Phytochemistry, 1964, Vol. 3, pp. 73 to 78. Pergamon Press Ltd. Printed in England

NEUROTOXIC COMPOUNDS OF THE SEEDS OF *LATHYRUS SATIVUS*

V. V. S. MURTI, T. R. SESHADRI
Department of Chemistry, Delhi University, Delhi-6
and
T. A. VENKITASUBRAMANIAN
Department of Biochemistry, V. P. Chest Institute, Delhi University, Delhi-6

(*Received* 9 *September* 1963)

Abstract—The seeds of *Lathyrus sativus* responsible for human lathyrism in India have been examined for toxic components. Four ninhydrin-positive substances were detected in the 30% aqueous ethanol extract by paper chromatography, of which two are present in appreciable amounts. The most prominent one has been isolated in crystalline form and studied for chemical and physiological (neurotoxic) properties and identified as (−)-β-oxalylaminoalanine. The second seems to be homoarginine. The other minor components have still to be identified.

INTRODUCTION

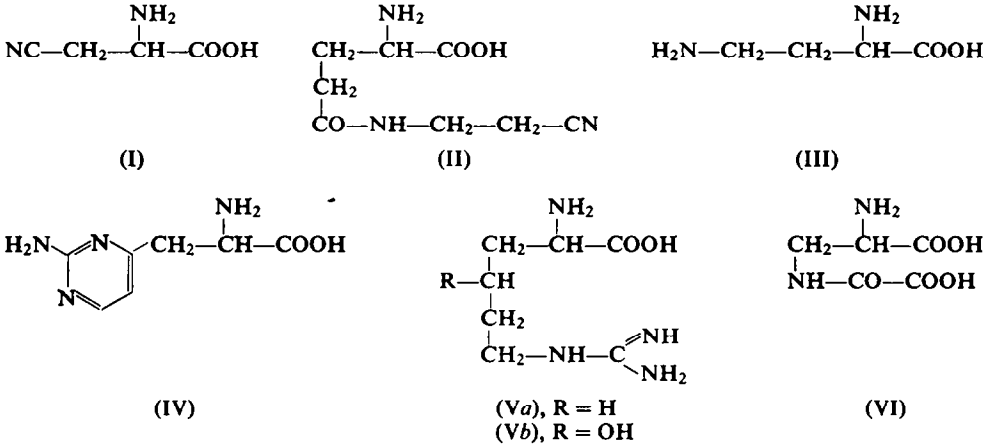
IT HAS been known for a long time that consumption of the seeds of certain *Lathyrus* species causes a disease known as "lathyrism" in animals and man.[1–6] This disease is widely prevalent in endemic form in many parts of Central India where the seeds of *Lathyrus sativus* are eaten by the poorer sections of the people; it has also been reported from other countries, viz. Spain, France, other Mediterranean countries and the U.S.A. where other species of *Lathyrus*, e.g. *L. odoratus*, *L. pusillus*, *L. latifolius*, *L. sylvestris* Wagneri are grown. Two distinct types of lathyrism are recognized: "neurolathyrism" characterized by nervous disorders such as hyperirritability, weakness and paralysis of leg muscles and convulsions, and "osteolathyrism" (odoratism) in which the bone structures, particularly of the spine, the ribs and the legs, undergo pathological changes and deformation. Different species of *Lathyrus* produce one or the other type of lathyrism though it is possible that some may cause both types of disease.

Because of the nutritional and economic importance of the problem considerable interest has been shown in identifying the toxic substances responsible for lathyrism.[1–6] Three main species of *Lathyrus* have been reported in India, viz. *L. sativus*, *L. aphaca*, and *L. pratensis*[7] and diet surveys indicated that *L. sativus* is the main species responsible for human lathyrism in India. However, later investigations resulted in considerable uncertainty and confusion; on the basis of animal experiments some workers reported that the seeds of *L. sativus* are

[1] F. M. STRONG, *Nutrition Revs* **14**, 65 (1956).
[2] H. SELYE, *Rev. can. biol.* **16**, 1 (1957).
[3] V. SUBRAHMANYAN, M. NARAYANA RAO and M. SWAMINATHAN, *Food Sci.* **6**, 156 (1957).
[4] A. F. GARDNER, *Am. J. Clin. Nutrition* **7**, 213 (1959).
[5] C. RESSLER, *J. Biol. Chem.* **237**, 733 (1962).
[6] P. R. ADIGA, G. PADMANABAN, S. L. N. RAO and P. S. SARMA, *J. Sci. Ind. Research* (India) **21C**, 284 (1962).
[7] R. N. CHOPRA, S. L. NAYAR and I. C. CHOPRA, *Glossary of Indian Medicinal Plants*, Council of Scientific and Industrial Research, New Delhi (1956).

toxic while others concluded that they are not. The toxic effects were variously attributed to the presence of selenium, manganese and phytic acid. There were also suggestions that lathyrism is a nutritional deficiency disease due to inadequate amounts of methionine, tryptophane and the B vitamins. Some workers were of the opinion that the toxicity is due to contamination with the seeds of leguminous weeds belonging to the *Vicia* species and this appeared to be supported by the botanical identification of the seeds of *V. sativa* in admixture with those of *L. sativus* collected from areas where lathyrism is widespread. Recently Ressler[5] found that the seeds of *V. sativa* and *V. angustifolia* contain β-cyano-L-alanine (I) which has neurotoxic properties.

The first important advance in identifying the lathyrogenic substances was due to Strong *et al.*[8,9] who isolated β(N-γ-glutamyl)-aminopropionitrile (II) from the seeds of *L. odoratus* and showed that it produces skeletal abnormalities in rats typical of osteolathyrism. Ressler *et al.*[10] identified αγ-diaminobutyric acid (III) as the neurolathyrogenic factor of *L. latifolius* and *L. sylvestris* Wagneri. Bell examined a number of *Lathyrus* species and found that β(N-γ-glutamyl)-aminopropionitrile and αγ-diaminobutyric acid are present in many of them;[12] in addition, three new amino acids, lathyrine[14] (tingitanine[15]) (IV), homoarginine[11,13] (V*a*) and γ-hydroxyhomoarginine[16] (V*b*) occur in some species.



(I) (II) (III)

(IV) (V*a*), R = H (V*b*), R = OH (VI)

Extensive studies of the clinical epidemiology of lathyrism[17] in India have indicated that it is mainly of the neurological type. The earliest attempts to isolate the lathyrogenic compound from *L. sativus* were made by Acton and Chopra[18] who claimed to have obtained a crystalline, water-soluble substance having neurotoxic activity. No further work on this aspect seems to have been done till recently. When this problem was referred to us about

[8] G. F. MCKAY, J. J. LALICH, E. D. SCHILLING and F. M. STRONG, *Arch. Biochem. Biophys.* **52**, 313 (1954).
[9] E. D. SCHILLING and F. M. STRONG, *J. Am. Chem. Soc.* **77**, 2843 (1955).
[10] C. RESSLER, P. A. REDSTONE and R. H. ERENBERG, *Science* **134**, 188 (1961).
[11] E. A. BELL, *Nature, Lond.* **193**, 1078 (1962).
[12] E. A. BELL, *Biochem. J.* **83**, 225 (1962).
[13] E. A. BELL, *Biochem. J.* **85**, 91 (1962).
[14] E. A. BELL and R. G. FOSTER, *Nature, Lond.* **194**, 91 (1962).
[15] E. NOWACKI and J. PRZYBYLSKA, *Chem. Abs.* **56**, 1725 (1962).
[16] E. A. BELL, *Nature, Lond.* **199**, 70 (1963).
[17] K. T. GANAPATHY and M. P. DWIVEDI, *Studies on Clinical Epidemiology of Lathyrism*, Indian Council of Medical Research (1961).
[18] H. W. ACTON and R. N. CHOPRA, *Indian Med. Gaz.* **57**, 412 (1922).

two years ago we undertook a systematic examination of the seeds of *L. sativus* and at first looked for water-soluble leucoanthocyanidins and other polyphenols which were not found in appreciable amounts; later we succeeded in isolating the toxic factor in crystalline form and determining its structure. When our work was nearly complete the preliminary observations of Sarma *et al.* appeared[6] and, in view of their recent paper[19] announcing the structure of the compound from *L. sativus*, we give here an outline of our findings and other additional information we have obtained.

RESULTS AND DISCUSSION

The powdered seeds of *Lathyrus sativus* were extracted with ether and then with 30% ethanol. The amino acids in the latter extract were adsorbed onto ion-exchange resin (IR-120 H form), and the acidic components eluted with water. Concentration of this fraction yielded a crystalline ninhydrin-positive solid which had different R_f values from substances previously isolated. Its infra-red spectrum showed bands for carboxyl, amide, and $-NH_3^+$ groups, but not for nitrile or aromatic groups. Hydrolysis with hydrochloric acid gave two products. The first proved to be (+)-diaminopropionic acid, the identity of which with an authentic specimen was proved by paper chromatography, preparation of derivatives[20] and infra-red spectrum.[21] It has been partially racemized during isolation.[22] The second product was shown to be oxalic acid by the preparation of derivatives,[23] and by specific colour tests.[24, 25]

From these data it was inferred that the *L. sativus* substance is an N-oxalyl derivative of (+)-$\alpha\beta$-diaminopropionic acid. One of the amino groups is free because the compound gives a positive ninhydrin reaction; since this reaction is not given after treatment with cupric nitrate reagent[26] it could be concluded that the free amino group is in the α-position to the carboxyl group. This was confirmed by the apparent pK values (2·4 and 9·0) typical of the α-amino acids;[27,28] in addition there is another pK value (3·25) corresponding to another free carboxyl group. These data are consistent with the structure of (−)-(β-N-oxalyl)-$\alpha\beta$-diaminopropionic acid (VI) for the compound.

When injected intraperitoneally into one-day-old chicks[29] (20 mg per chick) the substance produced nervous disorders, viz. the animals lost their activity, then became unable to keep head erect, developed paralysis of legs and convulsions, but they recovered after a few hours. When larger amounts (48 mg per chick) were used they died after a violent fit of convulsions. It was reported by Sarma *et al.*[19] that $\alpha\beta$-diaminopropionic acid did not produce neural symptoms in chicks. We have now found that injection of potassium oxalate at comparable levels did not bring about any nervous disorders; it thus appears that oxalic acid should be in combination with the diaminopropionic acid for toxic properties. When mice were

[19] P. R. Adiga, S. L. N. Rao and P. S. Sarma, *Current Sci. (India)* **32**, 153 (1963).
[20] I. Heilbron and H. M. Bunbury, *Dictionary of Organic Compounds*, Vol. 2, p. 42 (1953).
[21] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 3, p. 2497, John Wiley & Sons (1961).
[22] R. Gmelin, G. Strauss and G. Hasenmaier, *Hoppe-Seyler's Z. physiol. Chem.* **314**, 28 (1959).
[23] A. I. Vogel, *Practical Organic Chemistry*, Third edition, p. 366, Longmans (1956).
[24] J. Bergerman and J. S. Eliot, *Anal. Chem.* **27**, 1014 (1955).
[25] B. Haynes, *Qualitative Organic Analysis*, p. 48, Cleaver-Hume Press, London (1961).
[26] P. O. Larsen and A. Kjaer, *Biochem. Biophys. Acta* **38**, 148 (1960).
[27] E. J. Cohn and J. T. Edsall, *Proteins, Amino Acids and Peptides*, p. 75, Reinhold Publishing Corporation (1943).
[28] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. I, p. 486, John Wiley and Sons (1961).
[29] D. N. Roy, V. Nagarajan and C. Gopalan, *Current Sci. (India)* **32**, 116 (1963).

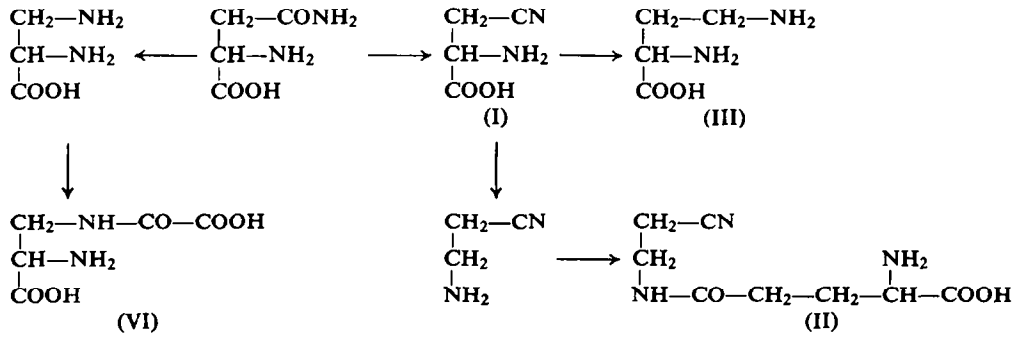
administered the *L. sativus* compound intraperitoneally (48 mg per mouse) they did not immediately develop any severe visible disorders but experienced difficulty of movement with dragging of legs; however, two of the three mice injected died the next day. Obviously there are considerable differences in susceptibility to the lathyrogenic substances. Furthermore, age and the size of the amino acid reserve may play an important part; one-day-old chicks are probably most susceptible because they have very little reserves of amino acids.

Rao *et al.*[30] reported the isolation of (+)-homoarginine from the seeds of *L. sativus*. We have also found evidence for its presence by paper chromatography. Preliminary experiments on the feeding of crude *L. sativus* seed concentrates to rats indicated that small but definite skeletal changes are produced, as could be observed from X-ray photographs. It appears that these seeds may also contain minor amounts of osteolathyrogenic compounds or that the oxalyl-diaminopropionic acid could also cause bone changes when fed in small doses for long periods.

CONCLUSIONS

It has been established that β-aminopropionitrile (BAPN) is the main toxic principle of the *L. odoratus* seeds and that the free amino group is essential for osteolathyrogenic activity. Recent work of Strong *et al.*[31] has shown that acyl derivatives of BAPN would be non-toxic unless hydrolysed enzymatically *in vivo*. Ressler[5] found that β-cyano-L-alanine is thrice as toxic as the D-isomer and that the closely related γ-cyano-L-α-aminobutyric acid has no appreciable toxicity. Another significant point is that while BAPN is an osteolathyrogen, its carboxy derivative (β-cyanoalanine) has pronounced neurotoxic action. β(N-oxalyl)-αβ-diaminopropionic acid occurring in *L. sativus* and αγ-diaminobutyric acid present in many *Lathyrus* species are also neurotoxic. These are indications of specificity generally found in compounds of high physiological activity. No generalizations about the mechanism of their action can yet be made. The discovery of the toxic properties of these substances, however, makes it imperative that a systematic search should be made for these and similar compounds in foodstuffs.

Ressler *et al.*[10] suggested that the osteolathyrogen BAPN and the neurolathyrogens β-cyanoalanine and αγ-diaminobutyric acid are synthesized *in vivo* from the same precursor, asparagine. It is attractive to suggest that αβ-diaminopropionic acid may also have its origin from the same source, the $-CONH_2$ group being replaced by a $-NH_2$ by analogy with the well-known Hofmann reaction. The subsequent step of acylation with oxalic acid is an acceptable biochemical process.



[30] S. L. N. RAO, L. K. RAMACHANDRAN and P. R. ADIGA, *Biochemistry* **2**, 298 (1963).
[31] L. A. EHRHART, S. H. LIPTON and F. M. STRONG, *Biochemistry* **2**, 300 (1963).

Now that the chief neurotoxic factor of the seeds of *L. sativus* has been isolated, and characterized, steps can be taken to combat lathyrism in India more effectively. Surveys have shown that mostly the poor and the under-nourished people with low reserves of protein suffer from this disease; severe depletion of the amino acid reserves is probably the main reason that makes one susceptible to it. One of the immediate relief measures, which has been suggested but not satisfactorily implemented, could be to boil the seeds with water and reject the water-extract. Such a method is commonly employed to make bitter foodstuffs edible. This measure, though it might mean the loss of other essential water-soluble factors, has at least the merit of removing the poisonous substances; at the same time the proteins would be retained to a large extent. In this context it might be profitable to develop suitable processing techniques just as they have been done in the case of the soyabeans.

EXPERIMENTAL

Coarsely powdered seeds of *L. sativus* (1 kg) were extracted with boiling ether (3 × 3 l., 4 hr each time) and the solvent was distilled off from the combined extracts. The dark brown concentrate was taken up in alcohol (200 ml) and allowed to stand when a light brown solid separated. On purification from glacial acetic acid it had m.p. 274–276° and its acetate melted at 166–168°. It gave a positive Molisch's test and Liebermann–Burchard reaction and was identified as β-sitosterol-D-glucoside.

The seed powder was then extracted with cold 30% ethanol (4 × 3 l., 16 hr each time), the combined extracts were filtered and concentrated to ca. 2 l. below 50°. Circular paper chromatography using three different solvent systems (*n*-BuOH–MeCOEt–H_2O, 2:2:1; phenol saturated with water; *n*-BuOH–HOAc–H_2O, 4:1:5) indicated the presence of four main ninhydrin positive substances; co-chromatography with a sample of β(N-γ-glutamyl)-aminopropionitrile kindly provided by Professor Strong showed that this substance is not present. The concentrate was passed through a column of Amberlite IR-120 resin column (H^+ form, 23 × 3 cm) and the initial coloured effluent was rejected. Further washing with distilled water (2 l.) gave an acidic eluate showing a purple–violet ninhydrin reaction. It was concentrated to ca. 100 ml, alcohol (100 ml) was added and the solution kept in the refrigerator. The crystalline solid that separated was purified by two recrystallizations from 50% alcohol. Yield, 1 g, m.p. 174–175° (with gas evolution) (Found: C, 32·77; H, 5·00; N, 15·39 $C_5H_8O_5N_2$, $\frac{1}{2}H_2O$ required: C, 32·43; H, 4·86 and N, 15·14%). $[\alpha]_D^{28}$ −28·1° (c, 1·99, 5N HCl).

The compound gives a blue–violet ninhydrin colour and is moderately soluble in water giving an acidic solution. Circular R_f values (28°): 0·23 (*n*-BuOH–HOAc–H_2O, 4:1:5, upper layer); 0·63 (75% ethanol); 0·26 (pyridine–water, 4:1); 0·28 (phenol saturated with water). The infra-red spectrum has the following bands (cm^{-1}): 3100 ($-NH_3^+$), 1690, 1370, 1230 (–COOH), 1640 (shoulder) (–CONH–); there is no nitrile or aromatic absorption.

Hydrolysis of the compound with 6 N hydrochloric acid for 22 hr at 100° gave (+)-αβ-diaminopropionic acid isolated as its monohydrochloride, m.p. 226–227° (decomp.) (Found: C, 25·36; H, 6·60; N, 19·55. Calculated for $C_3H_8O_2N_2$, HCl: C, 25·63; H, 6·41 and N, 19·94%). Its identity was established by paper chromatography, preparation of the diacetate and the dibenzoate[20] and characteristic infra-red spectrum.[21] However, it had a low rotation ($[\alpha]_D^{28}$ +10·7, 5 N HCl) which indicated that it was partially racemized (cf. [22]). The second product of hydrolysis was purified through its insoluble calcium salt; on recrystallization from water saturated with ether it had m.p. 185–186°. It was identified as oxalic acid by

mixed m.p., m.p. of its S-benzyl isothiuronium salt,[23] paper chromatography, ability to decolorize acidified potassium permanganate solution and characteristic colour reactions with indole[26] and diphenylamine.[25]

Acknowledgements—The authors are grateful to Dr. C. Gopalan, Director, Nutrition Research Laboratories, Hyderabad, for the supply of *L. sativus* seeds and Professor N. V. Subba Rao, Head of the Chemistry Department, Osmania University, Hyderabad, for the nitrogen analyses.