Associations of Ninhydrin-Reacting Compounds in the Seeds of 49 Species of *Lathyrus*

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Free amino acids occur in more than trace amounts in the seeds of many species of plant. The application of chromatographic methods to the analysis of seed extracts has increased our knowledge of their distribution and led to the discovery of a number of new ones (Fowden, 1959).

In some species a single amino acid may account for as much as 4% of the dry weight of the seed (Bell, 1958) and this suggests that it may constitute a reserve material for the developing embryo; for example, the concentrations of canavanine in the seeds of *Canavalia ensiformis* (Johnstone, 1956), *Colutea arborescens* (Tschiersch, 1959) and *Medicago sativus* (Bell, 1960) fall during germination.

Surveys made on the distribution of canavanine (Bell, 1958, 1960; Birdsong, Alston & Turner, 1960) have led Birdsong *et al.* (1960) to conclude that this compound, which is restricted to the Papilionoideae, is of taxonomic significance. The amino acid was not found in all the species or indeed in all the genera of Papilionoideae examined, yet its wide distribution within this subfamily and its apparent absence from all other species of plant appear significant. Commenting on its random distribution within the Papilionoideae Birdsong *et al.* (1960) state: 'It is not surprising that certain large and diverse genera, such as *Vicia*, *Astragalus*, and *Glycine*, contain species with canavanine as well as some without it. Complex synthesis requiring several precursors will not be completed if any critical enzyme is missing from the sequence.'

If the absence of canavanine from certain species of Papilionoideae is due to the absence of some critical enzyme or enzymes, and if canavanine is important as a source of nitrogen to the germinating seed, then, in those species of Papilionoideae that do not contain canavanine, the relevant precursor or some alternative nitrogen compound may be stored in its place.

A preliminary survey of a representative group of canavanine-free species of the Papilionoideae revealed the presence of the new amino acid lathyrine in acidified 50% (v/v) ethanol extracts of the seeds of certain species of *Lathyrus*. The amino acid ($C_7H_{10}N_4O_2$) which was isolated from *Lathyrus*

tingitanus is heterocyclic and gives a vivid red colour with ninhydrin (Bell, 1961). The ease of identification makes lathyrine an excellent subject for distribution studies.

In the present paper detailed chromatographic and ionophoretic analyses of the ninhydrin-reacting compounds present in the seeds of 49 species of *Lathyrus* are reported. The extremely good resolution of acidic and basic constituents achieved by high-voltage ionophoresis made this a most valuable analytical method.

EXPERIMENTAL

Preparation of seed extracts. Finely ground seed (100 mg.) of each species was stirred with ethanol (1 ml.) and 0.1 N-hydrochloric acid (1 ml.). After 17 hr. at room temperature the suspension was centrifuged and the supernatant used for subsequent analyses.

Chromatography. One-dimensional chromatograms were prepared by the descending technique on Whatman no. 1 paper, with 0.05 ml. of extract. Chromatograms were developed for 40 hr. with the butanol-acetic acid and lutidine, and for 17 hr. with the other solvents. The solvents used (see Table 2) were among those recommended by Smith (1960) for the separation of amino acids.

Two-dimensional chromatograms were prepared on Whatman no. 1 paper from each extract (0.25 ml.); the chromatograms were developed for 17 hr. with phenol-ammonia and for 17 hr. with lutidine.

Ionophoresis. Ionophoresis was conducted on Whatman no. 1 paper in buffer solutions of pH 1.9 [formic acid (98–100%)-acetic acid-water (33:148:1819, by vol.)], pH 3.6 [acetic acid-pyridine-water (5:0.5:95, by vol.)], pH 6.5 [acetic acid-pyridine-water (0.2:5:95, by vol.)] and pH 11.6 (sodium carbonate, 0.01 M). With the three buffers of lower pH, a horizontal-ionophoresis method essentially that of Gross (1961) was used, a potential difference of 75 v/cm. being applied for 30 min. At pH 11.6 a hanging-strip method was used, 5 v/cm. being applied for 17 hr.

Development of ninhydrin colours. Chromatography and ionophoresis papers were dipped in a 0.2% (w/v) solution of ninhydrin in aq. 95% (v/v) acetone and the colours developed at 80°.

RESULTS

The distribution of the major concentrations of lathyrine and other ninhydrin-reacting compounds is given in Table 1. The R_F values, ionic mobilities and reactions of most of the ninhydrin-positive

Table 2. *Approximate R_F values of ninhydrin-positive compounds, together with those of markers run under the same conditions*

The following solvents were used: 1, Butan-1-ol-acetic acid-water (60:15:25, by vol.); 2, 200 ml. of phenol-water (4:1, w/v) with the addition of 1 ml. of ammonia (sp.gr. 0.88) before use; 3, 200 ml. of phenol-ethanol-water (120:40:40, w/v/v) with the addition of 1 ml. of ammonia (sp.gr. 0.88) before use; 4, Ethanol-ammonia (sp.gr. 0.88)-water (18:1:1, by vol.); 5, Methanol-water-pyridine (40:10:2, by vol.); 6, Lutidine-water (132:60, v/v); 7, Butan-1-ol-pyridine-water (1:1:1, by vol.). Where the values (*) from Smith (1960) are quoted for the R_F of alanine, the other R_F values in that solvent system have been calculated from the R_{Alanine} values. S, Streak.

Solvent ...	1	2	3	4	5	6	7
Ninhydrin-positive compound							
Arginine	16	91	66	8	17	7	15
Lathyrine	17	83	60	7	35	15	29
Homoarginine	20	94	76	12	19	10	17
Glutamic acid	25	19	11	4	.	7	22
Aspartic acid	18	13	6	2	.	6	19
Alanine	30*	57	43	33*	57	23*	37*
B ₁	18	94	77	12	20	11	17
B ₂	11	68	45S	19	25S	12	24S
B ₃	15	87	72	8	16	8	15
B ₄	28	84	61	.	.	19	36
B ₅	8	70	53	7	.	8S	23
N ₁	36	36	16	5	57	.	25
N ₂	32	86	68	24	57	24	38
N ₃	30	83	64	24	53	34	43
A ₁	7	9	4	1	42	6	15
A ₂	8	13S	5S	1	42	6	15
A ₃	8	18S	5S	1	42	6	15

Table 3. *Ionic mobilities and colour reactions of ninhydrin-positive compounds*

	Group Ref.	Ionic mobilities	Ninhydrin colour reaction
Lathyrine	3	Positively charged at pH 1.9, moving fractionally slower than arginine. Moves between arginine and neutral amino acids at pH 3.6, is uncharged at pH 6.5 and negatively charged at pH 11.5	Red
B ₁	1, 3, 4 and 5	Positively charged at pH 1.9, 3.6 and 6.5, moving fractionally slower than arginine. Uncharged at pH 11.5. Fractionally faster than lathyrine at pH 1.9	Purple*
B ₂	2	Positively charged at pH 1.9, 3.6 and 6.5, moving faster than arginine. Negatively charged at pH 11.5	Brown-purple
B ₃	3	Positively charged at pH 1.9, moving fractionally slower than lathyrine. Moves with homoarginine at pH 3.6 uncharged at pH 11.5	Grey-purple*
B ₄	3	Moves between the neutral amino acids and lathyrine at pH 1.9 and 3.6. Uncharged at pH 6.5	Purple
B ₅	5	Strongly basic; moves faster than B ₂ at pH 1.9, 3.6, 6.5	Grey-purple
N ₁	3	An ampholyte uncharged at pH 6.5	Purple
N ₂	3, 4	An ampholyte uncharged at pH 6.5	Purple
N ₃	5	An ampholyte uncharged at pH 6.5	Purple
A ₁	1, 2	Strong acid, negatively charged at pH 1.9	Purple
A ₂	2	Strong acid, uncharged at pH 1.9, negatively charged at pH 3.6, moving fractionally slower at pH 3.6 than A ₁	Purple
A ₃	2	Strong acid, carrying small positive charge at pH 1.9. Negatively charged at pH 3.6, moving slightly slower than A ₂	Purple

* Also gives a positive Sakaguchi reaction.

other neutral amino acids by ionophoresis. The R_F values of this compound correspond to those given for β -(γ -glutamylamino)propionitrile (McKay, Lalic, Schilling & Strong, 1954; Schilling &

Strong, 1954), the toxic factor first isolated from one member of this group (*L. odoratus*).

The extracts of two of the remaining four species included in this group (*L. pisiformis* and *L. venosus*)

Fig. 1. Relative mobilities of ninhydrin-reacting compounds in extracts of species in groups 1-4, when subjected to ionophoresis at pH 3.6.

show weak patterns that are unlike those of the other species studied, and two (*L. angulatus* and *L. annuus*) contain no appreciable concentration of any ninhydrin-reacting compound.

DISCUSSION

The seeds of 49 species of *Lathyrus* examined contained ninhydrin-reacting compounds that could be extracted with acidified 50% (v/v) ethanol. All but four of the seeds contained appreciable concentrations (1% or more as judged by the intensity of the ninhydrin spots) of at least one of these compounds. The majority of species contained two, three or four.

The R_f values, ionic mobilities, and in four instances the colours given with ninhydrin, indicated that most of the compounds occurring in concentrations of this order were not amino acids normally found in protein hydrolysates.

Lathyrine was found as a major constituent in the extracts of 12 species, but of more interest was the discovery of other ninhydrin-positive compounds associated with it. The seeds of all but four of the remaining species, while containing little or no lathyrine, contained similar associations of other reacting compounds; within the genus there existed well defined groups of species that were

characterized, not by the presence of an arbitrary concentration of one specific ninhydrin-reacting compound, but rather by the presence of associated groups of such compounds. These groups of associated compounds appeared as characteristic patterns after the seed extracts had been chromatographed or subjected to ionophoresis on paper. In the extracts of most, but not all, of the species examined the spots forming the characteristic patterns were of comparable size and intensity.

Although the nature of the compounds stored varied, the total concentration of extractable ninhydrin-reacting material in the various species remained surprisingly constant. This finding is consistent with the suggestion made earlier that non-protein nitrogen compounds play an important role as storage products in the seeds of the Papilionoideae and also that such storage may take several different forms even within a single genus.

The most likely explanation of the associations found in the various groups is that the compounds concerned represent a final storage product together with its precursor or precursors. The relatively small number of spots in each pattern may perhaps reflect a simple relationship of this type, though a full understanding of these relationships must await the elucidation of the structures of the compounds concerned and the enzyme systems involved in their synthesis.

Although the taxonomic significance of these associations cannot be fully evaluated until more is known of similar associations in other genera, the associations cannot be ignored in studying relationships within this genus. Genetically as well as taxonomically the apparent existence of two subspecies (*L. laevigatus* subspecies *aureus* and *L. laevigatus* subspecies *occidentalis*) showing patterns characteristic of different groups is of particular interest.

Free protein amino acids were not found in high concentration in the seeds examined, except arginine, glutamic acid and aspartic acid, which were widely distributed in varying concentration in all the groups described. Their metabolic role may be fundamental and not necessarily or primarily associated with storage. Where these three amino acids were found they occurred in addition to those compounds forming the characteristic associations.

The non-protein amino acids probably undergo degradation and transformation during the germination of the seeds. Such changes will require their own enzyme systems, though the mobilization of nitrogen stored in this form may possibly be simpler than that of nitrogen stored as protein. The percentage of nitrogen in canavanine (31.8%) and in lathyrine (30.5%) is approximately twice that found in normal protein; among those compounds whose structures have not yet been determined

there are two guanidino compounds (B_1 and B_2) that are likely to be equally rich in this element. Therefore, although the total free non-protein amino acid nitrogen may be very much less than the protein nitrogen stored in a seed, it may nevertheless constitute a small, highly concentrated reserve immediately available to the embryo on germination.

SUMMARY

1. The genus *Lathyrus* may be subdivided into groups of species characterized by associations of ninhydrin-reacting compounds in their seeds.

2. Lathyrine is a major constituent in the seeds of 12 species of *Lathyrus*.

3. β -(γ -Glutamylamino)propionitrile, the toxic factor of *L. odoratus* seeds, has been identified chromatographically in the seeds of *L. hirsutus* and *L. roseus*.

4. A new naturally occurring guanidino amino acid occurring in the seeds of 36 species of *Lathyrus* has been identified by paper chromatography and ionophoresis as homoarginine.

5. Seven unidentified ninhydrin-reacting compounds in concentrations of about 1% have been found in the seeds of one or more of the species

examined. Details of colour reactions, R_f values and ionic mobilities are given.

6. Non-protein amino acids may constitute a highly concentrated and readily available form of nitrogen storage in leguminous seeds.

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Staphylococcal Penicillinase and the New Penicillins

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The isolation in quantity of 6-aminopenicillanic acid from penicillin fermentations (Batchelor, Doyle, Nayler & Rolinson, 1959) occasioned the synthesis of a large series of penicillins with different acyl side chains. A major objective was to prepare new drugs active against penicillin-resistant staphylococci. Since such organisms, as encountered clinically, invariably owe their resistance to their inducible penicillinase, it was evident that the success of a new compound depended upon its behaviour toward this enzyme; to be effective, it had either to be a worse substrate than penicillin G, or a worse inducer of the enzyme, or both.

The best of the new penicillins thus far reported is 2,6-dimethoxyphenylpenicillin (methicillin); all strains of staphylococci tested were inhibited by

it at concentrations ranging from 1 to 4 μ g./ml., whether they produced penicillinase or not (Rolinson, Stevens, Batchelor, Wood & Chain, 1960*b*; Stewart, 1960; Knox, 1960; Thompson, Harding & Simon, 1960; Branch, Rodger, Lee & Power, 1960), and almost all of a series of patients with severe penicillin-resistant staphylococcal infections were cured (Stewart, Nixon & Coles, 1960; Branch *et al.* 1960). It was reported that 2,6-dimethoxyphenylpenicillin was not hydrolysed (Rolinson *et al.* 1960*b*), or was only slightly hydrolysed (Stewart, 1960; Knox, 1960), by staphylococcal penicillinase, and that it was a good inducer of the enzyme (Branch *et al.* 1960; Knox, 1960; Rolinson *et al.* 1960*b*; Stewart, 1960). Its effectiveness was therefore considered to be due to its resistance to staphylococcal penicillinase. Two other new penicillins, α -phenoxyethyl- and α -phenoxypropyl-penicillin, were reported to be more

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