Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

6. CHANGES IN NITROGENOUS COMPOSITION DURING THE GROWTH OF *CONVALLARIA* AND *POLYGONATUM**

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(*Received* 6 *October* 1958)

Azetidine-2-carboxylic acid has been recognized as an important constituent of the 70% ethanol-soluble-nitrogen fraction of many liliaceous plants (Fowden, 1956; Fowden & Steward, 1957). *Convallaria majalis* (lily of the valley) and *Polygonatum multiflorum* (a Solomon's seal) are two common representatives of these plants in Britain, and part 5 of this series (Fowden & Bryant, 1959) described experiments in which various ^{14}C-labelled substances were supplied to tissues of these two plants to obtain information about the manner of biogenesis of azetidine-2-carboxylic acid. In this paper variations in the major nitrogenous fractions present in different organs of the plants during growth have been investigated to ascertain in another way where azetidine-2-carboxylic acid is synthesized and at what stage of the plant's development synthesis occurred. The importance of the imino acid in the general nitrogen economy of the plants is discussed.

EXPERIMENTAL

All plants were grown outdoors on the same location. Samples were collected at four stages of growth (S1, S2, S3 and S4). The first collection (S1) was made in February when the shoots remained as small underground buds. Collection S2 was made in mid-April. The *Convallaria* shoots were then 6–8 cm. high, the leaves being still rolled. The *Polygonatum* shoots were 12–15 cm. high, but the first leaves were not yet fully expanded. Collection S3 was made in late May when both plants were nearly mature. The *Convallaria* leaves were unfolded and had attained nearly maximum size, whereas the *Polygonatum* shoots had grown to their full height (60–80 cm.) and gained their maximum number of leaves. Both plants were in flower at this stage. The final collection S4 was taken in August when the shoots of both plants were becoming senescent and the leaves were browning. At each stage the plants were separated into root, rhizome and shoot components before analysis and their dry weight/fresh weight ratios determined. Since both plants are perennials the percentage increases in the shoot weights occurring during one season's growth were far higher than the corresponding increases for rhizome or roots.

* Part 5: Fowden & Bryant (1959).

Fractionation of the nitrogen constituents. Washed plant tissues were dried at 95° for 1 hr. and then at 65° to constant weight. The dried materials were ground to homogeneous powders upon which total nitrogen determinations were made. Other batches of plant material were macerated in an Ato-Mix Blender with 75% (v/v) ethanol (10 ml./g. fresh wt. of material). Extraction at laboratory temperature was continued for 24 hr. with shaking and, after separation of the extract and residue by centrifuging, the residue was twice re-extracted. The extracts were evaporated to dryness at temperatures below 50° and the residues were redissolved in water. Any material remaining insoluble at this stage was removed by centrifuging and discarded. Measured portions of these solutions were used for determinations of total soluble nitrogen and of individual free amino and imino acids.

Nitrogen determinations. Nitrogen was assayed by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943).

Amino acid determinations. Individual acids were determined after their separation on two-dimensional paper chromatograms. The methods used have been described previously (Fowden & Bryant, 1959; Fowden & Webb, 1958; Fowden, 1954). A preliminary separation of the cationic fraction (amino acids) from each extract was made (Fowden & Webb, 1958) before paper chromatograms were prepared. The volumes of the cationic fractions were adjusted so that 1 ml. was equivalent to 2 g. fresh wt. of plant material, and 50 μl. of each was applied to the chromatograms (Whatman no. 3MM chromatographic-grade filter paper). Phenol–aq. NH_3 soln. (Dent, 1947) was used as the first developing solvent, and was followed by butan-1-ol–acetic acid–water (Partridge, 1948). The final chromatogram size was 27 cm. × 27 cm. Location of the amino acid spots and the quantitative determination of each followed exactly the procedure described by Fowden (1954). Only the principal free amino acids were assayed; these included glutamic acid, glutamine, asparagine, alanine, serine, arginine and azetidine-2-carboxylic acid. The reported values are means of triplicate determinations and are subject to errors up to ±10% of the recorded values.

Although azetidine-2-carboxylic acid gives a brown spot after treatment of qualitative paper chromatograms with ninhydrin, the imino acid reacts to give a normal bluish purple colour (absorption maximum λ 570 mμ) under the controlled conditions used in quantitative colour development. Colour production from azetidine-2-carboxylic acid was, however, only about 25% of that obtained when an equivalent amount of an α-amino acid reacted with ninhydrin.

RESULTS

Both plants have perennial rhizomes and roots but produce completely new shoots each year. During one season's growth the percentage changes in the dry weight of rhizome and root components are very small compared with those of the shoots. For instance in *Convallaria* the dry weights of the shoot at S 2, S 3 and S 4 were approximately 2·6, 15 and 40 times the corresponding weight at S 1.

The total- and soluble-nitrogen contents of the plants' organs at the different stages of development are presented in Tables 1 and 2 respectively. The dry-weight/fresh-weight ratios of the tissues are given in parentheses in Table 1. Active growth of the plants began between S 1 and S 2 and was presumably associated with a marked uptake of nutrients which led to increased total nitrogen contents throughout the plant. This increase was particularly pronounced for *Polygonatum* shoots, which attained a very high 'crude protein' content (about 48 % of the dry weight) at S 2. As further growth of the shoot occurred and the leaves reached maturity their total nitrogen content rapidly decreased. Smaller decreases were observed for the rhizome and root tissues between S 2 and S 4.

In *Convallaria* the variations in the soluble-nitrogen contents of the tissues followed the same pattern as those of total nitrogen. Maximum values were generally reached at S 2. The increases observed for the shoot and root between S 1 and S 2 and the subsequent decreases to S 4 tended to be proportionately greater than those observed for the total nitrogen content of the same organ. Soluble nitrogen at S 2 therefore represented a larger percentage of the total nitrogen of these organs than it did at any other stage of growth (see parentheses, Table 2). The ratio soluble nitrogen/total nitrogen was generally higher in the rhizome than in the other organs. The most striking feature of the soluble-nitrogen data for *Polygonatum* was also the remarkably high levels found in the rhizome, particularly when expressed as percentages of the total nitrogen (see Table 2). These latter percentages were appreciably higher than the corresponding ones determined for *Convallaria* rhizome and somewhat higher than those recorded by Steward & Thompson (1954) for other typical storage organs, e.g. potato tuber, approximately 70 %, and carrot root, approximately 60 %.

The concentrations of individual free amino acids in the plant tissues are presented in Table 3. Well-marked trends in the concentrations were

Table 1. *Total nitrogen contents of* Convallaria *and* Polygonatum *tissues at different stages of growth*

Results are expressed as mg. of total nitrogen/g. dry wt. of tissue. Figures in parentheses are dry wt./fresh wt. ratios.

Sampling time	Root	Rhizome	Shoot
	Convallaria		
S 1; early February	24·5 (0·361)	15·2 (0·321)	47·6 (0·237)
S 2; mid-April	30·7 (0·226)	20·7 (0·268)	49·4 (0·134)
S 3; late May	21·2 (0·247)	16·3 (0·233)	34·6 (0·226)
S 4; August	12·9 (0·262)	12·3 (0·435)	17·6 (0·346)
	Polygonatum		
S 1; early February	21·6 (0·177)	16·7 (0·190)	51·0 (0·180)
S 2; mid-April	26·4 (0·159)	23·6 (0·161)	76·5 (0·153)
S 3; late May	19·4 (0·152)	14·2 (0·156)	42·1 (0·179)
S 4; August	15·5 (0·169)	13·7 (0·183)	27·5 (0·233)

Table 2. *The ethanol* 75 % *(v/v) soluble-nitrogen contents of* Convallaria *and* Polygonatum *tissues at different stages of growth*

Results are expressed as mg. of soluble nitrogen/g. dry wt. of tissue. Figures in parentheses show soluble-nitrogen contents as percentages of the total nitrogen content of tissue.

Sample	Root	Rhizome	Shoot
	Convallaria		
S 1	4·63 (18·9)	5·98 (39·3)	5·32 (11·2)
S 2	9·91 (32·3)	6·60 (31·9)	12·6 (25·7)
S 3	6·38 (30·1)	7·25 (44·5)	5·40 (15·6)
S 4	2·79 (21·6)	3·26 (26·5)	1·59 (9·0)
	Polygonatum		
S 1	5·63 (26·1)	13·2 (79·0)	13·3 (26·1)
S 2	3·90 (14·8)	19·5 (83·6)	9·93 (13·0)
S 3	4·14 (21·3)	9·1 (64·1)	4·33 (10·3)
S 4	4·61 (29·7)	11·0 (80·3)	2·10 (7·6)

Table 3. *Concentrations of free amino and imino acids of* Convallaria *and* Polygonatum *tissues at different stages of growth*

Results are expressed as μg. of nitrogen present in each acid/g. dry wt. of tissue. Figures in parentheses show nitrogen present in azetidine-2-carboxylic acid expressed as percentages of total soluble nitrogen.

Sample	Asparagine	Glutamic acid	Glutamine	Azetidine-2-carboxylic acid	Serine	Arginine	Alanine
			Convallaria				
Root:							
S1	160	135	44	289 (6·2)	38	115	30
S2	223	177	93	132 (1·3)	27	561	44
S3	348	129	154	173 (2·7)	50	442	67
S4	136	129	66	653 (23·4)	46	229	74
Shoot:							
S1	150	75	56	1470 (27·6)	40	149	38
S2	443	406	782	5040 (39·7)	200	34	237
S3	292	165	94	2290 (42·4)	70	15	99
S4	17	25	6	578 (36·3)	17	25	61
Rhizome:							
S1	241	131	56	314 (6·9)	41	231	28
S2	195	61	99	207 (3·1)	49	258	7
S3	484	156	113	180 (2·5)	62	518	84
S4	100	22	60	170 (5·2)	16	368	56
			Polygonatum				
Root:							
S1	82	239	166	734 (13·0)	94	127	62
S2	73	187	98	727 (18·6)	43	91	30
S3	113	189	85	677 (16·4)	39	239	41
S4	38	151	175	2260 (49·0)	47	157	96
Shoot:							
S1	81	276	407	5580 (41·9)	115	335	55
S2	27	333	243	2260 (22·8)	107	201	107
S3	8	253	92	810 (18·7)	124	291	78
S4	13	25	13	291 (13·8)	20	25	80
Rhizome:							
S1	296	249	461	4870 (36·9)	204	247	157
S2	205	117	363	9060 (46·5)	41	619	33
S3	116	167	214	5230 (57·4)	65	338	59
S4	142	238	767	7510 (68·3)	168	498	201

observed only in the shoots. In *Polygonatum* the concentrations of most amino and imino acids fell progressively during development from S1 to S4. In *Convallaria* an initial increase generally occurred in amino acid concentrations during the early growth of the shoot (S1 to S2); further growth was accompanied by decreased concentrations of most acids.

Azetidine-2-carboxylic acid was the most important constituent of the soluble-nitrogen fraction of all *Polygonatum* tissues, but in *Convallaria* it accumulated in very high concentrations only in the shoot. Arginine and the amides, glutamine and asparagine, were also important components of the soluble-nitrogen fraction of the plants at certain stages of development.

DISCUSSION

Analytical data of the present type can be presented in several ways; the amount of any single nitrogenous component can be expressed in terms of the plant's fresh weight, dry weight or total nitrogen content. It is not easy to decide which mode of expression is most useful. Values on a fresh-weight basis are valuable for most tissues in that they reflect the actual concentrations at which the nitrogen compounds are found within the cells. However, these values have less significance for tissues like rhizome where the dry-weight/fresh-weight ratio was relatively high. They also suffer from the disadvantage that the water content and hence the fresh weight of plant tissues may vary with changes in the plant's environmental conditions to a greater extent than the dry weight. A compromise was therefore used, all values being presented on a dry-weight basis and the dry-weight/fresh-weight ratios for all tissues were included in the tables. Values can be converted into a fresh-weight basis if this is desirable.

The fact that growth of both species beyond S2 was accompanied by progressively lower total

nitrogen contents agrees with similar observations made for many other species and several factors are probably operative in producing these decreases. During shoot growth between S 1 and S 2 the cells of the originally underground buds must have undergone a transition from a slow to a rapid state of division and this change was associated with a lowered dry-weight/fresh-weight ratio regarded as typical of meristematic tissue having a high rate of protein synthesis. Cell division after S 2 would be restricted to a smaller proportion of the cells of the shoot and the further growth to stages S 3 and S 4 was accompanied by progressively increased dry-weight/fresh-weight ratios, indicating that secondary thickening of many cell walls was occurring and producing increased carbohydrate to protein ratios. Decreases in the total nitrogen contents of the rhizomes and roots may in part be due to similar but less extensive changes in cellular activity in these organs. An extensive deposition, particularly in the rhizome storage tissue, of reserve carbohydrate products of photosynthesis would be expected to occur during the later part of the growing period and so this would be another factor leading to decreased nitrogen concentrations in the underground organs.

The decreases in the soluble-nitrogen concentration in the shoots between S 2 and S 4 are particularly striking. During this time the dry weight of the *Convallaria* shoot increased about 17-fold, whereas the total amount of nitrogen in the shoot increased only about six times. These figures may indicate that the supply of nitrogen to the shoot became limiting in the later stages of its growth and under these conditions there would be a tendency for available nitrogen to be incorporated into protein and so produce the observed marked decrease in soluble nitrogen compounds.

The exact reasons for the high concentrations of soluble nitrogen in the root and shoot of *Convallaria* at S 2 must be looked for in the individual free amino and imino acid concentrations (Table 3). Although the percentage increase for some of them (glutamic acid, glutamine and alanine) was larger than that of azetidine-2-carboxylic acid, it was the increase in the total amount of the imino acid that had a profound effect on the soluble-nitrogen level. Between S 1 and S 2 the soluble nitrogen increased by 7·3 mg./g. dry wt.; the increased concentration of azetidine-2-carboxylic acid accounted for about half of this (3·6 mg./g. dry wt.). In contrast, the increased soluble-nitrogen levels of the root at S 2 could not be attributed to changes of concentrations of azetidine-2-carboxylic acid, for although the concentrations of most other amino acids increased between S 1 and S 2 the imino acid levels actually decreased. Arginine showed the most marked increase of concentration and the increased amount of nitrogen present in the amino acid at S 2 compared with S 1 represented about one-third of the total increase of soluble nitrogen.

During the period S 1–S 2 the dry weight of the shoot increased 2·6 times, so that the total quantity of azetidine-2-carboxylic acid present in the shoot at S 2 was approximately nine times that at S 1. Although the decreases in the concentration of azetidine-2-carboxylic acid within the root and rhizome between S 1 and S 2 were small in comparison with the increase in the shoot, approximately half of the extra azetidine-2-carboxylic acid appearing in the shoot could have been transferred from the underground organs, since at S 2 their combined weights were about 20 times that of the shoot. This calculation assumes no synthesis of azetidine-2-carboxylic acid within the root or rhizome. If a net synthesis of the imino acid was occurring in one or both of these organs, then considerably more than half of the azetidine-2-carboxylic acid appearing in the shoot may have been deposited there after translocation. A further, approximately twofold, increase in the total amount of azetidine-2-carboxylic acid in the shoot occurred between S 2 and S 3, for although the concentration of the imino acid decreased during this phase of growth, the dry weight of the shoot increased about fivefold. However, at this stage the concentrations of azetidine-2-carboxylic acid in root and rhizome remained nearly constant. Therefore active translocation of the compound to the shoot would appear less likely at this stage, and synthesis of the imino acid was probably occurring within the shoot itself. This conclusion is in agreement with the observation of Fowden & Bryant (1959), who showed that excised leaves of *Convallaria* at this stage of development could photosynthetically assimilate $^{14}CO_2$ slowly into azetidine-2-carboxylic acid. Little change occurred in the total amount of azetidine-2-carboxylic acid within the shoot between S 3 and S 4, a further decrease in its concentration being offset by an increase in the shoot dry weight. It is then perhaps significant that in this period there was a very marked increase in the concentration of azetidine-2-carboxylic acid in the root, even though the concentrations of most of the other amino acids decreased. This observation suggests that in *Convallaria* the root may act as a major site of synthesis of azetidine-2-carboxylic acid, a fact that became apparent only at this late stage of growth when active translocation of the imino acid appeared to have ceased.

Reference to Table 3 shows that in *Polygonatum* azetidine-2-carboxylic acid was the major component of the soluble-nitrogen fractions of all tissues at all stages of growth. Particularly in the rhizome variations in the concentrations of other free amino acids were completely overshadowed by

the much larger changes in concentration of azetidine-2-carboxylic acid. The imino acid represented a higher proportion of the soluble-nitrogen fraction at S4 than at any earlier stage of growth, but it reached its highest concentration on a dry-weight basis at S2, when about 6·5% of the dry weight of the rhizome consisted of azetidine-2-carboxylic acid. This is a remarkably high concentration for a soluble-nitrogen compound; comparable concentrations have been observed only for certain alkaloids (see James, 1950). A further unusual finding was that the nitrogen present in azetidine-2-carboxylic acid of the rhizome generally exceeded that present in the protein. For instance at S4 the nitrogen of azetidine-2-carboxylic acid represented about 55% and protein nitrogen only about 20% of the total nitrogen.

Decreases in the concentrations of most amino acids occurred during the growth of the *Polygonatum* shoot, particularly between stages S3 and S4. The concentration of azetidine-2-carboxylic acid at S4 was only about 5% of that at S1. Since the increase in the dry weight of the shoot during the whole period of growth was only slightly greater than 20-fold no significant increase in the total amount of azetidine-2-carboxylic acid in the shoot occurred. The possibility that active translocation of the imino acid from the underground organs to the shoot occurred therefore appeared less likely than in *Convallaria*; however, it cannot be completely ruled out since shoots of *Polygonatum* may be able to degrade the compound. Information is lacking about this degradation in *Polygonatum* but preliminary studies with [^{14}C]azetidine-2-carboxylic acid indicated that degradation proceeded very slowly in *Convallaria* leaves (Fowden & Bryant, 1959).

Attention was drawn earlier to the increase in concentration of azetidine-2-carboxylic acid in *Convallaria* roots between S3 and S4. Table 3 shows that a similar rapid increase occurred for the imino acid in *Polygonatum* roots. Although some significance was attached to this finding in regard to the site of synthesis of azetidine-2-carboxylic acid in *Convallaria*, the position in *Polygonatum* is more obscure. The increase in concentration of azetidine-2-carboxylic acid in the roots was paralleled by an even larger increase in the amount of the imino acid present in the rhizome. Therefore the possibility that synthesis can proceed in both organs cannot be overlooked. Fowden & Bryant (1959) found that radioactivity supplied to roots and rhizome of *Polygonatum* in the form of [^{14}C]aspartic acid was incorporated into azetidine-2-carboxylic acid more rapidly in the former organ, although some azetidine-2-carboxylic acid was apparently also synthesized in the rhizome.

The role of azetidine-2-carboxylic acid in the nitrogen metabolism of liliaceous plants remains in doubt. The high concentrations in which it is encountered suggests that it is more likely to play the role of a storage compound rather than that of an actively metabolized intermediate compound. This conclusion receives some support from experiments with [^{14}C]amino acids, which showed that the rates of synthesis and degradation of the imino acid in these plants were slow (Fowden & Bryant, 1959). If azetidine-2-carboxylic acid is correctly regarded as a storage compound, then nitrogen so combined is probably less readily available for re-utilization than that present in other compounds such as glutamine and asparagine that are considered to play a similar role. The high concentrations and relatively low metabolic activity of azetidine-2-carboxylic acid in plants suggest that it may be more closely akin to the alkaloids than to the majority of amino acids. Indeed it is tempting to speculate that alkaloid-like compounds based on the four-atom heterocyclic ring of azetidine will ultimately be discovered in plants. A compound bearing the same structural relation to azetidine-2-carboxylic acid as that shown to proline by the alkaloid stachydrin would seem a distinct possibility.

If the idea is accepted that azetidine-2-carboxylic acid is synthesized largely in the root of *Convallaria* and then translocated and deposited as a storage compound in the leaves, a further similarity with certain alkaloids emerges. For example, a parallel exists with nicotine, which is elaborated by the roots of the tobacco plant (Mothes, 1928; Dawson, 1941, 1942). However, nicotine tends to be present in this organ in only low concentrations and is mainly transported to the leaves, where it is stored at much higher concentrations.

Our present understanding of the behaviour and function of azetidine-2-carboxylic acid in plants is certainly limited. The compound possesses features of metabolism which identify it closely in behaviour with a group of other compounds like alkaloids, floral pigments, glycosides, etc., the reason for whose existence in plants still remains unsolved.

SUMMARY

1. Variations occurring in the nitrogenous composition during the growth of *Convallaria majalis* and *Polygonatum multiflorum* plants were investigated. At four stages of growth the concentrations of total nitrogen, 75% (v/v) ethanol-soluble nitrogen and certain amino and imino acids were determined separately for roots, rhizome and shoots.

2. The soluble-nitrogen content of both plants, but particularly that of *Polygonatum*, was high

when expressed as a percentage of the total nitrogen. This observation is in agreement with those recorded previously for typical storage organs.

3. The two plants contained very large amounts of the imino acid, azetidine-2-carboxylic acid. Nitrogen present in azetidine-2-carboxylic acid represented more than 50 % of the total nitrogen of *Polygonatum* rhizome. The imino acid was also present in high concentrations in the young shoots of both plants, but its concentration in the mature shoots was much lower.

4. The analytical data indicate that the root may form a major site of synthesis of azetidine-2-carboxylic acid in *Convallaria*. It is more difficult to draw conclusions for *Polygonatum* since the very high concentrations present in its rhizome at all stages of growth tended to overshadow smaller changes of concentration elsewhere in the plant.

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Nitrogenous Constituents of the Thoracic Muscle of the African Migratory Locust (*Locusta migratoria migratorioides*)

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(*Received* 19 *September* 1958)

During recent years a considerable amount of information has become available concerning the non-protein nitrogenous constituents, and especially the amino acids in the haemolymph of various species of insects. Most of this is due to the work of Florkin and his colleagues, who have in the main used microbiological methods for their assays of amino acids (Florkin, 1954). It appears that the haemolymph of insects is characterized by a relatively high amino acid content, and in this respect they differ markedly from crustaceans such as lobsters which, though containing a high concentration of free amino acids in their muscles, have very little in the haemolymph (Kermack, Lees & Wood, 1955). Little or no information appeared to be available about the free amino acid content of insect muscle.

An insect which it seemed convenient to study in this respect was the migratory locust *Locusta migratoria migratorioides*. The insect order Orthoptera, to which the locust belongs, contains many of the larger insects, and a considerable amount of research has been carried out on this order. From the available data, the metabolism of orthopteran muscle appears to be similar to that of mammalian muscle; for example, the occurrence of glycolysis and the presence of the tricarboxylic acid cycle in the African migratory locust has been demonstrated by Rees (1954) and adenosine triphosphate has been isolated from the muscle of the locust *Gastrimargus musicus* (Calaby, 1951).

In the present work the nature of the non-protein nitrogenous constituents of locust muscle was investigated. Some of the present results have already been briefly presented (Kermack & Stein, 1955).

MATERIALS AND METHODS

Locusts. Fifth-stage nymphs of the African migratory locust, supplied by the Anti-Locust Research Centre, London, were kept in the Laboratory under the conditions advised by the Centre. The locusts, after undergoing their final moult to the adult form, were used as required. After starving overnight they were killed by placing for a few minutes in a jar containing chloroform, then cut open dorsally, the alimentary canal and fat body were removed and the thoracic muscles *in situ* washed free of haemolymph by squeezing 1% (w/v) NaCl from a Pasteur pipette between and underneath the muscle bands. Residual saline on the surface was removed with filter paper and the intact muscle excised.

Muscle extracts. Muscle from about six locusts was homogenized with 5% (w/v) trichloroacetic acid (5 ml.) for 5 min. in a Mickel homogenizer. The suspension was allowed to stand for 15 min. and centrifuged. The muscle residue was extracted a second time in the same way, and