 mi (lat. 77°30′S, long. 162°30′E). Small ponds occur in some of the kettle holes, although most of them are dry.

A small saline pond, approximately 25 ft long, 10 ft wide, and 1 ft deep, occurs in one of the kettle holes. The water is impotable and highly saline, as shown in the analysis presented in Table 1. From the values in the table, it can be seen that the salinity of the pond water is approximately four times that of sea water. The principal dissolved salt is sodium chloride. Magnesium chloride, calcium sulfate, magnesium sulfate, and calcium carbonate are also present.

The upper surface of the sediments at the bottom of the pond is light ocherous brown. Immediately below the surface and extending down, however, the sediment is black. Upon drying under oxidizing conditions, the black sediments become gray. Upon addition of dilute HCl to the black sediment, hydrogen sulfide gas is emitted. The black color is due to the presence of iron sulfide, probably of the type described as hydrotroilite ($FeS\text{-}nH_2O$), an amorphous, hydrous monosulfide of iron (*3*). The iron sulfide is precipitated by sulfate reduction induced by *Desulfovibrio* in the bottom muds. An energy source for the sulfate-reducing bacteria is readily available from decaying filamentous algae, diatoms, and other microplankton that occur in the pond waters. The existence of a rather complex biocenose, involving the sulfur cycle, under the ecological conditions currently prevailing in the pond is remarkable. In addition to the markedly high salinity of the water, the temperature regime, under which the sediments and their organic fraction are accumulating, is featured by changes from perhaps −60°F in winter to +40°F in summer (*4*).

The presence of living cells of *Desulfovibrio* in the pond sediments under consideration has been demonstrated in the laboratory by culturing in anaerobic sterile liquid media containing lactate as a carbon source. Replicate media were prepared by the use of both tap water and slightly saline water (NaCl). Better growth occurred in the saline medium. Cultures held at room temperature showed a more rapid rate of sulfate reduction than those held at 5°C. The precipitation of iron sulfide, as visually indicated, was used to determine viability of the cultures, and the rate of precipitation was used to indicate the relative rates of sulfate reduction. The laboratory cultures were prepared from samples collected by one of us (R.L.N.) on 9 January 1961. The samples were held under moist, relatively anoxic conditions until 1 February 1961, the time of inoculation of the media, a period of approximately 3 wk.

Table 1. Analysis of a sample of water from a small saline pond in the McMurdo Sound region of Antarctica (*p*H 7.8). (The analysis was made by the Water Analysis Laboratory of Metcalf and Eddy, Boston, Mass.)

Substance	Amount (mg/lit.)
Calcium as Ca	1,130.
Magnesium as Mg	4,890.
Sodium as Na	33,200.
Sulfates as SO_4	16,150.
Chlorides as Cl	58,000.
Bicarbonates as CO_3	330.
Sulfides as S	<0.1
Dissolved solids	132,620.

Pyritic sediments, similar to those described here, occur in other kettle holes in the Wright Dry Valley, on the marine beach on the south side of New Harbor, McMurdo Sound (lat. 77°35′S, long. 163°29′E), in the deposits of Green Lake, Cape Royds, Ross Island (lat. 77°32′S, long. 166°15′E) (*5*), and in a small pond in the elevated marine beaches at Marble Point, McMurdo Sound (lat. 77°26′S, long. 163°46′E). It is probable that further field and laboratory study will demonstrate that sulfate-reducing bacteria and pyritic sediments are widely distributed in Antarctica. It would be of interest to determine the optimum temperature for growth in strains of *Desulfovibrio* occurring in nature under these extreme environmental conditions (*6*).

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6. The field studies by one of us (R.L.N.) were made possible by the assistance of the National Science Foundation.

27 February 1961