PHYSIOLOGY OF PEA FRUITS

II. SOLUBLE NITROGENOUS CONSTITUENTS IN THE DEVELOPING FRUIT

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Summary

Soluble nitrogenous compounds in the seeds and hulls of developing fruits of the pea (*Pisum sativum*, var. Canner's Perfection) were studied at successive stages of growth during two seasons. Of the 26 compounds studied, some were undetectable in some samples and all decreased in the seeds during the period of intense protein synthesis. The results are discussed in conjunction with those of other workers.

I. Introduction

The experiments described in this paper were designed to identify soluble nitrogenous compounds in pea seeds and hulls at different stages of their development. These analyses were carried out on replicate samples of those used for determinations of the protein and soluble nitrogen, and other constituents (McKee, Robertson, and Lee 1955).

The main observations on the individual compounds represented in the non-protein nitrogen of pea fruits are due to Schulze and Winterstein (1910). They found in immature pods that the hulls contained asparagine (which accounted for about one-half of the total soluble nitrogen), arginine, histidine, tryptophan, leucine, choline, and trigonelline. In the seeds arginine was the main soluble nitrogenous compound, being accompanied by relatively small amounts of asparagine, choline, and trigonelline. Hyde (1953), using chromatographic methods, confirmed the predominance of asparagine in the hulls and of arginine in the seeds of developing peas and listed a number of other compounds. It may be noted that arginine is the chief amino acid liberated on the hydrolysis of protein from pea seeds (Holmes 1953). Snellmann and Danielsson (1953) recorded the presence in developing pea seeds of peptides containing two to six amino acid residues. Auclair and Maltais (1952) reported the occurrence of γ-aminobutyric acid in peas. They also tentatively identified an unknown spot as β-amino-*iso*butyric acid. Virtanen and Miettinen (1953) identified homoserine in peas and isolated it in crystalline form. Bisset (1954) recorded homoserine, citrulline, and α-aminobutyric acid in immature pea seeds.

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II. Materials and Methods

(*a*) *Sampling*

The peas (*Pisum sativum*, var. Canner's Perfection) used in the first season (1952) were grown at Boree Cabonne (17 miles west of Orange, N.S.W.). Uniformity of age in the material was obtained by using fruits from flowers tagged on a particular date. Seven samples were collected at 2- to 3-day intervals over a period from 11 to 27 days after tagging. The peas used in the second season (1953) were grown at Hawkesbury Agricultural College, Windsor, N.S.W., and nine samples were collected at 3- to 4-day intervals over a period from 14 to 40 days after tagging.

The seeds were separated from the hulls and known weights were placed in cans containing enough absolute ethanol to make the final concentration approximately 75 per cent.

(*b*) *Separation of Alcohol-Soluble Nitrogenous Compounds*

The seeds or hulls were blended in the alcohol for 3 or 5 min respectively in a Waring Blendor. The hulls contained fibrous material which was removed by squeezing the homogenate through muslin. The homogenates were filtered through Whatman No. 31 filter paper, yielding clear, bright green filtrates, containing the constituents soluble in 75 per cent. ethanol.

(*c*) *Preparation of the Extract for Chromatography*

In the first season, the method described by McKee and Urbach (1953) was used, except that the elution with sodium hydroxide was omitted. As the concentration of the basic amino acids, especially arginine, was very high in the first season, the samples in the second season were passed through "Zeo-Karb" 216, in its potassium form (Wieland 1944), to separate the basic amino acids. The neutral and acidic amino acids were then passed through "Zeo-Karb" 215 and treated as in the first season. Most of the excess hydrochloric acid was removed from the eluate containing the basic amino acids by six successive vacuum distillations to dryness.

(*d*) *Chromatographic Technique*

(i) *Standard Method.*—The ascending technique as described by Wolfson, Cohn, and Devaney (1949) was used. Generally, two-dimensional chromatograms were run, using phenol-water (4 : 1 w/v) in the first direction and *n*-butanol-acetic acid-water (4 : 1 : 1 by volume) in the second direction. The dry chromatograms were sprayed with an 0·1 per cent. solution of ninhydrin in 95 per cent. ethanol.

(ii) *Circular Chromatography.*—It was found that, with the standard two-dimensional method, the basic amino acids formed a streak across the chromatogram, whereas circular chromatography gave good separation. The technique of Zimmermann and Nehring (1951) as quoted by Cramer (1953) was used. Papers were run in phenol-water (4 : 1 w/v) for 6 hr. *n*-Butanol-acetic acid-water (4 : 1 : 1 by volume) was also tried, but the bands were more blurred than those run in phenol-water.

(*e*) *Specific Tests for Individual Substances*

(i) *Arginine.*—The presence of arginine was confirmed by the modified Sakaguchi reaction of Roche *et al.* (1951). The sodium bromide spray applied immediately after the first spray without drying the paper, gave satisfactory results.

(ii) *γ-Aminobutyric Acid.*—The copper carbonate method of Crumpler and Dent (1949) was used. γ-Aminobutyric acid gave a purple spot.

(iii) *Choline.*—The chromatograms were placed in glass tanks containing iodine vapour (Brante 1949). Some amino acids react with iodine but these had previously been developed with ninhydrin and their positions marked with pencil.

(iv) *Histidine and Tyrosine.*—These amino acids gave reddish pink spots with the modified Pauly reagent of Stepka (1952).

(v) *Homoserine, Pipecolinic Acid, and Proline.*—The papers were sprayed with 0·2 per cent. solution of isatin in *n*-butanol, containing 4 per cent. acetic acid (Acher, Fromageot, and Jutisz 1950). The chromatograms were heated for about 1 hr at 50°C and then for 4 hr at 90°C. Homoserine appeared as a pink spot. Methionine sulphone, which overlaps with homoserine, does not give a colour reaction with isatin. Pipecolinic acid appeared as a bluish green coloured spot, while proline appeared as a blue spot.

(vi) *The Sulphur-Containing Amino Acids.*—The method of Chargaff, Levine, and Green (1948) was used to detect the sulphur-containing amino acids. Spots appeared in the position of cysteic acid and methionine sulphoxide.

Cystine and cysteine, if present in the extracts, were oxidized during the experimental procedure to cysteic acid. Spots for methionine sulphone and methionine sulphoxide both appeared on the chromatograms. Oxidation of the extract with hydrogen peroxide resulted in a spot appearing in the position of methionine sulphoxide, as well as an increase in the size of the spot in the position of methionine sulphone. Therefore it appears that methionine sulphone and methionine sulphoxide are both present in the pea extracts, but either oxidation product of methionine could be an artifact arising during the preparation of the sample for analysis.

(vii) *Trigonelline.*—The phosphomolybdic acid-stannous chloride reaction of Munier (1951) was used. Trigonelline was observed to give a faint yellow spot with the phosphomolybdic acid reagent. This was reduced to a blue spot with stannous chloride.

(viii) *Tryptophan.*—The reaction with Ehrlich's reagent, *p*-dimethylaminobenzaldehyde (Block 1951), was used.

(ix) *Urea.*—The phenol-sodium hypochlorite method of R. J. Williams as described by Block, Le Strange, and Zweig (1952) was used.

The following amino acids also gave a colour reaction with the phenol-sodium hypochlorite reagent:

Threonine, α-alanine, methionine sulphone, methionine sulphoxide, arginine, serine, asparagine	Pale blue
Glycine, γ-aminobutyric acid, β-alanine, glutamine	Bright blue
Lysine	Blue-green
Histidine	Faint green.

III. Results

The following substances were detected on chromatograms in all samples of both hulls and seeds in both seasons: α-alanine, γ-aminobutyric acid, arginine, aspartic acid, glutamic acid, glycine, histidine, homoserine, "leucine",* methionine, methionine sulphone, methionine sulphoxide, serine, threonine, trigonelline, tyrosine, urea, and valine.

In seeds, pipecolinic acid disappeared at the 27th day in the first season and at the 26th day in the second season. Proline was detectable only in the first three samples (up to 17 days) in the first season and in the first sample (14 days) in the second season. In the second season, β-alanine disappeared after the 29th day. The large concentrations of basic amino acids which were not removed in the first season made glutamine difficult to demonstrate. In the second season, however, glutamine, though clearly present in the early samples, was not detected in the samples from the 26th, 29th, and 32nd days. Phenylalanine was present in all seed samples in both seasons and cysteic acid, undetectable in any samples in the first season, was present in chromatograms up to the 35th day in the second season. Asparagine, detectable in all seed samples in the first season, was undetectable in the samples from 32 and 35 days in the second.

In the hulls, the pattern of detectable amino acids was not the same as that in seeds. Phenylalanine, present in only the first four samples (i.e. up to 17 days) in the first season, was detected only in the samples from 26 and 29 days in the second season. Cysteic acid, undetected in the first season, appeared in chromatograms of the first three samples in the second season. Pipecolinic acid, proline, and β-alanine occurred in the first few samples in the first season but were undetectable in the second. Glutamine was present in all hull samples in both seasons except at the 27th day in the first and at the 26th day in the second. Asparagine was detected in all hull samples except for the first sample (14 days) in the second season. Lysine, undetected in the hulls in the first season, was detectable in all samples in the second.

Choline and tryptophan, found by Schulze and Winterstein (1910) in pea hulls, were not detected in hulls or seeds in either season. β-Amino-*iso*butyric acid (Auclair and Maltais 1952) and baikiain, ethanolamine, putrescine, and γ-methyleneglutamine, tentatively identified in pea fruits (Hyde 1953) were not detected in hulls or seeds.

In 1953, the relative amounts of the different amino acids were compared by running chromatograms with 1, 5, 10, 20, and 50 μl of each sample, and noting the minimum amount of sample necessary to give a spot with ninhydrin for a particular amino acid. The results so obtained agreed with the general

* "Leucine" may be a mixure of leucine, norleucine, and isoleucine.

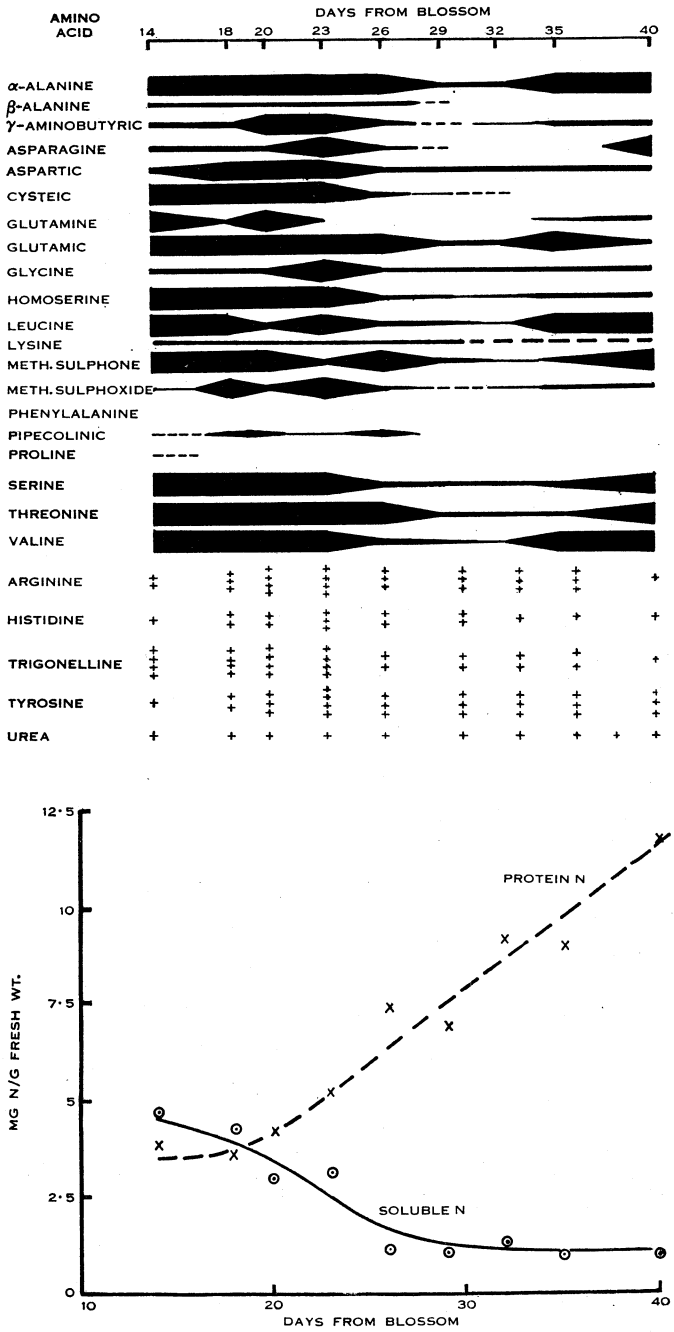

Fig. 1.—Changes in soluble nitrogen compounds in seeds with time from blossom. The width of the lines represents the relative amounts at different times (estimated from the reciprocal of the smallest amount of extract necessary to give a spot). Curves for soluble and protein nitrogen amounts are also shown.

appearance of the developed spots on the papers. By taking the reciprocal of the minimum amount necessary to give a spot, some idea of the relative amounts of the amino acids at different times can be obtained. This has been illustrated diagrammatically by the width of the lines for the different amino acids in Figure 1 for seeds and in Figure 2 for hulls; the width of the line is proportional to the reciprocal of the minimum amount of each sample to give a spot with ninhydrin. An estimate of the changes in arginine, histidine, trigonelline, tyrosine, and urea was obtained by comparison of the sizes of the spots and these are also given in Figures 1 and 2.

Though it must be emphasized that these diagrams cannot be regarded as quantitative, they give a useful indication of the changes in the various amounts of amino acids at different times. While, as has been noted, most of the amino acids occurred in all samples of the seeds and only β-alanine, asparagine, cysteic acid, glutamine, pipecolinic acid, and proline became undetectable at certain times, all the acids decreased in concentration, particularly over the period 23-32 days. Thus in that period α-alanine, β-alanine, γ-aminobutyric acid, asparagine, aspartic acid, cysteic acid, glutamine, glutamic acid, histidine, homoserine, leucine, lysine, the oxidation products of methionine, pipecolinic acid, serine, threonine, trigonelline, and valine all decreased in concentration in the seeds.

In the hulls, β-alanine, asparagine, cysteic acid, and glutamine were undetectable in some samples and other amino acids changed in amount, but there was no consistent change until the time of the last sample when all the amino acids except tyrosine decreased.

IV. Discussion

These results indicate that the changes in the concentration of soluble nitrogen in seeds and hulls, noted by McKee, Robertson, and Lee (1955) in the developing pea fruit, are accompanied by major changes in the individual amino acids. The concentration of soluble nitrogen in the 1953 samples is shown in Figures 1 and 2.

In the seeds, the combined effects of rapidly increasing cell size and increased rate of protein synthesis, with increasing protein concentration, resulted in a decrease in the concentration of soluble nitrogen constituents from about 3·5 mg/g fresh weight at about 20 days to about 1·2 mg/g fresh weight at 26 days and thereafter the soluble nitrogen remained low. About this time, the concentration of all amino acids decreased. Most of these amino acids are known to occur in protein molecules but two amino acids, β-alanine and pipecolinic acid, not known as constituents of protein, were present in the early picks, but not detected later.

The hulls showed a distinct contrast to the seeds. The soluble nitrogen concentration was never as high as in the seeds, and drifted slowly down from about 1·8 mg/g fresh weight on the 14th day to 0·9 mg/g on the 35th. This small change in soluble nitrogen is consistent with the observation that, except for a small rise in absolute amount during the period from 14 to 20 days, the absolute protein nitrogen per hull decreased during the period while, owing to the decreasing fresh weight of the hulls, the protein nitrogen per mg fresh

weight remained approximately constant. Changes in the amino acid pattern were slight in the hulls until the last sample, which corresponded with the biggest change in the soluble nitrogen with time between the 35th and 40th day, when the soluble nitrogen dropped to 0·2 mg/g fresh weight. This simul-

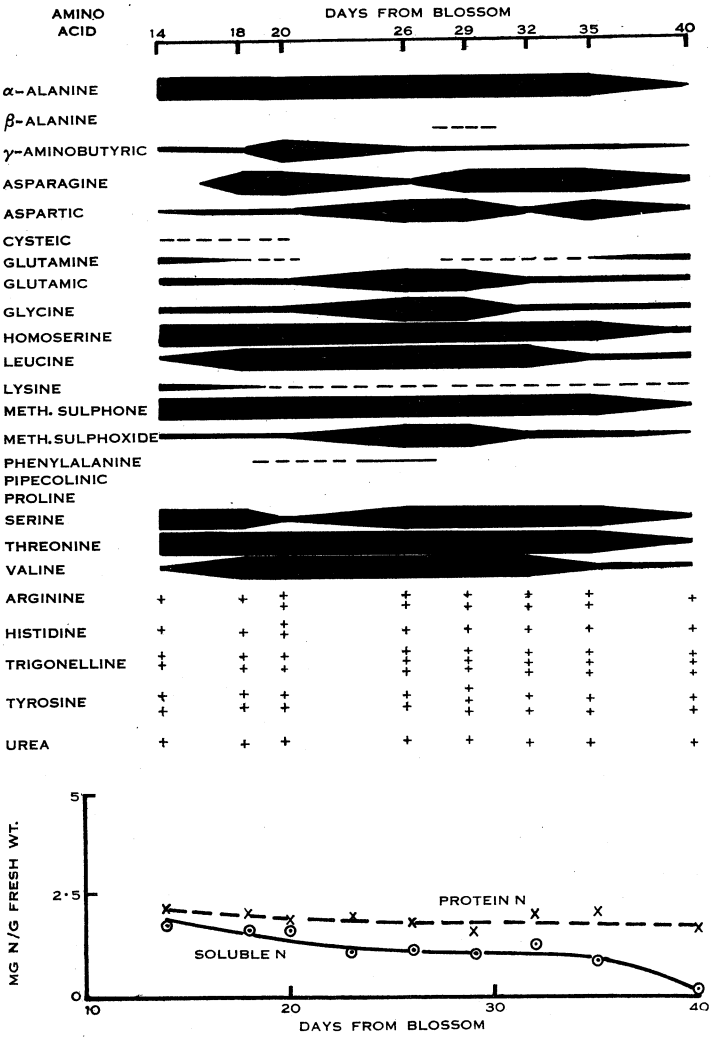

Fig. 2.—Changes in soluble nitrogen compounds in hulls with time from blossom. The width of the lines represents the relative amounts at different times (estimated from the reciprocal of the smallest volume of extract necessary to give a spot). Curves for soluble and protein nitrogen amounts are also given.

taneous decrease in soluble nitrogen and in the individual amino acids, unlike that in the seeds, cannot be attributed to protein synthesis in the hulls since the protein nitrogen per hull decreased over that period from 4·0 to 2·6 mg. The decrease in soluble nitrogen from 1·8 to 0·4 mg per hull must therefore

be attributed largely to export from the hulls. Assuming that the decrease in protein nitrogen was also due to export as soluble nitrogen, the total loss of soluble nitrogen per hull was 2·8 mg. Since each hull contained an average of six seeds, each seed could have gained 0·47 mg. As shown in Figure 1, the soluble nitrogen per seed does not change during the period but the protein nitrogen increases by about 1·8 mg, so about 26 per cent. of the gain in the seed might come from the hull. In this connection it is interesting to note that, while the total soluble nitrogen in the seeds did not increase during the period covered by the last two samples, one-half of the amino acids increased again. Some of these may have been contributed by the "pool" of amino acids in the hulls, which was decreasing simultaneously, but no balance sheet could be suggested on the data available, and full interpretation would require more knowledge than we have at present of the soluble nitrogen compounds which are translocated.

Some differences in occurrence of amino acids in the 1952 and 1953 crops were noted. Unfortunately the semi-quantitative observations on the individual amino acids were not carried out in the first season, but the soluble nitrogen was investigated quantitatively. In the second season the soluble nitrogen per seed reached its maximum on the 23rd day, and in the first season on the 21st day. The soluble nitrogen in the first season did not reach as low a level at the end of sampling as in the second season, though active protein synthesis had begun.

The reduction in concentration of the amino acids during the period of active protein synthesis in the seeds suggests that, for a time, their rate of translocation into the seed or their rate of formation from the translocated precursors was less than their rate of utilization. The complete disappearance of glutamine is interesting in view of the suggestions from work on several plant species that glutamine is used in protein synthesis in preference to the other simple nitrogenous compounds. In the apple, temporary absence of glutamine is one of the few qualitative changes in the amino acid picture as the fertilized ovary develops into the fruit (McKee and Urbach 1953).

The high concentration of soluble nitrogenous compounds in seeds including the pool of amino acids, which was reduced after the initiation of active protein synthesis, suggests that the initial low rate of protein synthesis was not limited by the concentration of the intermediates of nitrogen metabolism. This raises the interesting question of what controls the rate of synthesis which results in the increase after the 23rd day in 1953 and after the 17th day in 1952. It was found that the respiration rate was not correlated with the different rates of synthesis.

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