COMPOSITION OF THE NITROGEN FRACTION OF APPLE TRACHEAL SAP

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Summary

Organic nitrogenous compounds accounted for most of the nitrogen present in apple tracheal sap. Aspartic acid, asparagine, and glutamine were quantitatively the most important compounds. Glutamic acid and other amino acids were also present as well as a peptide-like substance. While apple variety, rootstock, or manurial treatment may have had effects on level of nitrogen in tracheal sap they seem to have had little effect on proportions of nitrogenous compounds present. Through the growing season, however, there was a definite change in proportions of some of the constituents. The composition of the soluble nitrogen fraction of leaves and fruits showed distinct differences from the composition of tracheal sap.

I. Introduction

Seasonal fluctuation in levels of nitrogen in tracheal sap extracted from the xylem of apple trees has already been described (Bollard 1953*b*). In the present paper a description of the nature of the nitrogenous compounds in this sap is given, and an account of some of the factors influencing their amount. The relation of these compounds to the soluble nitrogen fraction of leaves and fruit is also considered. A brief report of some of these results has already been given (Bollard 1953*a*).

II. Material and Methods

(*a*) *Extraction of Tracheal Sap*

Shoots consisting of only previous and current season's growth cut from mature apple trees were used for most sap extractions and all shoots were collected between 8 and 9 a.m. Each sap sample was extracted from shoots of a single tree by the methods previously described (Bollard 1953*b*). Early in the season, when total nitrogen content was high, about 10 ml of sap was enough for all determinations; later in the season, when total nitrogen had fallen, about 25 ml of sap was necessary.

(*b*) *Estimation of Nitrogen Fractions*

(i) *Total Nitrogen.*—Kjeldahl determinations were made on aliquots of usually 2 ml sap. No nitrate has been detected qualitatively in the sap and so no precautions were taken to include nitrate in the total nitrogen.

(ii) *Ammonia Nitrogen.*—Sap aliquots (2, 3, or 5 ml) were distilled *in vacuo* at 40°C with phosphate-borate buffer (Pucher, Vickery, and Leavenworth 1935). Ammonia was absorbed in dilute sulphuric acid and determined after redistillation from the Markham apparatus (Markham 1942).

(iii) *Glutamine-amide Nitrogen.*—Sap aliquots were subjected to mild hydrolysis (Vickery *et al.* 1935) by boiling with phosphate buffer at pH 6·5. Ammonia liberated

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was determined as above. Any increase in ammonia was due to glutamine breakdown and the nitrogen of this ammonia increment was designated glutamine-amide nitrogen.

(iv) *Asparagine-amide Nitrogen.*—Sap aliquots were hydrolysed in N sulphuric acid (Pucher *et al.* 1935). Ammonia liberated was determined as above. Any increase in ammonia nitrogen over that due to free ammonia and glutamine-amide was designated asparagine-amide nitrogen.

(v) *Amino Nitrogen.*—Amino nitrogen was determined in the Van Slyke manometric apparatus using nitrous acid (Milton and Waters 1949). Sap from which the ammonia had been previously removed was acidified before use. The value obtained was corrected for the presence of glutamine by subtracting 80 per cent. of the glutamine-amide nitrogen value (Vickery *et al.* 1935).

(vi) *Amide + Amino Nitrogen.*—This term is used for the sum of ammonia nitrogen, glutamine-amide nitrogen, asparagine-amide nitrogen, and amino nitrogen.

(vii) *Nitrate.*—The presence of nitrate in sap samples was tested for qualitatively after reduction to nitrite using sulphanilic acid and α-naphthylamine (Feigl 1947).

(*c*) *Chromatography of Sap Samples*

Aliquots of tracheal sap were frozen and concentrated *in vacuo* over sulphuric acid. This material was then ready for application to the chromatography paper. In general, enough concentrated sap to contain 10 μg nitrogen was used for one-way chromatograms and enough to contain 15–30 μg for two-way runs. Chromatography was also carried out on samples of sap which had been hydrolysed with 6N hydrochloric acid for 18 hr.

Chromatograms were run on Whatman No. 1 filter paper, using as solvents 80 per cent. phenol in water and butanol–acetic acid–water (5 : 1 : 4 v/v). Following chromatography, amino acid spots were revealed by spraying with ninhydrin (0·1 per cent. in *iso*propanol). After drying, the sheets of paper were heated in an oven at 80–85°C for 5 min.

Quantitative determinations of amino acids in sap samples were made using the method of Wellington (1952, 1953).

(*d*) *Chromatography of Leaf and Fruit Samples*

Samples of about 2 g of freshly collected leaf (or 6 g of fruit) were extracted for 3 min in six successive 25-ml portions of 87 per cent. ethanol in a "Nelco" homogenizer at room temperatures. All portions of alcohol were pooled and passed through a column of "Zeo-Karb 215" which had previously been washed with 87 per cent. ethanol. Amino acids were eluted from the column with 0·15N ammonia. Using this procedure it was possible to obtain the amino acids of leaf or fruit in solution free of sugar, colouring matter, etc. This elutate was reduced in volume at a temperature below 40°C and aliquots calculated to contain 30 μg nitrogen before application to the column (i.e. while chlorophyll etc. was still present) were chromatographed using the methods already described.

III. Nature of Nitrogenous Compounds Present in Tracheal Sap of Apple Trees

(a) *Nitrogen Balance Sheets*

Determinations of nitrogen present in ammonia, glutamine-amide, asparagine-amide, and amino forms as well as of total nitrogen were made on a series of apple tracheal saps collected at different times from several varieties on several rootstocks. Results are given in Table 1. It can be seen that the differences between amide plus amino nitrogen and total nitrogen are small and probably attributable to experimental error. No nitrate could be detected in the sap. Thus, over a considerable part of the growing season, the nitrogen in the tracheal sap of apple trees of a number of varieties on several stocks is present very largely as organic nitrogenous compounds. Asparagine and glutamine usually account for over 50 per cent. of the total nitrogen.

(b) *Amino Acids Present in Tracheal Sap*

The foregoing work showed that asparagine and glutamine were important compounds in apple tracheal sap and also indicated that other amino compounds were present. The nature of the unknown compounds has been elucidated using paper chromatography.

Chromatography of many sap samples from various varieties at different times and under different conditions (see Sections IV and V) has always revealed the presence of aspartic acid, asparagine, and glutamine in largest amounts. Lesser amounts of glutamic acid were present while also occurring were serine, threonine, arginine, methionine, valine, and leucine. Sometimes present in trace amounts were alanine, tyrosine, γ-aminobutyric acid, and phenylalanine. Quantitative measurements of most of these compounds are presented in Section IV.

After acid hydrolysis there were changes in amounts of some compounds. Apart from disappearance of the two amides, with corresponding increases in glutamic and aspartic acids, there was an increase in serine and alanine while glycine and, in lesser amounts, lysine, cystine, and, sometimes, cysteic acid appeared. These changes probably indicate that a peptide (or peptides) was originally present in the sap.

IV. Variation in the Level and Composition of the Nitrogen Fraction of Tracheal Sap

It was apparent in early work that changes occurred both in the level of nitrogen and nature of the nitrogenous compounds present in apple tracheal sap. Some of the factors likely to influence these were further investigated.

(a) *Variety*

No differences attributable to variety have been found in comparisons of the nitrogenous compounds present in the tracheal sap from trees of the following varieties: Delicious, Dougherty, Granny Smith, Gravenstein, Jonathan, and Sturmer.

TABLE 1

AMOUNTS OF VARIOUS NITROGENOUS COMPOUNDS AND TOTAL NITROGEN IN TRACHEAL SAP FROM APPLE TREES

Results expressed as μg nitrogen/ml of sap

Sampling Date	Source of Sap		Ammonia Nitrogen	Glutamine-amide Nitrogen	Asparagine-amide Nitrogen	Amino Nitrogen	Total Ammonia, Amide, and Amino Nitrogen	Total Nitrogen (determined by Kjeldahl method)
	Variety	Stock						
23.x.54	Gravenstein	Northern Spy	1	11	21	88	121	121
1.xi.54	Delicious	Malling XII	4	22	30	105	161	158
1.xi.54	Delicious	Malling XVI	6	16	32	103	157	155
1.xi.54	Gravenstein	Northern Spy	2	8	23	71	104	98
11.xii.54	Delicious	Malling XII	1	10	7	36	54	54
11.xii.54	Delicious	Malling XVI	1	4	20	56	81	77
11.xii.54	Gravenstein	Northern Spy	1	2	12	12	27	20
24.iii.55	Granny Smith	Malling XVI	0	1	2	13	16	17
24.iii.55	Granny Smith	Northern Spy	0	2	2	13	17	16
24.iii.55	Granny Smith	Malling XII	0	1	2	13	16	19

(*b*) *Time of Year*

It has already been shown shown (Bollard 1953*b*) that there is a considerable regular seasonal variation in level of nitrogen in apple tracheal sap. A low level (10 μg/ml) during the dormant period was found to be followed by a sudden rise (to 150 μg/ml) at blossom time. This high level was maintained for about 3 weeks and there was then a gradual decline till, by harvest time, the dormant level was again reached.

TABLE 2

PROPORTIONS OF VARIOUS NITROGENOUS COMPOUNDS PRESENT IN APPLE TRACHEAL SAP FOR GRANNY SMITH TREES AT SEVERAL TIMES DURING THE GROWING SEASON

Results expressed as per cent. nitrogen contributed by each compound. + = present but less than 1 per cent.

Compound	Sampling Date					
	12.x.54	19.x.54	26.x.54	29.xi.54	29.xii.54	26.i.55
Aspartic acid	12	10	22	31	58	41
Asparagine	65	59	52	65	34	48
Glutamic acid	+	+	+	1	2	3
Glutamine	20	27	24	4	6	7
Serine	+	1	+	+	+	2
Threonine	2	1	1	+	+	+
Methionine + valine	1	1	1	+	+	1
Leucine	1	+	+	+	+	+
Total nitrogen (μg/ml)	41	117	131	48	22	12
Percentage of total nitrogen contributed by aspartic acid + asparagine	77	69	74	96	92	89

Chromatography of a series of sap samples showed that the same nitrogenous compounds were generally present through the year. Aspartic acid and asparagine were the most important constituents quantitatively at all times. Glutamine tended to decrease relatively as the season progressed while glutamic acid tended to increase. Arginine showed a striking increase towards the end of the season. Serine, threonine, methionine or valine, and leucine were relatively abundant at blossom time, when nitrogen levels were high, but soon afterwards were scarcely detectable. The peptide-like substance was apparently present at all times as the same amino acids appeared on hydrolysis of each sap sample.

Proportions of individual compounds in a series of sap samples from Granny Smith trees were determined using the quantitative paper chromatographic method of Wellington (1952, 1953). Results are given in Table 2. These results show the same sort of relationships as those already indicated by the qualitative chroma-

tography. Asparagine and aspartic acid were clearly the most important compounds contributing from 69 to 96 per cent. of the total nitrogen.

(c) *Rootstock and Fertilizer Treatment*

Comparison of nitrogen contents of sap samples taken from trees on different rootstocks in two experimental blocks (Woodhead, McKenzie, and Farmer 1954; Woodhead and McKenzie 1955) and from trees receiving different fertilizer treatments (Tiller, Roberts, and Bollard, unpublished data) showed some significant differences at particular times especially in samples taken when nitrogen levels were high. More detailed data would be necessary to establish any overall differences attributable to rootstock or fertilizer treatment.

No differences in nitrogenous compounds present in the sap from trees on different rootstocks or receiving different fertilizer treatments could be detected. Examination of saps from a range of unworked stocks also revealed no differences.

V. Relation of Tracheal Sap Constituents to Composition of Soluble Nitrogen Fraction of Leaf and Fruit

It is evident that a substantial portion, at least, of the nitrogen reaching the shoot must travel from the root in the tracheal sap. Though McKee and Urbach (1953) have already given a full account of the amino acids present in fruit and leaves of the Granny Smith apple, it was considered of interest to compare the soluble nitrogenous substances present in leaves and fruit with the nitrogen compounds concurrently present in tracheal sap.

Tracheal sap, leaf, and fruit samples were collected on two occasions from the same group of Granny Smith trees on Malling XII stock as follows:

Sampling Date	Nitrogen Content of Tracheal Sap (μg N/ml)	Average Weight of Fruit (g)
Nov. 22	57	2·9
Jan. 12	24	56·0

Examinations were made of two-way chromatograms run using tracheal sap and alcoholic extracts from leaf and fruit.

The first samples were taken at a time when there were comparatively large amounts of glutamine and asparagine in the sap in addition to aspartic acid, and other amino acids were present to only a slight extent. The amino acids in the leaf were present in very different proportions from those found in the sap. In the leaves, alanine and glutamic acid were most important quantitatively, with aspartic acid and serine next most abundant. The two amides were not prominent. In the fruit, asparagine was unquestionably the most abundant amino acid while only a trace of glutamine was present. Alanine, serine, glutamic acid, and aspartic acid were also prominent.

The second samples were taken at a time when aspartic acid had become more important in the tracheal sap. In the leaf during the same period, this compound was

not so important quantitatively as alanine, serine, or glutamic acid. In the fruit, asparagine was again the most important compound with alanine and serine also prominent.

These results with leaf and fruit extracts are in close agreement with those recorded by McKee and Urbach (1953) but it is interesting to note that glycine was present in the leaf and fruit and cysteic acid in the leaf at both sampling times. These compounds were not reported by McKee and Urbach nor did they occur free in the tracheal sap.

VI. Discussion

It would seem that the nitrogen in tracheal sap of apple trees is mostly present in organic compounds. Ammonia nitrogen was present in small proportion only and nitrate nitrogen has not been detected. These results are in accordance with those of Thomas (1927) who showed that nitrate could be detected in only the thinnest roots of apple trees, and with those of many workers who have shown that nitrates are not normally present in apple foliage. With heavy applications of nitrate Stuart (1932) found some in apple leaves but its presence was usually associated with damage. This is of interest in view of the fact that nitrate apparently occurs normally in all parts of many plants.

Work with radioactive tracers (Stout and Hoagland 1939) has confirmed the older view that upward translocation of, at least, some elements takes place in the xylem. The movement of nitrogen in the xylem has not been confirmed in this way but the present work has shown that nitrogen occurs abundantly in tracheal sap and a substantial portion, at least, of the nitrogen reaching the shoots must come through this channel. The occurrence of organic nitrogenous compounds in a sap from the xylem is perhaps surprising as the older view was that the transpiration stream simply carried upwards absorbed but unassimilated inorganic nutrients.

It is clear that, in the apple tree, stages in the assimilation of nitrogen are separated morphologically. Normally, incorporation of inorganic nitrogen into organic compounds would seem to be largely accomplished in the roots. The root system then supplies upper parts of the tree with a defined range of amino acids, amides, and peptide-like substances. Further metabolism of nitrogen in leaves and fruit can then occur using the compounds supplied in tracheal sap as starting materials.

It is not certain that nitrogenous compounds present in tracheal sap are the immediate products of incorporation of inorganic nitrogen. The root of an apple tree is a bulky perennial organ and there is ample opportunity for storage of nitrogen and some or all of the nitrogen present in tracheal sap may well result from hydrolysis of storage proteins. There is some evidence (Steward and Thompson 1954) that asparagine may be closely associated with protein breakdown. There is no doubt that, in apple tracheal sap, aspartic acid and asparagine are quantitatively more important than glutamic acid and glutamine and this may indicate that protein breakdown occurs in roots. It is not impossible that all nitrogen absorbed and assimilated by apple roots passes through a transitory storage phase.

The pattern of amino acids etc. present was remarkably constant through a range of varieties, stocks, and manurial treatments. The presence of amino acids such as serine, threonine, etc. raises the question of whether these compounds are necessarily synthesized in the root or whether the leaf is also capable of their synthesis. The peptide-like substance is constantly present but there is no indication as to what role, if any, it plays in further nitrogen metabolism. There is some evidence that nitrate cannot be metabolized by apple leaves but there is little other indication of how specific are the needs of upper parts of the plant. Changes in the proportions of different amino acids present in tracheal sap occurred through the growing season. It is not possible to say if these are simply changes consequent on changes in root growth or metabolism and of little importance to the shoot.

Comparison of tracheal sap constituents with compounds present in soluble nitrogen of leaves and fruit has produced interesting differences, which are difficult to explain in the present state of our knowledge. While it is reasonable to believe that the leaves receive a significant proportion of their nitrogen from tracheal sap one cannot be sure about fruits. It may be that tracheal sap supplies some nitrogen directly to developing fruits but it is also possible that some of the fruit nitrogen arrives after passage through, and possible transformation in, leaves.

There have been suggestions recently (Steward and Thompson 1954) that, despite the analogous nature of glutamine and asparagine, these two compounds are physiologically dissimilar. The present work has shown that, in apple, these two compounds seem to have different roles. While both were present in tracheal sap asparagine was more important quantitatively as a translocatory substance at all times, and particularly after the initial high nitrogen levels had dropped. In leaves, both amides were present in comparatively low concentration, glutamic acid and other amino acids being present in greater quantity. Trees more adequately supplied with nitrogen might have shown higher concentrations of one or both amides, or it may be that it is a result of translocation of these compounds away from leaves. In this connection it is interesting to note that in leaves soluble nitrogen is usually only 3–5 per cent. of total nitrogen while in fruit it is usually about 50 per cent. In fruits only a trace of glutamine was present and asparagine was the predominant compound.

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