The role of tingitanine in nitrogen metabolism of *Lathyrus tingitanus*

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INTRODUCTION

In 1961 a new free amino acid was isolated from seeds of *Lathyrus tingitanus*. This amino acid was isolated and partly described, independently and at the same time, by Bell (1961) who termed it lathyrine, and Nowacki and Przybylska (1961) who suggested for it the term tingitanine. In 1962 Bell and Foster (1962) presented the structural formula of the new amino acid.

tingitanine — lathyrine

Accumulation of large amounts of the new amino acid in seeds of *Lathyrus tingitanus* and the large proportion of nitrogen per molecule of this compound indicated its possible role in the storage of nitrogen.

Primary research into the role of tingitanine in nitrogen metabolism of *Lathyrus tingitanus* was carried out on etiolated plants (Przybylska 1962a, 1962b). It was found that during plant development in darkness the amount of tingitanine in cotyledons gradually drops, while in cotyledonless plant it rises. After 18-day period of growth the level of tingitanine in cotyledonless plant was much higher than in seed. These data prove that in etiolated plants there occurs a synthesis of tingitanine which performs a definite role of fixing ammonia.

This paper presents the investigations on the role of tingitanine in nitrogen metabolism of green plants.

MATERIALS AND METHODS

Analyses were carried out on *Lathyrus tingitanus* plants grown in the field in 1961. Cotyledons, roots, root-nodules, stems, leaves, flowers, seedless pods (pericarps) and seeds were analysed separately. Cotyledons were sampled from 3-week-old plants (22.V.). Leaves, stems and roots were sampled from 3-week-old plants, then at the period of flowering, and later every 3—5 weeks. In all, six samplings took place. Except

during the first sampling when all the leaves were collected, leaves were taken from the highest stem. The same material was the source of stem samples. Root-nodules, were sampled at the 2nd, 3rd and 4th collecting of roots, as during early phase of plant development they were not fully grown and towards the end of the growing season they were dead and unfit for analysing. Flowers were collected a single time.

Table 1
List of analysed samples

Date of sampling	Analysed organs
22.V.1961	cotyledons, roots, main (longest) stems* leaves (3-week-old plants)
1.VII.1961	roots, root-nodules, stems, leaves, flowers
7.VII.1961	very young pods — seeds not visible yet
13.VII.1961	seedless pods (pericarps), seeds
24.VII.1961	roots, root-nodules, stems, leaves, seedless pods, seeds
1.VIII.1961	seedless pods, seeds
8.VIII.1961	seedless pods, seeds
16.VIII.1961	roots, root-nodules, stems, leaves, seedless pods, seeds
23.VIII.1961	seedless pods, seeds
2.IX.1961	seedless pods, seeds
18.IX.1961	seedless pods, seeds
25.IX.1961	roots, stems, leaves, seedless pods, seeds
2.X.1961	seedless pods, seeds
16.X.1961	seedless pods, seeds
23.X.1961	roots, stems, leaves, seedless pods, seeds
30.X.1961	seeds (harvest)
18.VII.1962	stems, defoliated side-shoots, peduncles, upper leaves, mid-stem leaves, bottom leaves

* Hereafter the main (longest) stems will be termed stems.

Seeds and seedless pods were sampled every 7th or 8th day. During certain periods however, owing to the retarded growth rate, seedless pods and seeds were sampled less frequently. In all, 14 samples of seedless pods and 14 of seeds were taken.

In 1961 the weather regime was atypical. The mean temperature in July and August was exceptionally low and rainfall in July was much above the average. These conditions induced the plants to produce a large bulk of green mass followed in turn with a much delayed seed setting and pod formation. In effect, pods ripened in October instead of at the beginning of September.

Some of the organs were additionally analysed in 1962. Sampling took place during the peak bloom (18.VII). Main (longest) stems, defoliated side-shoots and peduncles were analysed separately. Leaves of different

ages were sampled at the same time. They were collected from the longest stems and divided into three groups: 4 youngest leaves from the upper stem, medium-sized leaves from the mid-stem, and 4 eldest leaves from the bottom part of the stem. The three groups were analysed separately. The terms of samplings and analysed organs are listed in Table 1.

For the purpose of finding the free amino acids, into which inorganic nitrogen compounds introduced into plants become incorporated, a special trial was laid out. On Dec. 5, 1962, seeds of *Lathyrus tingitanus* were planted in pots with soil from the trial field. The pots were kept in a greenhouse at around 18°C. At the beginning all plants received tap-water. On Jan. 1, 1963, the plants were arranged into three groups: 1) control plants supplied with distilled water, 2) plants supplied with a 0.1% solution of ammonium nitrate, 3) plants supplied with a 0.5% solution of ammonium nitrate. After 20 days the plants were analysed in separate lots of roots, stems and leaves.

All the samples of field-grown plants were analysed for dry matter, total nitrogen, α -amino nitrogen, amido nitrogen and tingitanine nitrogen. Two-dimensional paper chromatography was applied to determine free amino acids. Quantitative analyses of all the amino acids were carried out in extract of young pods sampled on July 7, 1961, by means of an automatic fraction analyser (Phoenix P.P.I., mod. K-5000 A) according to Spackman, Stein and More* (1958).

Samples collected from the pot trial (control plants and those supplied with ammonium nitrate) were only analysed for free amino acids by means of two dimensional paper chromatography. Detailed descriptions of the methods are given in the previous paper (Przybylska 1962b).

The amino acid appearing on two-dimensional chromatograms in the place of homoserine was isolated as follows: free amino acids of an extract of young *Lathyrus tingitanus* pods (sampled on July 7, 1961) were separated by means of paper electrophoresis (Wagner 1958). A fraction of neutral amino acids was eluted with 50% ethanol and separated chromatographically on Whatman 3 paper with the use of Partridge's solvent (butanol: acetic acid: water in a 4: 1: 5 ratio) according to the ascending technic. After developing the chromatogram four times the band corresponding to homoserine was eluted, and the eluate subjected to a chromatographic resolution with solvent according to Wolfe (1957) — methyl ethyl ketone: butanol: 17N ammonia: water in a 3:5:1:1 ratio — on Whatman 3 paper, using the descending technic. Following a development repeated three times the chromato-

* These analyses were performed by J. Kowalczyk Sc. M. Dept. of Agricultural Technology of the Poznań College of Agriculture.

graphic band corresponding to homoserine was eluted, the eluate evaporated and the remainder treated with a given volume of water.

The identity of the dissolved substance with authentic homoserine was proved by means of the following methods:

1. Both samples were subjected to cochromatography with the use of the circular technic and Whatman 3 paper (Przybylska, Kociałkowski, Wiewiórowski 1958) in four solvents (Smith 1960): a) ethanol: water: ammonia (0.88) in a 180: 10: 10 ratio (by volume); b) phenol: ethanol: water: ammonia (0.88) in a 120: 40: 40: 1 ratio (by volume); c) methanol: water: pyridine in a 160: 40: 8 ratio (by volume); d) 200 ml of the mixture of phenol and water (4: 1, w/v) with 1 ml of ammonia (0.88).

In each case a coincidence of the R_f values of the compared substances was revealed.

2. Color reactions of both samples with different developers were compared. Reactions with ninhydrin (0.4% in acetone), ninhydrin stabilized with CoCl_2 (0.4% solution of ninhydrin in acetone with an addition of 0.2% CoCl_2), isatine (0.4% solution in acetone) and alloxan (0.2% solution in acetone) were all identical.

3. Products of an acid hydrolysis of both samples were compared. The hydrolysis was performed with 3N HCl in sealed glass ampoules at 120°C for 24 hrs. The acid was removed by repeated evaporation at 60°C under a lower pressure and the dry remainder was dissolved in water. The products of hydrolysis were analysed by means of the two-dimensional paper chromatography and paper electrophoresis. Both on chromatograms and on electropherograms of both hydrolyzates developed with ninhydrin two spots were to be seen. The color and position of one of them corresponded to homoserine. The second spot appeared in the position of strong bases and was of an intense-yellow coloration, which soon turned brownish-violet. According to the reference work (Greenstein, Winitz 1961) homoserine in an acid medium easily forms lactone, and so the additional substance is a lactone of homoserine.

4. Synthetic homoserine and the isolated substance were treated with 2N HCl for 24 hrs. at a room temperature and thus partly converted into lactone. A cochromatographic resolution of both mixtures was carried out with the use of 9 solvents (Block, Durrum, Zweig 1958; Smith 1960: 1) butanol: acetic acid: water (120: 30: 50); 2) butanol: 2N acetic acid (1: 1); 3) butanol: ethanol: acetic acid: water (4: 1: 1: 10); 4) butanol: formic acid: water (600: 50: 50) + 300 ml of water (cf. Block *et al.* (1958); 5) butanol: propanol: 0.1N HCl (1: 1: 1); 6) tert-butanol: formic acid: water (70: 15: 15); 7) butanol: propionic

acid: water (1264 : 620 : 974); 8) phenol: butanol: acetic acid: water (20 : 20 : 8 : 40); 9) phenol: ethanol: water (120 : 40 : 40).

In each case a coincidence of the R_f values of the isolated compound and its derivative with synthetic homoserine and its lactone was found.

5. The analysis of free amino acids in the extract of young pods, performed with an automatic fraction analyser, revealed the presence of homoserine.

For the purpose of finding out, whether or not, tingitanine or homoserine are part of the seed protein, or protein of vegetative tissues, a fraction of crude protein from roots, stems (harvested at the period of flowering) and mature seeds was isolated and products of its hydrolysis analysed.

The fraction of crude protein was isolated as follows: defatted seed meal, or fresh stems and roots, were ground in a mortar with a phosphate buffer (pH 7.2) to a uniform, thin paste and left standing for an hour. Next the extract was centrifuged, decanted, added with 2 parts of 96% ethanol and heated to precipitate proteins. There were centrifuged and rinsed three times with small portions of 70% ethanol to remove all the nonprotein amino acids. The fraction of crude protein thus obtained was washed on a filter with ether, dried and ground (Fowden 1954). The isolated fraction of crude protein was subjected to enzymatic digestion, and to acid and basic hydrolysis.

The enzymatic digestion was carried out with the help of trypsin and pepsin. 200 mg samples of each protein were treated first with 10 mg of trypsin in 10 ml of pH 8.5 phosphate buffer for three days at 37°C under toluene. The activity of the trypsin was destroyed by placing the samples in a boiling water bath for 15 minutes, the solutions were acidified to pH 2, to each sample 10 mg of pepsin were added and hydrolysis allowed to proceed for a further period of three days at 37°C. After removal of the toluene layer two volumes of ethanol were finally added to remove the enzymes by precipitation. The supernatants obtained after centrifuging were evaporated to dryness and the residues dissolved in 50 ml of distilled water.

Acid hydrolysis was performed as follows: 20 mg samples of each protein were boiled with 25 ml of 6N HCl on a sand bath under reflux for 24 hrs. The excess HCl was removed by low pressure evaporation at 60°C to dryness, and the residues dissolved in 10 ml of distilled water.

Basic hydrolysis was carried out with NaOH: 20 mg samples of each protein were refluxed with 25 ml 5N NaOH on a sand bath for 12 hrs. The sodium ions were removed on Dowex 50 $\times$ 4 (H^+) columns. Amino acids were eluted with a 0.1 M aqueous solution of piperidine. The eluates were evaporated to dryness at 60°C, and the residues dissolved in 10 ml of distilled water.

RESULTS

The results are discussed in two chapters. First of them is concerned with the changing level of dry matter, total nitrogen and different forms of soluble nitrogen; the second represents the results of chromatographic analyses of free amino acids, and of products of crude protein hydrolysis.

A. Changes in the levels of dry matter, total nitrogen, α -amino nitrogen, amido nitrogen and tingitanine nitrogen in various organs of *Lathyrus tingitanus* during the growing season

Cotyledons. Cotyledons from three-week-old plants had an appearance of used-up organs. A relatively high level of α -amino and amido nitrogen indicates a much advanced proteolysis, while the level of tingitanine nitrogen is much lower (Tab. 2).

Table 2

Contents of dry matter, total nitrogen, α -amino nitrogen, amido nitrogen and tingitanine nitrogen in cotyledons of *Lathyrus tingitanus* (22 May, 1961)

Dry matter %	Total N in dry matter %	α -Amino N as percentages of		Amido N as percentages of		Tingitanine N as percentages of	
		dry matter	total N	dry matter	total N	dry matter	total N
6.90	5.95	1.48	24.88	0.81	13.54	0.35	5.92

Table 3

Changes in dry matter and total nitrogen in roots, stems and leaves of *Lathyrus tingitanus* during the growing season (1961)

Date of sampling	Roots		Stems		Leaves	
	dry matter %	total N in dry matter %	dry matter %	total N in dry matter %	dry matter %	total N in dry matter %
22.V	6.72	3.18	11.45	4.30	15.52	5.19
1.VII	13.92	2.39	16.48	1.82	17.59	4.03
24.VII	16.60	2.61	15.01	1.91	13.34	4.64
16.VIII	16.57	2.92	15.18	2.03	13.77	5.21
25.IX	17.53	2.98	17.02	1.74	16.45	4.37
23.X	18.32	2.81	21.93	1.48	17.34	4.11

Roots. Changes in the dry matter and total nitrogen level in roots are listed in Table 3. The relative amount of dry matter in roots definitely rises throughout the growing season, most intensely however during the early phases of development. The percentage of total nitrogen drops at first, to rise again slightly and remain on an equal level.

Changes in the different forms of soluble nitrogen in roots are a characteristic feature (Diagram 1a, b). During the period prior to flowering, the level of all forms of soluble nitrogen on a dry matter basis definitely drops: of α -amino and tingitanine nitrogen to the similar extent, of amido nitrogen to a lesser degree. In more advanced phases the contents of α -amino and amido nitrogen remain more or less unchanged: in mid-August they slightly rise, and then gradually drop. The level of tingitanine nitrogen follows a different pattern, most evident since late August: the level of α -amino and amido nitrogen drops then, while tingitanine nitrogen rises in amount very markedly,

Diagram 1. Percentage of α -amino nitrogen, amido nitrogen and tingitanine nitrogen in roots of *Lathyrus tingitanus* during the growing season (1961).

1 — α -amino N; 2 — amido N; 3 — tingitanine N; a — in respect to dry matter; b — in respect to total nitrogen.

exceeding the remaining forms of soluble nitrogen. During the period of drying up of the pods the relative amount of tingitanine nitrogen rapidly falls; — in respect however to α -amino and amido nitrogen it remains on a higher level. In considering the levels of soluble nitrogen on a total nitrogen basis the changes are of a like manner.

Root nodules. Table 4 represents the analyses results of root nodules. The dry matter percentage is not much different in samples collected at different times, whereas the total nitrogen level varies. Root nodules sampled on Aug. 16 (last collecting) were found to have a lower proportion of total nitrogen than the two earlier lots.

In root nodules the proportion of the examined forms of soluble nitrogen in respect to total nitrogen is clearly different than in roots analysed

at the same terms. In root nodules α -amino nitrogen is the main form of soluble nitrogen; the level of amido nitrogen is lower and of tingitanine nitrogen lower still. Relative amounts of the individual forms do not show great changes in dependence on the terms of sampling (Table 4).

Table 4

Contents of dry matter, total nitrogen, α -amino nitrogen, amido nitrogen and tingitanine nitrogen in root-nodules of *Lathyrus tingitanus* at various terms of sampling (1961)

Date of sampling	Dry matter %	Total N in dry matter %	α -amino N as percentages of		Amido N as percentages of		Tingitanine N as percentages of	
			dry matter	total N	dry matter	total N	dry matter	total N
1.VII	17.24	5.87	0.51	8.75	0.24	4.15	0.06	1.07
24.VII	14.67	5.66	0.45	8.02	0.25	4.38	0.08	1.42
16.VIII	14.77	4.79	0.39	8.15	0.19	3.96	0.12	2.41

Stems. As shown in Table 3 the percentage of dry matter in stems rises evidently during the period prior to flowering, to drop slightly and then rise again at maturity. The negligible drop in the dry matter level in July and August may be due to the abundant rainfall during these months. Total nitrogen at first rapidly drops in amount, to remain on a more or less equal level, and drop again towards maturity.

Table 5

Contents of dry matter, total nitrogen, α -amino nitrogen, amido nitrogen and tingitanine nitrogen in stems, defoliated side-shoots, peduncles and leaves of different ages of *Lathyrus tingitanus* (18 July, 1962)

Analysed organs	Dry matter %	Total N in dry matter %	α -amino N as percentages of		Amido N as percentages of		Tingitanine N as percentages of,	
			dry matter	total N	dry matter	total N	dry matter	total N
Stems	12.93	2.36	0.40	16.95	0.19	8.11	0.02	0.64
Defoliated side-shoots	14.27	2.44	0.34	13.88	0.16	6.49	0.01	0.38
Peduncles	12.14	2.49	0.32	12.97	0.13	5.35	0.09	3.47
Upper leaves	15.97	7.08	0.70	9.85	0.16	2.57	0.08	1.09
Mid-stem leaves	13.97	4.54	0.32	6.98	0.13	2.75	0.04	0.78
Bottom leaves	10.66	3.12	0.32	10.33	0.17	5.57	0.01	0.28

α -amino nitrogen of the forms of soluble nitrogen represents the highest level, amido nitrogen a much lower level, and tingitanine nitrogen — with the exception of the first and last sampling — the lowest level (Diagram 2a, b). Prior to flowering, a considerable drop in the

content of α -amino and tingitanine nitrogen is found, both on a dry matter and on a total nitrogen basis. Likewise the percentage of amido nitrogen becomes less, to a smaller degree however. At the more advanced phases of development the level of α -amino and amido nitrogen remains on an equal level, save a negligible rise in mid-August. The slightly fluctuating level of tingitanine nitrogen rises at maturity.

Diagram 2. Percentage of α -amino nitrogen, amido nitrogen and tingitanine nitrogen in stems of *Lathyrus tingitanus* during the growing season (1961).

1 — α -amino N; 2 — amido N; 3 — tingitanine N; a — in respect to dry matter, b — in respect to total nitrogen.

While representing changes in the level of different forms of soluble nitrogen in stems, analyses results from main stems, defoliated side-shoots and peduncles, sampled in 1962, must be provided (Table 5). These analyses were made to compare relative amounts of the examined forms of nitrogen in different conducting plant organs. It follows that no greater difference between the organs was found in the percentage of total nitrogen. As regards α -amino nitrogen — the principal form of soluble nitrogen in all three types of organs — its proportion is greater in main stems than in defoliated side-shoots and peduncles. The proportion of amido nitrogen is higher in the main stem, lower in defoliated side-shoots and lowest in peduncles. In contrast with these findings, tingitanine nitrogen is highest in peduncles and lowest in defoliated side-shoots.

Leaves. The percentage of dry matter in leaves changes throughout the growing season at first rising, to become lowered and rise again towards maturation. The level of total nitrogen changes in a direction opposite to that of dry matter (Table 3).

In 3-week-plant leaves, α -amino nitrogen and tingitanine nitrogen remain on the same level, while amido nitrogen content is lower. Considered on a dry matter basis, tingitanine nitrogen rapidly drops prior to flowering (Diagram 3a). Other forms of soluble nitrogen fall

Diagram 3. Percentage of α -amino nitrogen, amido nitrogen and tingitanine nitrogen in leaves of *Lathyrus tingitanus* during the growing season (1961).

1 — α -amino N; 2 — amido N; 3 — tingitanine N; a — in respect to dry matter; b — in respect to total nitrogen.

less at that time. In later phases of plant development soluble nitrogen forms do not change much in amount — α -amino nitrogen being highest. A similar pattern is found when results are expressed on a total nitrogen basis (Diagram 3b).

In view of the fact that leaf samples consisted of differently aged leaves (total number of leaves of the main stem) it was found advisable to compare the levels of soluble nitrogen in leaves of different ages.

Analyses of leaves sampled in 1962 and divided into three age groups revealed a highest level of dry matter and total nitrogen in youngest leaves, and a lowest level in the most aged leaves; middle-aged leaves were intermediate in this respect (Table 5).

In all three groups of leaves α -amino nitrogen was the principal form of soluble nitrogen; the level of amido nitrogen was lower; and the level of tingitanine nitrogen lowest of all. Proportions however of the different forms of nitrogen in respect to total nitrogen changed in dependence on the age of leaves. The relative amount of α -amino nitrogen is higher in youngest and eldest leaves, than in the middle-aged ones. The percentage of amido nitrogen is highest in eldest leaves. On the contrary, tingitanine nitrogen is highest in the youngest leaves.

Flowers. In flowers, α -amino nitrogen, constituting over 70% of total nitrogen, is the main form of soluble nitrogen (cf. Table 6). The level of amido nitrogen is much lower and the proportion of tingitanine nitrogen does not even amount to 1% of the total nitrogen.

Table 6

Contents of dry matter, total nitrogen, α -amino nitrogen, amido nitrogen and tingitanine nitrogen in flowers of *Lathyrus tingitanus* (1 July, 1961)

Dry matter %	Total N in dry matter %	α -amino N as percentages of		Amido N as percentages of		Tingitanine N as percentages of	
		dry matter	total N	dry matter	total N	dry matter	total N
13.00	3.54	2.56	72.40	0.26	7.24	0.03	0.82

Table 7

Changes in fresh matter, dry matter and total nitrogen in seedless pods and seeds of *Lathyrus tingitanus* during pod development (1961)

Date of sampling	Seedless pods					Seeds				
	Fresh matter per single g	dry matter		total N		Fresh matter per single mg	dry matter		total N	
		per single g	%	per single mg	in dry matter %		per single mg	%	per single mg	in dry matter %
7.VII*	0.14	0.02	14.29	1.34	6.70	—	—	—	—	—
13.VII	1.11	0.22	19.82	6.74	3.06	8.1	1.6	19.75	0.13	8.13
24.VII	1.20	0.26	21.67	6.72	2.58	15	2.9	19.33	0.23	7.93
1.VIII	1.23	0.27	21.95	6.61	2.45	17	3.4	20.00	0.24	7.05
8.VIII	1.30	0.32	24.62	6.61	2.07	53	11	20.76	0.72	6.55
16.VIII	1.64	0.35	21.34	6.22	1.78	119	26	21.85	1.58	6.07
23.VIII	1.73	0.38	21.97	6.23	1.64	153	37	24.18	2.11	5.70
2.IX	1.72	0.38	22.09	5.17	1.36	174	43	24.71	2.40	5.58
18.IX	1.96	0.40	20.41	3.95	0.99	231	65	28.14	3.65	5.62
25.IX	2.00	0.41	20.50	3.71	0.91	235	79	33.62	4.28	5.42
2.X	2.02	0.44	21.78	3.52	0.80	235	87	37.03	4.60	5.29
9.X	2.04	0.47	23.04	3.01	0.64	264	105	39.77	5.50	5.24
16.X	1.85	0.46	24.87	2.28	0.50	238	98	41.18	5.26	5.37
23.X	0.60	0.40	66.67	2.09	0.52	168	98	58.33	5.30	5.41
30.X	—	—	—	—	—	125	108	86.40	6.57	6.08

* On 7 July very young pods with as yet invisible seeds were analysed.

Seedless pods. Table 7 represents changes in the levels of fresh and dry matter, and of total nitrogen in seedless pods throughout their development. Fresh and dry matter calculated per pod indicate a rapid growth at the early phase of pod development. Later the rate of increase of fresh and dry matter falls. Since mid-October, when the pods ripen and dry up, the level of fresh matter rapidly drops, while that of dry matter only slightly falls. In accordance with these changes the dry matter percentage in pericarps rises evidently during the first few days of pod development, to remain on an equal level throughout further growth, and rise again in the phase of pod drying-up.

Diagram 4. The content of α -amino nitrogen, amido nitrogen and tingitanine nitrogen in seedless pods of *Lathyrus tingitanus* during the pod development (1961).

1 — α -amino N; 2 — amido N; 3 — tingitanine N; a — in per cent of dry matter, b — in per cent of total nitrogen, c — in μg per one seedless pod.

Changes in total nitrogen throughout the pod development are a characteristic feature. Calculated per pod, the level of total nitrogen rapidly rises during the first days of pod development (beginning of July) and remains on an equal level until the end of the first decade of August to fall towards maturity. On a dry matter basis, the percentage of total nitrogen drops rapidly during the early phase of pod development, and continues to fall gradually as the pods develop.

Changes in the levels of different forms of soluble nitrogen in developing seedless pods are an interesting feature. They are plotted in Diagrams 4a—c. It is clearly evident that α -amino nitrogen is the main form of soluble nitrogen, amido nitrogen represents a lower level and tingitanine nitrogen occurs in smallest amount. Nevertheless, all the forms of soluble nitrogen follow the same pattern of changes during the pod development.

Plotted results per pod (Diagram 4c) reveal an evident rise in the levels of all three forms of soluble nitrogen during the first few days of pod development. Soon however their levels fall, fluctuations not hampering the continuous tendency to drop. Last-term analyses reveal very low levels of all three forms of soluble nitrogen.

Percentages of the three forms of soluble nitrogen on a dry matter basis become lower as the pods develop (Diagram 4a). The fall is greatest at the early phase of pod development and is related to the high growth rate and rapid increase in the dry matter content at that time.

Proportion of all three forms of soluble nitrogen in total nitrogen, especially of α -amino nitrogen, fluctuates, rather appreciably, to fall decidedly in the phase of pod drying-up (Diagram 4b).

Seeds. As shown in Table 7, during the first weeks of pod development seed growth is relatively slow; only since the beginning of August a larger increase in the fresh and dry matter content is found. In September the dry matter content of seeds continuously rises, while the fresh matter content gains less. Since the first decade of October water losses related to ripening and drying-up of the seeds bring down the fresh matter level, while that of dry matter remains in general unchanged. In accordance with these changes the percentage dry matter of seeds is more or less equal until mid-August then gradually increases to rise rapidly in late October.

During the early phase of seed development the level of total nitrogen rises at a slow rate related to the slow growth of seeds. Since the beginning of August, the seed growth rate rises and so does the rate of increase in the content of total nitrogen per seed.

Total nitrogen content on a dry matter basis drops in the first weeks of seed development, then it remains on more or less equal level, to rise slightly towards maturity.

Changes in the levels of different forms of soluble nitrogen during seed development are an exceptionally characteristic feature. Diagrams 5a—c show the different patterns of the three forms. In following changes per seed (Diagram 5c) a considerable rise in the level of tingitanine nitrogen is observed; α -amino nitrogen at first rises more rapidly

Diagram 5. The content of α -amino nitrogen, amido nitrogen and tingitanine nitrogen in seeds of *Lathyrus tingitanus* during the pod development (1961).

1 — α -amino N; 2 — amido N; 3 — tingitanine N; a — in per cent of dry matter, b — in per cent of total nitrogen, c — in μg per one seed.

than the latter form but next remains on an equal level and finally drops. The level of amido nitrogen also rises at first, but not so rapidly, and soon becomes settled. In mature seeds tingitanine nitrogen is the main form of soluble nitrogen; α -amino nitrogen represents a much lower level; and amido nitrogen occurs at even lesser amounts.

Changes in the contents of each particular form of soluble nitrogen on a dry matter basis (Diagram 5a) and on a total nitrogen basis (Diagram 5b), follow a similar general pattern. Each form however has its peculiar run. During the earlier phases of pod development (until beginning of September) the level of α -amino nitrogen is much higher than that of the other two forms. The percentage of α -amino nitrogen, whether on a dry matter or total nitrogen basis, at first rises, but soon starts to drop rapidly. The relative amounts of amido nitrogen fall gradually throughout the entire period of seed growth and development. The percentage of tingitanine nitrogen remains on a more or less unchanging level.

B. Free amino acids in various organs of *Lathyrus tingitanus*

Analyses of free amino acids, carried out with the use of paper chromatography, found individual organs to differ greatly, both in respect to the quantitative and qualitative composition of free amino acids. The changes in proportions of individual amino acids are also different in particular organs. For this reason it was necessary to give the results separately for each tested organ.

Cotyledons. In three-week-old plants arginine and aspartic acid with asparagine were the main free amino acids of the cotyledons. Glutamic acid with glutamine, serine and alanine occurred in lesser amounts. The other amino acids, i.e. tingitanine, cystine, lysine, glycine, histidine, proline, tyrosine, methionine and/or valine, threonine, isoleucine, leucine, and phenylalanine appeared in small or trace amounts (Table 8).

Roots. The following free amino acids were found in all analysed samples of roots: aspartic acid*, glutamic acid*, tingitanine, alanine, γ -aminobutyric acid, serine, homoserine, proline, methionine and/or valine, isoleucine, leucine and phenylalanine. In addition, at certain periods of development small or trace amounts of arginine, histidine and threonine were found (Table 8; Phot. 1a). On the chromatograms of all analysed root samples a pale spot X appeared (Phot. 1a). After a development of the chromatogram with a solution of ninhydrin and CoCl_2 the

* Aspartic and glutamic acid represent the sum of these amino acids and their amids (cf. Methods, Przybylska 1962 b).

Table 8

A rough estimate of free amino acids in cotyledons, roots, root-nodes, stems and leaves of *Lathyrus tingianus* at various terms of sampling (1961)

Analysed organs	Date of sampling	Tingianine *	Aspartic acid **	Glutamic acid **	Lysine	Arginine	Histidine	Serine	Alanine	γ -Aminobutyric acid	Proline	Tyrosine	Methionine and/or valine	Isoleucine	Leucine	Phenylalanine	Cystine	Homoserine	Unidentified compounds	
																			X	X ₁ t***
Cotyledons	22.V	2 1	5	4	1 1	5	1 1	3	3	—	1 1	1	1	1	1	1	—	—	—	—
	22.V	5	3 1	3	—	—	—	1	2	2	—	—	—	—	—	—	1	1	—	—
	1.VII	3 1	4	3 1	—	—	—	2	3 1	1 1	1 1	—	—	—	—	—	—	2 1	—	—
	24.VII	4	5	4	—	—	—	1 1	2	2	—	—	—	—	—	—	—	3	—	—
	16.VIII	5	5	5	—	1	—	2	4	3	1 1	—	1	—	—	—	—	1 1	—	—
Roots	25.IX	5	5	3 1	—	—	—	2	3 1	2 1	—	—	—	—	—	—	—	—	—	—
	23.X	5	5	3	—	—	—	2	3	2	1 1	—	—	—	—	—	—	1	—	—
	1.VII	2	5	4	—	—	—	2	4	3 1	—	—	—	—	—	—	—	—	—	—
	24.VII	3 1	5	4	—	—	—	2	4	2 1	—	—	—	—	—	—	3 1	—	—	—
	16.VIII	3	5	3 1	—	—	—	2	3	3	—	—	—	—	—	—	3	—	—	—
Stems	22.V	5	5	2	—	—	—	1 1	1	—	—	—	—	—	—	—	—	—	—	—
	1.VII	1 1	5	4	—	—	—	2	3	1	—	—	—	—	—	—	—	—	—	—
	24.VII	1 1	5	4	—	—	—	2	3	1	—	—	—	—	—	—	—	—	—	—
	16.VIII	2	5	3 1	—	—	—	1 1	2	1	—	—	—	—	—	—	—	—	—	—
	25.IX	2	5	3	1	—	—	1 1	3	2 1	—	—	—	—	—	—	—	—	—	—
Leaves	23.X	3	5	3	1	—	—	2	3	—	—	—	—	—	—	—	—	—	—	—
	22.V	5	2	3	1 1	—	—	1 1	2 1	—	—	—	—	—	—	—	—	—	—	—
	1.VII	2	3	4	1	—	—	1	2 1	—	—	—	—	—	—	—	—	—	—	—
	24.VII	1 1	3 1	4	1	—	—	1 1	1	—	—	—	—	—	—	—	—	—	—	—
	16.VIII	2	4	4	1	—	—	—	3	2	—	—	—	—	—	—	—	—	—	—
	25.IX	2	2	3	1	—	—	1	2	1	—	—	—	—	—	—	—	—	—	—
	23.X	2	3	3	1	—	—	1	2	1	—	—	—	—	—	—	—	—	—	—

Conventional values:

a) for amino acids:

5 — very large amount
 4 — large amount
 3 — average amount
 2 — small amount
 1 — very small amount
 $\frac{1}{2}$ — trace amount; — — — unfound

b) for unidentified compounds:

+++ — intense spot
 ++ — medium-intense spot
 + — pale spot

* — The spot of tingianine overlaps on the chromatograms partly the glycine spot (Przybylska 1962 b). All spots evaluated under „Tingianine + glycine” gave colour reaction with ninhydrin typical for tingianine.

** — Aspartic and glutamic acids are given as the sums of these amino acids and corresponding amides.

*** — The unidentified compound t gives a specific colour reaction with ninhydrin like tingianine.

Remark: $\frac{1}{2}$ placed after a whole number denotes an intermediate value.
 The same explanations concern Tables: 9, 10 and 12.

spot was at first brownish-violet but soon turned to purplish-violet. On the electropherogram, the substance X was situated at the position of neutral amino acids. Besides spot X, chromatograms of all root samples were found to reveal a pale, unidentified spot t (Table 8; Phot. 1a), seen already on chromatograms of etiolated plants (Przybylska 1962 b). On chromatograms of samples taken at later phases of development a very pale, unidentified spot X₁ was observed (Phot. 1a).

Throughout the entire period of plant development aspartic acid and tingitanine are the main free amino acids of roots. Glutamic acid remains on a relatively lower level. In the first-taken samples alanine appears in small amount which however rises to a considerable level in later samples. γ -Amino butyric acid follows a similar pattern, at a lower level however. Serine appears in small amounts and its content remains on an equal level. Methionine and/or valine as well as proline also occur in small or trace amounts and do not show marked changes.

Root nodules. In the root nodules aspartic acid is the main free amino acid; glutamic acid, cystine, alanine and γ -aminobutyric acid are found also in great quantities. In the latest terms of sampling the relative amounts of tingitanine are also rather large. All samples contained serine, methionine and/or valine and the unidentified substance X (Table 8).

Stems. In stems, aspartic acid is the main free amino acid (Table 8). Tingitanine appears in large amounts, but only in stems of three-week-old plants. Further samples contained much less of this amino acid which remained on the low level for a long period. Only towards maturity the level of tingitanine was found to be higher. The proportion of homoserine follows the same pattern though on a lower level. Different changes are found in glutamic acid, alanine, γ -aminobutyric acid and serine — listed in the order of diminishing amounts. In young stems concentrations of these amino acids are relatively low; they rise as the plants develop. Following amino acids occur in stems in small or trace amounts: proline, cystine, methionine and/or valine, isoleucine, leucine, phenylalanine and lysine.

Stems are characterized by the appearance of rather large amounts of the unidentified substance X, found also in roots and root nodules.

Chromatographic analyses of main stems, defoliated side-shoots and peduncles sampled in 1962 revealed in all these organs the following free amino acids: aspartic acid, glutamic acid, homoserine, alanine, serine, γ -aminobutyric acid, tingitanine, lysine, methionine and/or valine, isoleucine, leucine, phenylalanine and the unidentified substance corresponding to the spot X. Relative amounts of the various amino acids are different in each of the organs (Table 9). In the main stem, as in 1961, aspartic acid is the main amino acid. In the defoliated

Phot. 1a-d. Chromatograms of free amino acids of different organs of *Lathyrus tingitanus*: a) roots (sampled 25.IX.1961), b) very young pods—seeds not visible yet (sampled 7.VII.1961), c) seeds (sampled 13.VII.1961), d) seeds (sampled 30.X.1961).

A — starting point. Particular spots correspond to the following amino acids: 1 — tingitanine + glycine, 2 — aspartic acid, 3 — glutamic acid, 4 — lysine, 5 — arginine, 6 — histidine, 7 — serine, 8 — alanine, 9 — γ -aminobutyric acid, 10 — methionine and/or valine, 11 — threonine, 12 — isoleucine, 13 — leucine, 14 — phenylalanine, 15 — homoserine, X, X₁, t — unidentified compounds.

Table 9

A rough estimate of free amino acids in stems, defoliated side-shoots, peduncles and leaves of different ages of *Lathyrus tingitanus* (18 July, 1962)

Analysed organs	Tingitanine* + glycine*	Aspartic acid**	Glutamic acid**	Lysine	Arginine	Histidine	Serine	Alanine	γ -Amino butyric acid	Proline	Tyrosine	Methionine and or valine	Threonine	Isoleucine	Leucine	Phenylalanine	Cystine	Homoserine	Unidentified compound	X
Stems	1 1/2	5	4	1	—	—	1 1/2	2 1/2	1	1 1/2	—	1 1/2	1 1/2	1	1	1	—	3	—	++
Defoliated side-shoots	1	3 1/2	3 1/2	1	—	—	1 1/2	2 1/2	2	1 1/2	—	1 1/2	—	1 1/2	1 1/2	1 1/2	—	3 1/2	—	++
Peduncles	2	3	3 1/2	1	1 1/2	—	1 1/2	2 1/2	2	1 1/2	—	1 1/2	—	1 1/2	1 1/2	1 1/2	—	4 1/2	—	++
Upper leaves	2	3 1/2	3 1/2	1	1 1/2	—	1 1/2	2 1/2	2	1 1/2	—	1 1/2	—	1 1/2	1 1/2	1 1/2	—	3	—	++
Mid-stem leaves	1 1/2	3	4	1	1	—	1 1/2	3 1/2	2 1/2	1 1/2	—	—	—	1	1	1	—	2	—	++
Bottom leaves	—	3	3	1	1 1/2	—	2	3 1/2	2	1 1/2	—	2	—	1	1	1	—	1 1/2	—	++

All explanations are given in Table 8.

side-shoots, aspartic acid, glutamic acid, homoserine and the substance X remain on a similar level. In peduncles, homoserine and the unidentified substance X are the main amino acids.

Leaves. Within leaves of three-week-old plants tingitanine is decidedly the main free amino acid, homoserine appears in fairly large amounts; while glutamic acid in lesser quantities (Table 8). Aspartic acid, alanine, methionine and/or valine, threonine, isoleucine, leucine, phenylalanine, proline, serine, lysine, arginine and γ -aminobutyric acid occur in small or trace amounts. During the more advanced phases of plant development the level of tingitanine evidently drops, while the contents of aspartic acid, glutamic acid, alanine and γ -aminobutyric acid rise. The level of homoserine in leaves remains more or less equal throughout the plant development; only the last two samplings reveal a slight rise. At that time homoserine is the main free amino acid of leaves, the levels of aspartic acid, glutamic acid and alanine having become lower.

As in stems and roots, the unidentified substance X appears also in leaves.

Analyses of differently aged leaves provide more information than analyses of all main-stem leaves. Table 9 shows a marked difference to occur in their amino acid composition, mainly in the level of homoserine. In youngest leaves homoserine is one of the main amino acids; in the eldest leaves it only appears in trace amounts; medium-aged leaves show intermediate amounts of this amino acid.

Flowers. Homoserine is the main free amino acid of flowers. Aspartic acid, alanine and proline occur in mean quantities; while glutamic acid, serine, cystine, threonine, lysine, methionine and/or valine and the unidentified substance X appear in the order of diminishing amounts (Table 10).

Seedless pods. First samples of "seedless pods" represented very young pods with as yet invisible seeds. In this case too, homoserine was the main free amino acid (Table 10; Phot. 1b). Aspartic and glutamic acids appeared in lesser amounts. Tingitanine, alanine, serine, methionine and/or valine, threonine, lysine, γ -aminobutyric acid and proline were on a still lower level. A quantitative analysis of free amino acids of young pods, performed by means of an automatic fraction analyser, proved that homoserine made up 50% of the total amount of α -amino nitrogen, and its lactone (of a secondary origin) — over 8% (Table 11).

All through the pod development relative amounts of individual amino acids remain more or less the same, with the exception of homo-

Table 10

A rough estimate of free amino acids in flowers and seedless pods of *Lathyrus tingitanus* at various phases of pod development (1961)

Analysed organs	Date of sampling	Tingitanine* + glycine*	Aspartic acid**	Glutamic acid**	Lysine	Arginine	Histidine	Serine	Alanine	γ -Amino butyric acid	Proline	Tyrosine	Methionine and/or valine	Threonine	Isoleucine	Leucine	Phenyla- lanine	Cystine	Homoserine	Unidentified compounds	
																				x	x ¹
Flowers	1.VII	—	3	2	1	—	—	1 ^{1/2}	3	—	3	—	1 ^{1/2}	1	—	—	—	1 ^{1/2}	5	+	—
Pods ¹	7.VII	2 ^{1/2}	4	3	1	—	—	1 ^{1/2}	1 ^{1/2}	1 ^{1/2}	1 ^{1/2}	—	1 ^{1/2}	1	—	—	—	—	5	—	—
	13.VII	1 ^{1/2}	3 ^{1/2}	3	—	1	—	1 ^{1/2}	1 ^{1/2}	—	1 ^{1/2}	—	1	1 ^{1/2}	—	—	—	—	4	+	—
	24.VII	2	3 ^{1/2}	3	—	1 ^{1/2}	—	1	2 ^{1/2}	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	3	+	—
	1.VIII	2	3 ^{1/2}	3	—	1	—	1	2	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	3	+	—
	8.VIII	2	3 ^{1/2}	3 ^{1/2}	—	2	—	1	2 ^{1/2}	—	1 ^{1/2}	—	1	1 ^{1/2}	—	—	—	—	2 ^{1/2}	+	—
	16.VIII	2	3 ^{1/2}	3 ^{1/2}	—	1	—	1 ^{1/2}	2 ^{1/2}	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	2 ^{1/2}	+	—
	23.VIII	2	4	3 ^{1/2}	—	1 ^{1/2}	—	1	2	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	2	+	—
	2.IX	2	4	3 ^{1/2}	—	1	—	1 ^{1/2}	1 ^{1/2}	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1 ^{1/2}	+	—
	18.IX	2	4	3 ^{1/2}	—	1	—	1	1 ^{1/2}	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1	+	—
	25.IX	2	4	3 ^{1/2}	—	1	—	1	1	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1	+	—
	2.X	1 ^{1/2}	3 ^{1/2}	3 ^{1/2}	—	1	—	1	1	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1	+	—
	9.X	1 ^{1/2}	3 ^{1/2}	3 ^{1/2}	—	1	—	1	1	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1 ^{1/2}	+	—
	16.X	1 ^{1/2}	3 ^{1/2}	3 ^{1/2}	—	1	—	1	1	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1 ^{1/2}	+	—
	23.X	1 ^{1/2}	4	2	—	1	—	1	1	—	1 ^{1/2}	—	1 ^{1/2}	—	—	—	—	—	1 ^{1/2}	+	—

¹ Very young pods — seeds not visible yet.

All other explanations are given in Table 8.

serine, the content of which gradually drops to a very low level. As regards the unidentified substances, spots X and X₁ appear on chromatograms of seedless pods.

S e e d s. Definite changes in levels of alanine, homoserine and tingitanine are observed during seed development (Table 12). For a long

Table 11

Percentual share of α -amino nitrogen of particular free amino acids in total α -amino nitrogen of young pods of *Lathyrus tingitanus* (7 July, 1961)

Amino acid	α -amino-N in per cent of total α -amino-N
Tingitanine	not determined
Aspartic acid	12.38
Glutamic acid	4.54
Lysine	0.66
Arginine	traces
Histidine	0.44
Serine	2.35
Alanine	1.52
γ -Aminobutyric acid	not determined
Proline	traces
Tyrosine	—
Methionine	traces
Valine	1.88
Threonine	1.25
Isoleucine	0.70
Leucine	0.43
Phenylalanine	traces
Cystine	traces
Homoserine	50.05
Homoserine lactone	8.63

period alanine is the main free amino acid (Phot. 1c), towards maturity however its level rapidly drops (Phot. 1d). In quite young seeds (first sampling) homoserine is on a rather high level, soon however its relative amount falls and it is not found in samples from the four latest terms. Changes in the level of tingitanine are reversed: its level rises continuously throughout the entire period of seed development.

Relative amounts of the remaining amino acids do not show conspicuous changes. In mature seeds tingitanine is the main free amino acid; aspartic and glutamic acid occur in rather large amounts, arginine, lysine, alanine, methionine and/or valine, threonine, isoleucine, leucine and proline appear in small or trace amounts (Phot. 1d). Seed extracts also contained the unidentified substances corresponding to the spots X, X₁ and t.

Table 12

A rough estimate of free amino acids in seeds of *Lathyrus tingitanus* at various phases of their development (1961)

Date of sampling	Tingitanine + glycine *	Aspartic acid **	Glutamic acid **	Lysine	Arginine	Histidine	Serine	Alanine	γ -Amino- butyric acid	Proline	Tyrosine	Methionine and or valine	Threonine	Isoleucine	Leucine	Phenyla- lanine	Cystine	Homoserine	Unidentified compounds		
																			$\times$	$\times 1$	t^{***}
13.VII	2 1/2	3	3	2	1 1/2	—	2	5	1	2	—	1 1/2	1	1	1	—	—	3 1/2	+	—	—
24.VII	2	3	3	2	2	—	2	4 1/2	1	1	—	1	1 1/2	1 1/2	1 1/2	—	—	2 1/2	—	—	—
1.VIII	2	3	3	2	2	—	2	4 1/2	—	1 1/2	—	1	1 1/2	1 1/2	1 1/2	—	—	2 1/2	—	—	—
8.VIII	2	3	3	2	2	—	2	5	—	1 1/2	—	1	1 1/2	1 1/2	1 1/2	—	—	2	—	—	—
16.VIII	2 1/2	3	3	1 1/2	2	—	2	4	—	1 1/2	—	1 1/2	1	1 1/2	1 1/2	—	—	1	—	—	—
23.VIII	2 1/2	3	3	1 1/2	2 1/2	—	2	4 1/2	—	1 1/2	—	2	1	1 1/2	1 1/2	—	—	1	—	—	—
2.IX	2 1/2	3	3	1 1/2	2	—	1	4 1/2	—	1 1/2	—	1 1/2	1 1/2	1 1/2	1 1/2	—	—	1 1/2	+	—	—
18.IX	3 1/2	3 1/2	3 1/2	—	2	—	1	3	—	1 1/2	—	1 1/2	1 1/2	1 1/2	1 1/2	—	—	1 1/2	+	—	—
25.IX	3 1/2	3	3	—	2	—	1	3	—	1 1/2	—	1 1/2	1 1/2	1 1/2	1 1/2	—	—	1 1/2	+	—	—
2.X	4	3	3	—	2	—	—	2 1/2	—	1 1/2	—	1	1 1/2	1 1/2	1 1/2	—	—	—	+	—	—
9.X	4 1/2	3	3	—	2	—	—	2 1/2	—	1 1/2	—	1	1 1/2	1 1/2	1 1/2	—	1	—	+	—	—
16.X	4 1/2	3	3	—	2	—	—	2 1/2	—	1 1/2	—	1	1 1/2	1 1/2	1 1/2	—	1	—	+	—	—
23.X	5	2 1/2	3 1/2	—	2	—	—	2	—	1 1/2	—	1 1/2	1 1/2	1 1/2	1 1/2	—	1	—	+	—	—
30.X	5	2	3	1	2	—	—	1	—	1 1/2	—	1 1/2	1 1/2	1 1/2	1 1/2	—	—	—	+	—	—

All explanations are given in Table 8.

Free amino acids in plants supplied with ammonium nitrate

It was found that plants additionally fertilized with ammonium nitrate carried higher levels of most of the amino acids than check plants. The situation was most evident in a combination supplied with 0.5% solution of ammonium nitrate. In some of the organs certain amino acids amounted to exceptionally high levels. E.g. in roots, extraordinary large amounts of tingitanine, asparagine and arginine were found; in stems, asparagine and arginine were on a much higher level than in the check plants; in leaves, tingitanine and asparagine greatly exceeded the amounts found in unsupplied plants.

Hydrolyzates of seed, root and stem crude proteins

Chromatographic and electrophoretic analyses of products of enzyme digestion, as well as of acid and basic hydrolysis of seed, root and stem crude protein failed to reveal the presence of tingitanine or homoserine. Two-dimensional chromatograms of enzyme-digestion products carried, beside protein amino acids, spots corresponding presumably to undecomposed peptides.

DISCUSSION OF RESULTS

The present work was intended to establish the role of the new free amino acid tingitanine in nitrogen metabolism of normally developing plants of *Lathyrus tingitanus*. According to the analyses of hydrolyzates of seed, root and stem proteins, tingitanine is not a component of protein. What function does it then perform? As mentioned before, the accumulation of tingitanine in seeds and the high proportion of nitrogen in tingitanine itself suggest it is a nitrogen storing substance. Investigations of etiolated plants (Przybylska 1962a, b) enable to assume that free ammonia fixation may be attributed to tingitanine. Studies of the changes in the level of tingitanine nitrogen in different organs of *Lathyrus tingitanus* throughout the entire growing season, on the basis of changes in dry matter and total nitrogen contents, and in comparison with α -amino and amido nitrogen, were aimed at obtaining more information, both on the role and on the site of synthesis of this compound. It was also considered purposeful to compare the changes of tingitanine with other free amino acids.

In comparing the levels of tingitanine in roots, stems and leaves of *Lathyrus tingitanus* a remarkably high content in the roots is noticeable. Solely in 3-week-old plants high levels of tingitanine nitrogen were found also in leaves.

A gradual increase in the content of tingitanine nitrogen in roots,

from the beginning of July to the end of September when it amounts to 14 per cent of total nitrogen, is a very peculiar feature. Total α -amino nitrogen constitutes then under 9 per cent, and amido nitrogen hardly amounts to 5 per cent of total nitrogen. At the same time the level of tingitanine nitrogen in stems and leaves is found to be much lower than the level of total α -amino nitrogen. Only towards the end of the growing season a relative increase in the amount of tingitanine nitrogen in stems, accompanied by a drop in the roots is revealed. These data suggest the following: the relatively high level of tingitanine in roots and leaves of 3-week-old plants is probably due to the inflow of this amino acid from decomposing cotyledons; it is also possible however that a synthesis of tingitanine in roots had already commenced. During later plant development the high level of tingitanine in roots may be accounted for by a synthesis *in situ*. Ammonia absorbed and assimilated by the roots might become incorporated into tingitanine. Tingitanine formed in roots is probably transported upwards to the leaves where it might be a nitrogen source for protein synthesis. The relatively low level of tingitanine nitrogen in stems does not suggest however that tingitanine is of great importance in the transport of nitrogen. A partial at least therefore transformation of tingitanine in roots already, or perhaps in stems, needs to be assumed. Tingitanine nitrogen in leaves, and likewise in stems, is on a low level in comparison with the other forms of soluble nitrogen. This may be either due to the limited supply of tingitanine to leaves — e.g. in case of an earlier conversion of tingitanine into a different compound (or compounds) — or to a dynamic equilibrium between tingitanine inflow and consumption. The comparatively higher content of tingitanine nitrogen in the youngest leaves, to which the supply is most abundant, is a characteristic feature.

The pattern of changes in the level of tingitanine nitrogen in seedless pods and seeds is a feature of great interest. The seedless pods i.e. pericarps are a temporary store of soluble nitrogen compounds transported to the seeds. At very early phase of pod development, when seeds are not yet visible, the pericarps accumulate reserves of soluble nitrogen to pass at the right time to the seeds. For this reason the composition of soluble nitrogen in seedless pods is a feature of interest — especially during very early phase of pod development — and throws light upon forms under which nitrogen is supplied to the seeds. Experimental data show that tingitanine nitrogen in seedless pods remains throughout the entire period of pod development on a low level, much lower than α -amino nitrogen, and even than amido nitrogen, so that tingitanine cannot be the main form of nitrogen supply for the metabolic processes of seeds.

Studies on changes in the tested forms of soluble nitrogen in developing seeds reveal a relatively equal level of tingitanine nitrogen

in relation to the dry matter and total nitrogen. Relative amounts of α -amino and amido nitrogen definitely drop throughout the development of seeds, thus indicating their contribution to protein synthesis. A certain rise in the level of α -amino nitrogen at the early phase of seed development should be interpreted by the predominance of the supply of free amino acids over their consumption in protein synthesis. Changes in the level of tingitanine nitrogen suggest a passive role of this amino acid in maturing seeds: It seems to be transported to seeds from other organs and simply stored there (in this place attention must be drawn to the slightly higher level of tingitanine nitrogen in peduncles as compared with stems and defoliated side shoots). The other supplied forms of soluble nitrogen are used for the seed protein synthesis and so their level falls, tingitanine is not involved in these processes and therefore its content in seeds rises and it becomes the main free amino acid in ripe seeds.

In view of the fact that tingitanine is not the main form of nitrogen transport for metabolic processes, which compound performs this role? An answer to this question is provided to a certain degree by the analyses of free amino acids discussed below.

In roots, beside tingitanine highly concentrated throughout the entire period, aspartic acid + asparagine appear in large quantities and glutamic acid + glutamine in slightly smaller amounts. Probably these compounds are, like tingitanine, the main forms of ammonia assimilation. γ -Aminobutyric acid appearing in rather large quantities may originate from the decarboxylation of glutamic acid. The different arrangement of free amino acids in root nodules is an interesting feature: aspartic acid + asparagine are the main compounds, while tingitanine and other amino acids appear in much lesser amounts. In root nodules of *Pisum sativum* high concentrations of asparagine were likewise found (Miettinen 1955) so it is possible that aspartic acid + asparagine constitute in these species the primary products of nitrogen fixation. Relatively high levels of γ -aminobutyric acid in root nodules may originate from a decarboxylation of glutamic acid. In nodules, high content of cystine, higher than in other analysed organs, is also a peculiar feature.

In stems, aspartic acid + asparagine is the main free amino acid throughout the entire period of development, and this suggests an important role in transporting nitrogen for it. Tingitanine appears in fairly large amounts only in stems of very young plants, which may be accounted for by the inflow of reserves stored in cotyledons; later it remains on a rather low level to rise slightly only towards maturity. The latter increase in amount may result from a passage of this amino acid from roots (see tingitanine losses in roots) accompanied by a check in its outflow to other organs (end of growing season).

In stems of young plants a relatively high level of homoserine must be pointed out, in spite of its future drop. Analyses of stems, defoliated side-shoots and peduncles, sampled at the same time in 1962, showed that in defoliated side-shoots, and above all in peduncles, homoserine constitutes a larger proportion in the total free amino acid pool. In the peduncles, which are the route of nitrogen supply for flowers, *eo ipso* for seeds, homoserine is the main free amino acid. The unidentified substance X comes next in amount (in judging by the spot intensity). Homoserine therefore must play a large part in supplying nitrogenous compounds for seed protein synthesis. This assumption is confirmed by a high concentration of homoserine in flowers and young pods. Quantitative analyses of free amino acids in young pods with as yet invisible seeds found homoserine to be their principal free amino acid, occurring in much larger quantities than all the other amino acids, even than aspartic acid + asparagine. Homoserine soon drops in the pods, but this may be due to its outflow to the seeds. Changing levels of homoserine in seeds seem also to indicate its involvement in the synthesis of seed protein. In contrast with tingitanine, the relative amount of which in seeds (in relation to other amino acids) remains at first on an equal level to rise subsequently, homoserine appears in rather large amounts at the outset of seed development but soon its concentration rapidly falls; towards maturity no traces of homoserine were to be found.

It is very possible that homoserine plays an indirect role not only in the protein synthesis of seeds, but also of leaves. Analyses of leaves differently aged revealed that in young leaves homoserine and the unidentified compound X are the principal substances giving a positive reaction with ninhydrin.

The importance of homoserine in amino acid metabolism of various organisms is a well known phenomenon nowadays. It was found to be a common precursor of methionine and threonine in *Neurospora crassa* (Teas, Horowitz, Filing 1948). Enzymes involved in the transformation of aspartic acid into threonine through homoserine were investigated in *Escherichia coli* and yeast (Cohen, Hirsch 1953, 1954; Hirsch, Cohen 1953; Nisman, Cohen, Wiesendanger, Hirsch 1954; Black, Gray 1953; Black, Wright 1954, 1955).

Homoserine is a common amino acid in seed plants, generally however it appears in small amounts (Fowden, Bryant 1958). Until recently it was only known in free form, since then Vähätalo and Virtanen (1957) revealed it in a bound form in berries and leaves of *Vaccinium vitis idaea* and in berries of *Oxycoccus quadripetalus*.

Interesting observations were made on free homoserine in *Pisum sativum*. Virtanen *et al.* (1953) found that homoserine arises rapidly during seed germination in *Pisum sativum*, so that already after 72 hrs

it becomes the main free amino acid in the young plants. Similar observations were made by Miettinen (1955): in 12-day-old plants of *Pisum sativum* homoserine was the main free amino acid, later however its level dropped considerably. In analyzing free amino acids of the pericarps and seeds of *Pisum sativum*, Hyde (1954) found homoserine to be one of the principal amino acids; here too its level lowered as the plants developed. In 1958, Grobbelaar and Steward (1958) detected O-acetylhomoserine in various organs of *Pisum sativum* — exceptionally large quantities appeared in the pericarps.

In considering on grounds of the above given findings results obtained in this work a certain analogy must be noticed. In *Lathyrus*, as in *Pisum sativum*, homoserine fails to accumulate in ripe seeds. It appears in large quantities during the vegetative development of young plants to take part in the transport of nitrogen to developing seeds. During the period of seed development and maturation its lowering level indicates transformation which it obviously undergoes. It is either possible that homoserine is a precursor of other free amino acids which accumulate nitrogen in seeds, or that it becomes indirectly involved in the protein synthesis.

It is interesting that high levels of homoserine were recently found to occur in a few species of the genus *Lathyrus* which accumulate in ripe seeds various atypical free amino acids. As shown, homoserine is the principal free amino acid in flowers, young pericarps and young, immature seeds of *Lathyrus sativus*, *L. ochrus*, *L. silvester* (Przybylska 1963) and *L. latifolius**. None of these species contained homoserine in ripe seeds: in the two first mentioned species homoarginine is the principal free amino acid of ripe seeds, while in the two last mentioned — α , γ -diaminobutyric acid. On grounds of these data it seems likely that homoserine is the key substance in the amino acid metabolism of various species of *Lathyrus*. Possibly it is a common precursor of unusual amino acids which appear within the genus *Lathyrus*, or else a common product of their decomposition.

The relations of homoserine with characteristic nonprotein amino acids are not limited to the genus *Lathyrus*. According to Johnstone (1956) canavanine, accumulated in seeds of many legumes belonging to different tribes (Przybylska, Hurich 1960; Tschiersch 1961) decomposes during the germination of *Canavalia ensiformis* supposedly through homoserine. Homoserine is also the assumed precursor of azetidine-2-carboxylic acid recently detected in the family *Liliaceae* (Fowden, Bryant 1958).

As regards tingitanine it is interesting to compare its role with that of other nonprotein amino acids in plants.

* J. Przybylska — unpublished results.

Roles of the following new free amino acids were closely investigated: that of γ -methyleneglutamic compounds in *Arachis hypogea* (Fowden 1954), of citruline in *Corylus avellana* (Reuter 1957a, 1957b) and of canavanine in nitrogen metabolism of certain legumes (Johnstone 1956; Tschiersch 1959). From the standpoint of the role in plants, canavanine seems to be closest to tingitanine. A comparison of the roles of these two compounds is rather difficult however because of the trials not being comparable. Tingitanine was tested in annual herbs, while canavanine mainly in woody perennials. For this reason the comparability of results is limited. Tingitanine, and likewise canavanine, accumulates in seeds and becomes the main free amino acid. During germination of seeds of *Canavalia ensiformis* canavanine decomposes, presumably through homoserine (Johnstone 1956), and serves as a source of nitrogen for young plants. Tingitanine is assumed to perform the same function, and by analogy it might be suggested that homoserine is here the product of decomposition; beside tingitanine, homoserine is the most characteristic free amino acid of *Lathyrus tingitanus*.

In *Caragana arborescens*, in wintertime, an exceptionally high level of canavanine was found in roots (Tschiersch 1959) — this too suggests an analogy with tingitanine. Nevertheless the latter must be considered incongruent. In woody plants roots are largely storing organs and an accumulation of canavanine within them during the resting stage should be interpreted from this standpoint.

Changes in levels of both amino acids in question in maturing seeds are on the other hand comparable and very similar. Canavanine in *Colutea arborescens* (Tschiersch 1959) and tingitanine in *Lathyrus tingitanus* — both rise gradually in parallel to dry matter as the seeds develop, thus proving their metabolic inactivity.

Both these amino acids fix free, toxic ammonia. As regards tingitanine, trials with etiolated plants and those with feeding plants with ammonium nitrate proved this function; as regards canavanine, analyses of rooted leaves of *Canavalia* proved the same. During the relatively slow rate of growth of rooted leaves the supplied nitrogen was stored in the form of canavanine, which accumulated in roots and leaf mid-ribs (Tschiersch 1959).

Studies on the metabolism of citruline in *Corylus avellana* (Reuter 1957a, 1957b), and on γ -methyleneglutamic compounds in *Arachis hypogea* (Fowden 1954) suggest an essential difference in respect to tingitanine. Both citruline and γ -methyleneglutamine, beside the storage function they share in common with tingitanine, are the main, perhaps even the sole forms of nitrogen transport in plants. Investigations however into *Tulipa gesneriana* (Fowden, Steward 1957) show that functions of individual substances may not be generalized. γ -Methyleneglutamic compounds do not play an important part in the development.

of *Tulipa gesneriana* and no interrelationship between them and protein metabolism is found. Exclusively the role or function of a given compound in a given plant can be considered.

Summarizing the results of studies carried out so far on the role of tingitanine in nitrogen metabolism of *Lathyrus tingitanus* following conclusions can be drawn:

1. Tingitanine accumulated in seeds during the processes of germination and of development of the young plants is transported from cotyledons to seedling axes; evidence comes from the disappearance of tingitanine in cotyledons and its relatively high level in all the tested organs (roots, stems, leaves) of 3-week-old plants.

2. At later phases of plant development, in view of an exceptionally high level of tingitanine, roots are the hypothetical site where tingitanine is synthesized.

3. Relatively low proportion of tingitanine in stems — with the exception of very young, 3-week-old plants — disproves an essential role of this compound in the transport of nitrogen for metabolic processes. A partial decomposition of tingitanine is assumed, whether in roots or stems, into products subsequently transferred to developing organs.

4. Relatively low levels of tingitanine in seedless pods throughout the entire period of development and maturation of pods show this compound not to play an important part in transporting nitrogen for the protein synthesis of developing seeds.

5. Changing levels of α -amino nitrogen, amido nitrogen and tingitanine nitrogen in developing seeds reveal the particular role of the new amino acid in respect to the common free amino acids and their amides. The role of tingitanine in seeds seems to be limited to nitrogen storage.

6. On the basis of results of free amino acid analyses the important role of homoserine in nitrogen metabolism of *Lathyrus tingitanus* was discovered: this particular amino acid seems to contribute to the transport of nitrogen to developing organs, in main to seeds, where presumably it plays an indirect role in protein synthesis. The hypothetical interrelationship between homoserine and tingitanine needs to be re-examined.

Added in proof: Further studies on the genus *Lathyrus* showed that at least a part of the free homoserine found in the extracts arises from a labile compound in course of analytical procedure.

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REFERENCES

- Bell E. A., 1961, *Biochim. Biophys. Acta* 47:602.
 Bell E. A., Foster R. G., 1962, *Nature* 194:91.
 Black S., Gray N. M., 1953, *J. Am. Chem. Soc.* 75:2271, 5766.
 Black S., Wright N. G., 1954, *Federation Proc.* 13:189.
 Black S., Wright N. G., 1955, *J. Biol. Chem.* 213:27, 39, 51.
 Block R. J., Durrum E. L., Zweig G., 1958, *A. Manual of Paper Chromatography and Paper Electrophoresis*, New York.
 Buchanan D. L., 1957, *Anal. Chem.* 29:1877.
 Cohen G. N., Hirsch M., 1953, *Compt. rend. Acad.* 236:1302.
 Cohen G. N., Hirsch M., 1954, *J. Bact.* 67:182.
 Fowden L., 1954, *Ann. Bot.* 18:417.
 Fowden L., Bryant M., 1958, *Biochem. J.* 70:626.
 Fowden L., Steward F. C., 1957, *Ann. Bot.* 21:69.
 Greenstein J. P., Winitz M., 1961, *Chemistry of the Amino Acids*, New York.
 Grobbelaar N., Steward F. S., 1958, *Nature* 182:1358.
 Hirsch M., Cohen G. N., 1953, *Compt. rend. Acad.* 236:2338.
 Hyde T. G., 1954, *Proc. Roy. Soc. Edinburgh*, 65. B:229.
 Johnstone J. H., 1956, *Biochem. J.* 64:21.
 Miettinen J. K., 1955, *Biochemistry of Nitrogen A.II.60*. Suomalainen, Tiedeakatemia, Helsinki, p. 620.
 Nisman B., Cohen G. N., Wiesendanger S. B., Hirsch M., 1954, *Compt. rend. Acad.* 238:1342.
 Nowacki E., Przybylska J., 1961, *Bull. Acad. Polon. Sci., Ser. sci. biol.* 9:279.
 Przybylska J., 1962a, *Bull. Acad. Polon. Sci., Ser. sci. biol.* 10:157.
 Przybylska J., 1962b, *Acta Soc. Bot. Polon.* 31:471.
 Przybylska J., 1963, Homoserine in different species of *Lathyrus*, *Bull. Acad. Polon. Sci., Ser. sci. biol.* (in press).
 Przybylska J., Hurich J., 1960, *Bull. Acad. Polon. Sci., Ser. sci. biol.* 8:505.
 Przybylska J., Kociałkowski Z., Wiewiórowski M., 1958, *Polish Agr. Annual.* 79-A-1:453.
 Reuter G., 1957a, *Naturwiss.*, 44:45.
 Reuter G., 1957b, *Flora.* 145:326.
 Smith I., 1960, *Chromatographic and Electrophoretic Techniques*. London.
 Spackman D. H., Stein W. H., Moore S., 1958, *Anal. Chem.* 30:1190.
 Teas H. J., Horowitz N. H., Filing M., 1948, *J. Biol. Chem.* 172:651.
 Tschiersch B., 1959, *Flora* 147:405.
 Tschiersch B., 1961, *Flora* 150:87.
 Vähätalo M., Virtanen A. I., 1957, *Acta Chem. Scand.* 11:747.
 Virtanen A. I., Berg A. M., Kari S., 1953, *Acta Chem. Scand.* 7:1423.
 Wagner I., 1958, *Naturwiss.*, 45:110.
 Wolfe N., 1957, *Biochim. Biophys. Acta.* 23:186.

Rola tingitaniny w metabolizmie azotowym Lathyrus tingitanus

Streszczenie

Celem pracy było zbadanie, jaką rolę spełnia nowy wolny aminokwas — tingitamina w metabolizmie azotowym *Lathyrus tingitanus*.

Przedmiotem badań były rośliny rosnące w warunkach polowych w sezonie

wegetacyjnym 1961 r. Osobno analizowano: liścienie (3-tygodniowych roślin), korzenie (w sześciu terminach), brodawki korzeniowe (w trzech terminach), łodygi (w sześciu terminach), liście (w sześciu terminach), kwiaty, strączyny (w czternastu terminach) i nasiona (w czternastu terminach).

Dodatkowo analizowano niektóre części roślin z 1962 r., a mianowicie: główne (najdłuższe) łodygi, pędy boczne (bez liści), szypułki kwiatowe oraz liście różnego wieku. Próby te pobrano do analiz w okresie pełni kwitnienia roślin.

Celem wykazania, w jakie wolne aminokwasy wbudowuje się wprowadzany do roślin *Lathyrus tingitanus* azot związków nieorganicznych, założono doświadczenie doniczkowe, obejmujące kombinacje dokarmiane azotem amonu (w dwóch dawkach). Osobno analizowano korzenie, łodygi i liście.

We wszystkich próbach pobranych z roślin z uprawy polowej oznaczono zawartość suchej masy, azotu ogólnego, azotu α -aminowego, azotu amidowego i azotu tingitaniny. Przeprowadzono również jakościową analizę wolnych aminokwasów przy pomocy dwukierunkowej chromatografii bibułowej.

Dla prób pobranych z doświadczenia doniczkowego przeprowadzono jedynie jakościową analizę wolnych aminokwasów.

Celem przekonania się, czy tingitanina lub homoseryna nie są składnikami białka *Lathyrus tingitanus*, wyodrębniono frakcję surowego białka korzeni, łodyg oraz dojrzałych nasion, a następnie analizowano produkty jego kwaśnej i zasadowej hydrolizy oraz trawienia enzymatycznego.

W podsumowaniu przeprowadzonych badań wyciągnięto następujące wnioski:

1. Nagromadzona w nasionach tingitanina w trakcie kiełkowania nasion i rozwoju młodych roślin zostaje odprowadzona z liścieni do osi siewek, o czym świadczy zanik tingitaniny w liścieniach i jej wysoka stosunkowo zawartość we wszystkich badanych organach (korzenie, łodygi, liście) młodej 3-tygodniowej rośliny (Tab. 2, Diagram 1, 2, 3).

2. W późniejszym okresie rozwoju roślin przypuszczalnym miejscem syntezy tingitaniny jest korzeń, w którym stwierdza się szczególnie wysoką zawartość tego aminokwasu (Diagram 1, Fot. 1).

3. Stosunkowo niska zawartość tingitaniny w łodygach (Diagram 2) — z wyjątkiem młodych łodyg, 3-tygodniowych roślin — wskazuje, że związek ten nie spełnia zasadniczej roli w transporcie azotu dla celów metabolicznych. Wsuwa się przypuszczenie, że tingitanina w korzeniach, bądź w łodygach, ulega częściowemu rozkładowi, którego produkty doprowadzane są do rosnących części rośliny.

4. Stosunkowo niska zawartość tingitaniny w strączynach w ciągu całego okresu rozwoju i dojrzewania strąków (Diagram 4) świadczy, że związek ten nie odgrywa zasadniczej roli w doprowadzaniu azotu dla syntezy białka rozwijających się nasion.

5. Kształtowanie się poziomu azotu α -aminowego, azotu amidowego i azotu tingitaniny w rozwijających się nasionach (Diagram 5) wskazuje na odrębność funkcji nowego aminokwasu w stosunku do pospolitych wolnych aminokwasów i ich amidów. Rola tingitaniny w nasionach zdaje się ograniczać wyłącznie do magazynowania azotu.

6. Na podstawie wyników analizy wolnych aminokwasów (Tab. 8—12) stwierdza się znaczną rolę homoseryny w metabolizmie azotowym *Lathyrus tingitanus*; aminokwas ten zdaje się brać udział w transporcie azotu do nowych rozwijających się organów, a przede wszystkim nasion, gdzie prawdopodobnie bierze pośredni udział w syntezie białek. Sugerowane powiązania między homoseryną i tingitaniną wymagają dalszych badań.