Nitrogen Mobilization in Pea Seedlings. II. Free Amino Acids[1,2]

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In an earlier report from this laboratory (10) the changes in protein nitrogen and various forms of nonprotein nitrogen—amino, amide, peptide, ammonia, and urea—were reported for the different tissues of peas and pea seedlings during the early stages of growth. The data emphasize the quantitative importance of the free amino acids, particularly in the roots and shoots of these young seedlings. Although they contribute only about 3 % to the total nitrogen of the seed, their contribution rises to 10 % in the 5-day seedling. In root shaft and shoot shaft tissue of the 5-day seedling, from 40 to 45 % of the nitrogen is in the free amino acids. The work did not make clear the role these amino acids play in the conversion of the storage proteins of the seed to the functional proteins of the young plant. Clearly the first information needed for this purpose is a detailed picture of the changes in the concentration of the individual amino acids.

The cotyledons and embryonic axis tissue of pea seeds before and after one day's germination, and the cotyledons, root shafts, shoot shafts, root tips, and shoot tips of 3- and 5-day seedlings have been analyzed for their free amino acid content. The results give evidence for extensive interconversions and metabolism of amino acids during seedling growth, and show much similarity between the compositions of seedling root and shoot tissue. The very high levels of homoserine in certain tissues are documented.

Materials and Methods

Pea seeds (*Pisum sativum* L. var. Unica) were germinated and the seedlings processed as previously described (10). Briefly, they were surface sterilized, planted in vermiculite, germinated, and grown beneath the surface for up to 5 days at 18°, harvested, and dissected into the several individual tissues, which were frozen rapidly on dry ice, dried by lyophilization, and ground. Initial seed samples were dissected without preliminary softening by soaking. The free amino acids were extracted by thorough treatment with 70 % (v/v) ethanol solution.

The free amino acids were chromatographically separated by the method of Moore et al. (11), using 150 cm, 50 cm, and 15 cm columns and a fraction collector. The determinations were later repeated on at least one sample of each type using the Technicon automatic analyzer and the method of Piez and Morris (12) on extracts which had been held at −18° for one to two years.

For the former method, the extracts were evaporated to dryness under vacuum, and taken up either in water followed by adjustment to about pH 2 with a few drops of HCl, or in citrate buffer of pH 2.35 (11). At this stage, the cotyledon extracts were filtered and made to a convenient volume for the ion exchange chromatography. The other extracts, however, gave better chromatographic separations, if they were first extracted with petroleum ether before filtration and dilution to a convenient volume.

The method of Rosen (13) was used with minor modifications to determine the ninhydrin color yield of the fractions from the column. The concentration of the ninhydrin reagent was increased to 5 %, since the 2 ml fractions from the fraction collector resulted in increased dilution of the reaction mixture. Twice the recommended volume of the 50 % isopropanol diluent was used. We found the cyanide-acetate solution not to be stable (7) in that a lower ninhydrin color was obtained if the solution used was more than 4 hours old. Hence the cyanide and acetate components were mixed just before use. The fractions, except for the less acidic ones from the 15 cm column for the basic amino acids, were partially neutralized with 2 drops of 2 N NaOH before treatment with the reagents. Standard leucine samples were run at regular intervals to check the performance of the method.

For the automatic method, the extracts were evaporated to dryness under vacuum, and simply dissolved up to a convenient volume in pH 2.91 citrate buffer (12). In general, the results by the 2 methods agreed well, with the exceptions mentioned below, and were averaged together for the results presented in table I.

Amino acids were determined on hydrolysates of some of the residues from the 70 % alcohol extractions. The residues containing 8 to 25 mg of nitrogen were hydrolzed by autoclaving them at 121° for 5 hours with 25 ml of 3 N HCl. The amino acid contents of aliquot portions were determined using the manual (fraction collector) method.

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Results

The results are given in table I in terms of μmoles of amino acid per 100 seeds or seedlings. The figures for all tissues except the seed root-shoot axis (0-day) represent averages of determinations on 2 or more collected samples of the type. The amino acid peaks were identified by comparing their time of emergence from the column with that for standards. When runs were made on the same sample by each method, and the quantitative results agreed, there remained little doubt as to the identification. These methods sufficed for the familiar amino acids, and for certain less familiar ones which were well separated and present in sufficient quantity for reasonably precise determination. The identification of others remains uncertain. The position of peaks was less reproducible in some parts of the chromatograms in the case of the manual method.

Figure 1, the chromatogram obtained by the automatic method for one of the 5-day root shaft samples, shows the position of the various identified, tentatively identified, and most of the unidentified ninhydrin-positive compounds which appeared in this study.

Asparagine and glutamine overlap serine but not threonine in the manual method, whereas they over-

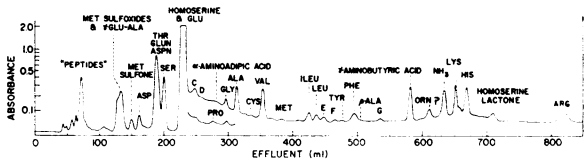

FIG. 1. Chromatogram of the free amino acids of a 5-day root shaft sample. The homoserine peak was off scale.

Table I

Free Amino Acids of Pea Seedlings

	Cotyledons				Root-shoot axis		Shoot shafts		Root shafts		Shoot tips		Root tips	
Age (Days)	0	1	3	5	0	1	3	5	3	5	3	5	3	5
Dry wt (g per 100 seedlings)	21.1	21.9	21.0	17.4	0.25	0.30	0.58	1.42	0.50	0.90	0.13	0.14	0.04	0.03
							*Amino Acids**							
Glutamic acid	248	310	250	210	5	7	7	4	2	1	5	4.1	1.2	0.6
Glutamine	5	100	160	300	0.3	3.7	12	32	5	10	2	1	0.3	0.3
γ-Aminobutyric acid	6	23	420	435	0.2	1.8	18	32	11	18	4.5	6	0.6	0.2
Proline	14	24	87	40	0.1	1.2	6	14	4	6	0.6	0.6	0.1	0.4
Histidine	5	8	19	26	0.1	0.7	9	32	5	10	2	1	0.3	0.3
Aspartic acid	65	45	10	21	0.3	0.5	3	10	3	5	0.4	0.5	0.1	0.1
Asparagine	230	315	155	145	3.8	3.4	47	220	64	120	1.6	3.0	0.2	0.3
Arginine	230	385	350	250	17	21	12	30	7	14	3.5	4.8	0.8	0.2
Glycine	41	24	56	35	0.8	0.8	2	4	2	5	1.2	0.8	0.3	0.1
Serine	17	73	270	240	0.4	2.4	16	59	17	34	3.0	5	0.7	0.6
Alanine	24	88	150	140	1.8	12	17	32	14	18	4.6	5	1.4	0.8
Tryptophan	8	0	9	15	0	0	2	6	7	3	T	T	0	0
Methionine	5	10	24	16	0.2	0.2	12	22	12	19	0.9	1.0	0.3	0.2
Cystine	T	T	26	6	0	T	1	2	1	0.4	0.1	0.1	0	0
Homoserine	0	32	710	540	0.2	1.3	270	1380	350	900	20	21	10	5
Threonine	41	100	76	74	1.2	2.3	9	32	9	15	6	46	1.2	0.5
Valine	9	23	72	64	0.2	1.0	9	49	8	22	0.6	0.8	0.2	0.2
Isoleucine	3	12	33	30	0.1	0.3	3.2	18	3.7	6	0.3	0.4	0.1	0.1
Leucine	6	13	34	34	0.1	0.6	2.6	10	2.8	4	0.3	0.4	0.1	0.04
Phenylalanine	7	14	44	38	0.1	0.3	4.0	31	2.8	6	0.2	0.3	0.1	0.1
Tyrosine	10	16	23	12	0.1	0.8	1.2	3	2.3	T	0.1	0.1	0.03	0.04
Lysine	32	17	40	39	0.5	0.7	10	46	7	21	0.5	1.0	0.1	0.1
α-Aminoadipic acid	2	14	32	15	0	0.3	1.2	2	0.3	0.9	0.1	T	0.04	0.01
Pipecolic acid	2	3	2	1	0.04	0	0.8	2	0.8	1.2	0.1	0	0	0
Compound A**	T	T	31	27	...	0.6	1.9	T	1.3	2.2	0.3	0.2	0.05	0.05
Compound B***	T	16	29	34	...	1.5	24	5	1.7	5	0.6	0.8	0.2	0.2
Compound C	17	0	10	T	0	0	3.0	15	4	10	0.3	1.0	0.3	0.5
Compound D	T	T	T	T	0	0	1	4	T	T	0.3	0.4	0	0
Compound E	0	0	0	0	...	0	1	3	3	3	0	T	0	0
Compound F	0	0	0	0	...	0	1	3	2	1	0.1	0.1	0	0
Compound G	0	0	0	0	...	0	1	4	2	1	0	T	0	0
Peptides	110	110	110	70	2.0	3	8	270	120	130	16	30	4	2.3

* Micromoles per 100 seedlings. T = trace. Zero means that the compound was undetectable under the analytical conditions.

** Probably β-alanine

*** Possibly ornithine

lap threonine but not serine in the automatic method. Hence threonine was determinable by the former, and serine by the latter procedure. To determine asparagine and glutamine, the fractions containing them and serine in the manual method were pooled, and deionized by adsorption on a 0.9 by 10 cm column of Dowex 50 × 8, 100-200 mesh, in the H^+ form, and eluted with 3N NH_4OH. The eluate was concentrated by a stream of warm air from a hair dryer and the amides were hydrolyzed by heating in a boiling water bath with 1 N HCl for 3 hours (3). After evaporation to dryness on a steam bath the material was redissolved in pH 2.35 citrate buffer and rechromatographed. Serine, glutamic acid, and aspartic acid appear as separate peaks and were calculated as serine, glutamine, and asparagine. Control experiments showed that about 10 % of a glutamine standard is hydrolyzed while passing through the column of acidic ion exchange resin at 30°. The results reported for glutamine and glutamic acid include a correction for this reaction.

In both methods, the homoserine and glutamic acid peaks partially overlap. In cases where one is present in very small quantity relative to the other, the lesser member of the pair could not be determined. In such cases, the chromatography was rerun with the 150 cm column held at 30° (11). In this case, separation was good enough for both these amino acids to be determined, although certain other amino acids were less well separated. The homoserine-glutamic acid area also coincides with that of the disulfide form of glutathione. Standard disulfide glutathione appears as a broad peak with a low color yield. Glutathione is known to be present in peas (6, 18) but in relatively small amount, and no correction for its presence was felt necessary.

Homoserine is slowly converted to the lactone form under mildly acid conditions (1). The peak corresponding to the resulting basic compound was identified following ammonia from the 15 cm column in the manual method, and following histidine in the automatic method. The correction in the homoserine assay was minor and has been made.

Aspartic acid was determined by the automatic method only, since in the manual method a number of other peaks emerged at about the same time and identification was usually uncertain.

Methionine as reported in table I was determined by the manual method. The results by the automatic method were lower, apparently because of loss of methionine by oxidation. The oxidized products could not be determined because the peak for the sulfoxides coincides with that for γ-glutamylalanine, which is known to be present in pea seedlings (8, 19).

The levels of pipecolic acid were too low for detection by the column chromatographic methods, and too low for determination with any precision by the method of Silberstein et al. (17), which was used for the reported results.

The identification of α-aminoadipic acid seems reliable since it emerged just before glycine in the manual method and just after proline in the automatic method as do standards. The identification of compound A as β-alanine depends on its regular appearance in its characteristic position for the automatic method immediately after phenylalinine. The quantity was calculated using the low color yield figure determined for standard β-alanine. The identification of compound B as ornithine is based only on its regular emergence in the automatic method between γ-aminobutyric acid and ammonia. In the manual method, the 15 cm column was used to determine the basic amino acids. In this case ornithine would overlap lysine. Since results for lysine (as well as the other basic amino acids) by the 2 methods agree, the identity of compound B remains uncertain. Calculations are in terms of ornithine, since its color yield is normal.

Recognition of the other listed unknown compounds was also based mainly on the automatic method because random fluctuations of the baseline were absent. Figures for the quantity present are based on the leucine color yields. Compounds C and D appeared between glutamic acid and proline. In the manual method, unknown peaks appear with many of the samples between proline and α-aminoadipic acid, and may well represent the same compounds. Compounds E and F emerged between leucine and tyrosine, and compound G followed β-alanine. Citrulline would be present in the region of C and D, norleucine in that of E and F, and β-aminoisobutyric acid in that of G, but we do not intend any implication as to identity. Other unidentified ninhydrin-reactive materials appeared in various locations interspersed between the identified amino acids, usually in only trace amounts. The more frequent of these came between valine and methionine (where cystathionine emerges), between lysine and histidine (where 1-methyl histidine emerges), and just after aspartic acid.

A number of peaks appeared early before aspartic acid, and 2 of these could be accounted for by methionine sulfoxides, sulfone and probably γ-glutamylalanine. Frequently, one or two of these early unidentified materials were present in considerable quantity. When several of the alcoholic extracts (fresh enough to contain no oxidized products of methionine) were acid-hydrolyzed, the early peaks disappeared with concomitant increases in the concentration of various amino acids. They have not yet been studied further, but it appears probable that they are peptides soluble in 70 % alcohol or compounds of some other type containing bound amino acids. The figures given in table I as peptides have been calculated from the manual runs by summing all the peaks before serine, subtracting the separately-determined aspartic acid, and using the leucine color yield to calculate μmoles.

A comparison of the total free amino nitrogen determinations as previously reported (10) and the sum of the concentration of the individual amino

Table II

Amino Nitrogen Recovery

Type of tissue	Total free amino N	Sum of amino acids (as amino N)
	mg per 100 seedlings	
Seed cotyledons	19	16
Seed root-shoot axis	0.47	0.48
1-day seedling cotyledons	23	25
1-day seedling root-shoot axis	0.92	0.94
3-day seedling cotyledons	42	45
3-day seedling shoot tips	1.1	1.1
3-day seedling shoot shafts	8.8	8.2
3-day seedling root shafts	9.9	9.4
3-day seedling root tips	0.4	0.3
5-day seedling cotyledons	36	40
5-day seedling shoot tips	1.0	1.3
5-day seedling shoot shafts	33	33
5-day seedling root shafts	20	20
5-day seedling root tips	0.3	0.2

acids (including unknown peaks), calculated as amino nitrogen, is in table II. For part of this work, the same alcoholic extracts as in the previous study were used, but for much of the material new seedlings were grown. The recoveries are satisfactory.

Discussion

Semiquantitative analyses by paper chromatography have been reported for many of the free amino acids in pea seeds (9, 16, 20), whole seedlings at various growth stages (20) and seedling cotyledons and axis tissue at different growth stages (16). These workers generally agreed that glutamic acid is the predominant amino acid of seeds and of seedlings in the first few days of germination. In the complete analyses reported here, there was at least as much arginine and asparagine present as glutamic acid in the seeds initially and after one day's germination, after which homoserine rose to predominance, as has been noted by others (15, 20). The changes in amount of some of the more quantitatively important amino acids in the total seedling tissue have been summed up in table III. The total amino N values for whole seedlings were calculated from results obtained in the earlier study (10).

Table III

Free Amino Acid Changes for Whole Seedlings

	Age (Days) 0	1	3	5
Glutamic acid	254	320	324	332
Arginine	247	406	373	299
Asparagine	234	318	268	488
Homoserine	0	33	1360	2846
γ-Aminobutyric acid	6	25	454	491
Glutamine	5	103	122	233
Serine	17	75	307	339
Alanine	25	100	187	196
Threonine	42	102	101	126
Aspartic acid	65	45	17	37
Total Amino N	1390	1710	4440	6450

These values are in μmoles per 100 seedlings, with seed coats not included.

It appears that the amino acids initially predominant (glutamic acid, arginine, and asparagine) show no striking increase during early seedling growth and actually decrease relative to the total amino nitrogen, while another group (homoserine, γ-aminobutyric acid, glutamine, serine, and alanine) show a very rapid absolute and relative increase.

Among the nonprotein amino acids, besides homoserine, the occurrence of γ-aminobutyric acid has repeatedly been observed in pea seedlings (8, 16) and in other pea plant tissues. α-Aminoadipic acid has been isolated from seedlings and identified (8). β-Alanine has been observed in paper chromatography (8).

Considering the data in table I in more detail, the reversal in the relative amounts of asparagine and glutamine in the cotyledon tissue as growth proceeds confirms the observations of Shirakawa and Otakara (16). They found no γ-aminobutyric acid and more aspartic acid than glutamic acid in axis tissue. However, they reported amounts of most of the amino acids in relative terms, so detailed comparison is not possible. Studies of smooth and wrinkled peas (14) and of aphid-resistant and susceptible peas (2) indicate there are considerable differences between varieties.

There appear to be very characteristic free amino acid compositions for particular types of tissue. Shoot shaft and root shaft tissue made up of mature conductive cells have similar amino acid profiles, while shoot tips and root tips with a high proportion of dividing cells are quite comparable with each other. This is indicated in figure 2 where the con-

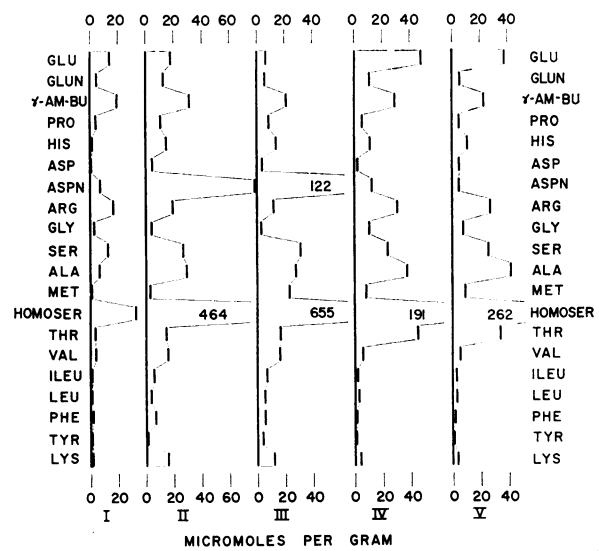

FIG. 2. Free amino acid profiles for 3-day pea seedlings. Micromoles per gram dry tissue for cotyledons (I), shoot shafts (II), root shafts (III), shoot tips (IV), and root tips (V).

centrations are given on a dry weight basis to aid in comparisons. In the 3-day seedlings, root and shoot shaft tissues contain much more γ-aminobutyric acid than glutamic acid, while the reverse is true for root and shoot tips. There is relatively more serine and less alanine and threonine in the former as compared with the latter. The differences might be more clear-cut, except for the considerable proportion of mature conductive cells in the root and shoot tip samples (10). Considering the analyses on a dry weight basis emphasizes certain points. For example, 655 μmoles of homoserine per gram in 3-day root shafts (fig 1) represents 78 mg per gram; and 980 μmoles per gram in 5-day root and shoot shafts is a little less than 12% of the dry weight. As there is practically none in the seed, a tremendous change has occurred. Also notable are the relatively high levels of arginine in the embryonic root-shoot axis, γ-aminobutyric acid in 3- and 5-day cotyledons, and asparagine in 3- and 5-day root and shoot shaft tissue.

The data can be considered from the view point of utilization of the protein reserves of the cotyledons and provision of the proper array of amino acids for synthesis of protein in the new tissue. The change in the free amino acids (considering only those usually thought of as occurring in proteins) during the first day's germination is not at all similar to the amino acid composition of the seed hydrolysate (mainly from protein) as is shown in table IV; or to the composition of individual pea seed proteins (5), as might be expected if certain proteins were utilized before others (4).

The last 2 columns of the table show that the composition of root tip protein is also quite different from that of the free amino acids present. The same is true for the shoot tips. In fact, the composition of the proteins of root tips, shoot tips, root shafts, and shoot shafts is very uniform. It seems probable that the amino acids are very extensively interconverted and otherwise metabolized in cotyledons, as well as in the other types of tissue.

Summary

Peas were germinated in vermiculite at 18° and the seedlings grown for 1, 3, and 5 days. The harvested samples were dissected into cotyledons and embryonic root-shoot axes for the initial and 1-day samples; and into cotyledons, root tips, shoot tips and root and shoot shafts for the 3- and 5-day seedlings. The free amino acid composition of 70% ethanol extracts of these samples were determined by ion exchange column chromatography. Amino acids were determined also on hydrolysates of residues from some of these extractions.

The more notable observations were these. Homoserine increased from being practically absent in the seed to very high levels by the 3rd and 5th day, particularly in the root and shoot shaft tissue where it amounted to almost 12% of the dry weight in the 5-day seedling. At these stages, next to homoserine, γ-aminobutyric acid was present in the largest amount in the cotyledons, and asparagine in the root and shoot shafts. Arginine was outstanding in the seeds and 1-day seedlings, particularly in the axis tissue. The free amino acid compositions of root and shoot shaft tissue were very similar and the same was true for root and shoot tip tissue. The amino acid compositions of the total proteins of all 4 types of tissue were almost the same. Differences between free and protein amino acid compositions and between the free amino acids of different types of tissue suggest extensive interconversions and metabolism of amino acids. α-Aminoadipic acid and

Table IV

Comparative Amino Acid Compositions

	Increased free amino acids in cotyledons during 1 day's germination	Amino acids from hydrolysates for alcoholic extracts of residues					Free amino acids of 3-day root tips
		Whole peas	3-day shoot shafts	3-day root shafts	3-day shoot tips	3-day root tips	
Glutamic acid + Glutamine	25.6	15.5	10.8	10.2	10.8	10.2	17.7
Proline	1.7	6.1	6.1	6.5	6.7	6.5	1.7
Histidine	0.5	2.3	1.8	2.6	2.3	2.6	5.6
Aspartic acid + Asparagine	11.3	14.8	12.1	11.8	12.2	11.8	4.4
Arginine	27.0	4.7	4.0	3.8	4.3	3.8	10.9
Glycine	−3.0	7.5	9.6	8.7	9.0	8.7	4.0
Serine	9.7	4.4	6.5	6.3	6.0	6.3	10.4
Alanine	11.1	10.0	9.5	9.1	9.0	9.1	19.3
Methionine	0.9	1.5	1.1	1.4	1.1	1.4	2.8
Threonine	10.3	3.7	5.5	5.1	5.1	5.1	13.9
Valine	2.4	4.8	6.2	5.5	5.6	5.5	2.8
Isoleucine	1.6	3.5	4.0	4.9	4.4	4.9	1.2
Leucine	1.2	7.6	8.6	9.0	8.7	9.0	1.6
Phenylalanine	1.2	4.1	3.5	3.9	3.9	3.9	1.0
Tyrosine	1.0	2.0	2.4	2.5	2.6	2.5	0.4
Lysine	−2.6	7.5	8.1	8.6	8.5	8.6	2.0

These values are per cent of total μmoles of listed amino acids.

β-alanine were probably present in all samples as was another peak which may have been ornithine. Numerous other unidentified compounds occurred in many of the samples.

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