THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

V. SOLUBLE NITROGEN CONSTITUENTS

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Summary

The ninhydrin-reacting compounds of the apple fruit have been studied during its development on the tree. The compounds present show little variation, but glutamine, conspicuous in very young and in over-mature apples, disappears at intermediate stages. The increase in soluble nitrogenous compounds occurring in over-mature apples left on the tree (Pearson and Robertson 1953) is confirmed qualitatively. An account is given of the chromatographic behaviour of several unidentified ninhydrin-reacting substances found in extracts of apple fruits, leaves, and stems.

I. Introduction

Previous communications from this laboratory (Robertson and Turner 1951; Pearson and Robertson 1953) have reported that in the developing Granny Smith apple cell division is completed about 6 weeks after blossoming, subsequent growth being due to cell enlargement. Total nitrogen and protein nitrogen per cell are correlated with cell surface rather than cell volume, suggesting that the enlarging cell maintains a layer of protoplasm of approximately constant thickness.

Up to the time at which the fruit is normally picked there is a high correlation between the amounts of protein nitrogen and of soluble nitrogen per cell, but in fruits left longer on the tree soluble nitrogen increases faster than protein nitrogen (Pearson and Robertson 1953). The present paper reports a qualitative study of the ninhydrin-reacting substances of the Granny Smith apple during its development on the tree. These substances were extracted with alcohol, adsorbed on "Zeo-Karb" 215, eluted with ammonia, and identified by paper chromatography; the technique used detected only large changes in concentration. Similar studies were made with leaves and branches.

II. Material and Methods

The work was carried out in the 1950-51 and 1951-52 seasons, results in both being similar. The data cited in the paper, unless otherwise stated, refer to the later season.

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(*a*) *Sampling*

All the fruits, and some of the leaves and branches analysed in this work, came from one tree at Bathurst, N.S.W. This tree also supplied part of the fruit used in earlier studies. Sampling dates and other details are shown in Table 1.

TABLE 1

SAMPLING DATES

Pick No.	Date 1950-51	Days from Full Blossom	Date 1951-52	Days from Full Blossom
1	26.xi.50	54	3.x.51	0
2	18.xii.50	76	10.xi.51	38
3	15.i.51	104	7.i.52	96
4	12.ii.51	132	18.ii.52	138
5	12.iii.51	160	25.iii.52	174
6	9.iv.51	188	5.v.52	215
7	7.v.51	216	23.vi.52	264
8	4.vi.51	244	7.vii.52	278
	18.vi.51 Tree bare, fruits and leaves picked up from the ground	258		

The sampling technique was designed (Pearson and Robertson 1953) to provide a statistically random sample of fruits from all parts of the tree. Leaves for analysis were picked immediately behind fruits on the experimental tree or on other Granny Smith trees. At 244 days from full blossom (1950-51), when most of the leaves had already fallen, the sample of leaves was taken from all parts of the tree. When the last sample was taken in 1950-51 the tree was already bare of fruits and leaves, and those analysed were picked up from the ground beneath it, the leaves being already brown and moist.

(*b*) *Extraction*

The material was dropped into absolute ethanol as soon as possible after cutting. At 0 days the complete flowers were used, and at 34 days the whole fruit less seeds, but later the cortex was analysed separately. About 100-400 g of tissue was taken for analysis of mature fruits and ethanol added to a final concentration of about 75 per cent. The tissue was then ground in the alcohol for 5 min in a Waring Blendor and the homogenate filtered through a Whatman No. 31 filter-paper on a Buchner funnel without pressure.

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

The ethanolic extract was passed down a column of "Zeo-Karb" 215 (10 g). The presence of ethanol did not interfere with adsorption of the amino acids. The column, prepared by the method of Partridge and Westall (1949), was first treated with aqueous alcohol of the same concentration to prevent precipitation of chlorophyll. It was eluted with ammonium hydroxide (approximately

1N) until the eluate no longer gave a reaction with ninhydrin. The column was then eluted with 2.5N sodium hydroxide and the eluate tested for arginine (Sakaguchi 1925). The ammonia eluate was evaporated to dryness under reduced pressure, taken up in 2 ml of water per 100 g wet material, and filtered. The filtrate was then ready for chromatography.

(*d*) *Protein Hydrolysis*

The residue filtered from the ethanolic extract was washed several times with 75 per cent. aqueous ethanol and hydrolysed under reflux for 24 hr with 5N hydrochloric acid. The hydrolysate was filtered, evaporated to dryness, taken up in about 20 ml of distilled water, refiltered, and passed through a "Zeo-Karb" 215 column to remove excess hydrochloric acid and other non-basic impurities. Subsequent procedure was similar to that described above for tissue extracts.

(*e*) *Chromatographic Technique*

(i) *Solvents.*—The ascending method of Williams and Kirby (1948) was used as modified by Wolfson, Cohn, and Devaney (1949). For two-dimensional chromatograms 80 per cent. phenol was followed by a mixture (4 : 1 : 1 by volume) of butyl alcohol, acetic acid, and water (Reed 1950); in early work the non-aqueous phase of the corresponding 4 : 1 : 5 mixture (Partridge 1948) was used. Aqueous ethanol (75 per cent.) (Patton and Foreman 1949; Bentley and Whitehead 1950) was occasionally used as second solvent. Phenol was removed by drying at 40-50°C. The butyl alcohol-acetic acid was removed by heating similarly, or by air-drying for several hours. The papers were then sprayed with ninhydrin (0.1 per cent. in absolute ethanol) and placed at 40°C, the temperature being raised to about 100°C in 45 min. The chromatogram was examined frequently, each spot being outlined when it first appeared in order to separate spots which overlapped on further heating.

(ii) *Filter Paper.*—Whatman No. 1 paper was used whenever available. Ekwip No. 1, stated by the manufacturers (Industrial Equipment (A/asia.) Pty. Ltd., Paddington, N.S.W.) to be identical with Whatman No. 1, gave only about two-thirds of the rates of solvent ascent with Whatman No. 1, being equivalent to Whatman No. 2. The map of spots, however, was similar on all three papers.

(*f*) *Estimation of Amide Nitrogen*

Amide nitrogen was estimated by the method of Vickery *et al.* (1935) on aqueous extracts of dried tissue.

III. Results

(*a*) *Identification of Individual Amino Acids*

The R_F values found with butyl alcohol-acetic acid are shown in Table 2. These values agree well with the unpublished data, for the same solvent, of Dr. T. M. Reynolds, of this Division.

Individual amino acids were identified using authentic specimens. A substance was considered to be identified only when it gave a single spot with an authentic specimen on two two-dimensional chromatograms prepared with

phenol followed respectively by butyl alcohol-acetic acid and by 75 per cent. aqueous ethanol.

TABLE 2

R_F VALUES OF VARIOUS AMINO ACIDS IN THE NON-AQUEOUS PHASE OF BUTYL ALCOHOL-ACETIC ACID-WATER (4 : 1 : 5)

Amino Acid	R_F
Asparagine	0·09
Arginine	0·14
Aspartic acid	0·20
Serine	0·23
Glycine	0·24
Threonine	0·25
Glutamic acid	0·25
β-Alanine	0·27
α-Amino-*n*-butyric acid ..	0·37
α-Amino-*iso*butyric acid ..	0·37
Valine	0·47
Phenylalanine	0·57
Leucine	0·62

The colour reactions of isatin (Grassman and von Arnim 1935; Acher, Fromageot, and Jutisz 1950; Hulme and Arthington 1952) with amino acids were also studied, results being shown in Table 3.

(*b*) *Unknown Ninhydrin-reacting Substances*

In the various extracts examined, three unidentified ninhydrin-reacting substances occurred regularly, and several other spots appearing occasionally are believed to indicate further unidentified constituents. Those occurring regularly will be cited as unknowns *A*, *B*, and *C*. They form distinct spots when chromatographed with added anserine, carnosine, citrulline, ornithine, arginine, lysine, histidine, N-methylhistidine, sarcosine, theanine (ethylglutamine), γ-methyleneglutamine, baikiain (1,2,3,6-tetrahydropyridine-2-carboxylic acid), nipecotinic acid, and putrescine.

In phenol *A* runs just ahead of valine, and in butyl alcohol-acetic acid level with threonine; it gives a yellow colour with ninhydrin and a blue colour with isatin. *A* also gives a blue spot with a spray containing nitroprusside and acetaldehyde (Feigl 1939). These colour reactions are all consistent with its being an imino acid. In phenol *B* runs level with *A* and just ahead of it in butyl alcohol-acetic acid; it gives a purple colour with ninhydrin. In phenol *C* runs just behind *B* and in butyl alcohol-acetic acid level with asparagine; it gives a yellow-brown colour with ninhydrin.* A purple spot overlapping *C* on standard ninhydrin-sprayed two-dimensional chromatograms, but displaced in phenol-ammonia to a position ahead of proline, is attributed to arginine. This

* Note added April 1953: recent tests with diazotized *p*-bromaniline indicate that *C* is histidine.

identification was made after most of the work reported in this paper had been completed. It is based on the overlapping of the spot in question with that of added arginine in three separate solvents, and on the deep red spot formed in the same positions on chromatograms sprayed with a modified Sakaguchi reagent (Roche *et al.* 1951). Arginine is incompletely eluted from "Zeo-Karb" 215 by ammonium hydroxide. Its distribution in the samples examined is therefore uncertain, but the greatest concentrations are found in the bark.

TABLE 3

COLOUR REACTION OF VARIOUS AMINO ACIDS WITH ISATIN

Amino Acids	Colour
Aspartic acid .. .. Glutamic acid	Dark purplish blue
Threonine .. .. Serine .. .. .. Tyrosine	Light brown
β-Alanine .. .. γ-Aminobutyric acid ..	Dark blue
Proline .. .. Hydroxyproline ..	Bright blue
Pipecolinic acid ..	Bluish green
Asparagine, glutamine, valine, leucine, glycine, alanine	Pink

(*c*) *Individual Amino Acids in Fruit*

The free amino acids detected in the apple fruit at various times during its development are shown in Table 4. The most prominent in every sample was asparagine, although it crystallized from concentrated extracts before chromatography. The other prominent amino acids were aspartic acid, glutamic acid, serine, threonine, and alanine. Valine, proline, γ-aminobutyric acid, methionine sulphoxide, and "leucine" were also present in all samples; "leucine" is one or both of isoleucine and leucine, which were not separated by the technique used. Nearly all the amino acids showed a high concentration at 0 days (blossom), dropping sharply at 38 days, with a slight further drop at 96 days, after which little change appeared until 264 days, when there was a considerable increase, though not reaching the initial level. Thus over the period of increasing cell size (Robertson and Turner 1951) their concentration remains approximately constant. The late increase in concentration occurs after cell enlargement ceases.

Minor components were demonstrated in chromatograms of more concentrated extracts of fruit from some picks, and are recorded in Table 4. As asparagine crystallized out before chromatography, changes in its concentration cannot be deduced from the chromatograms.

TABLE 4

AMINO ACIDS DETECTED AT VARIOUS STAGES OF THE DEVELOPMENT OF THE GRANNY SMITH APPLE FRUIT (1951-52 SEASON)

Days after Full Blossom	Aspartic Acid	Glutamic Acid	Asparagine	Glutamine	Serine	Alanine	Tyrosine	Valine	Leucine and/or Isoleucine	Proline	β-Alanine	Threonine	γ-Aminobutyric Acid	Hydroxyproline	Pipecolinic Acid	Methionine Sulphoxide	Phenylalanine	Glycine	Unknowns		
																			A	*B*	*C*
Sample Fruit																					
0	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
38	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	×
96	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
138	×	×	×	—	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
174	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
215	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	×
264	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
278	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	—	—	×	×	×
Leaves																					
0	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	—	—	×	×	—
38	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	—
96	×	×	×	×	×	×	×	×	×	×	—	×	×	—	—	×	—	—	×	×	—
138	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	—
174	×	×	×	×	×	×	×	×	×	×	×	×	×	—	—	×	×	—	×	×	—
215	×	×	×	×	×	×	—	×	×	×	—	×	×	—	—	—	×	—	×	×	—
Branches																					
215	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
Hydrolysate of protein from fruit 264 days	×	×	—	—	×	×	×	×	×	×	—	×	—	×	—	×	×	×	—	—	—

× = Present; — = not detected by technique used.

The trend described above holds for aspartic acid, alanine, serine, threonine, and γ-aminobutyric acid, and particularly for "leucine" and valine. Glutamine shows the trend in an exaggerated form, being prominent in early and late picks, and faint at other times; it could not be demonstrated, even in concentrated extracts, at 138 days. Pipecolinic acid was present in all samples but was prominent only at 0 and 38 days. Methionine sulphoxide was always present; methionine sulphone sometimes occurred in small amounts. Both

these may be artifacts produced by oxidation of methionine. Methionine sulphoxide increased steadily relative to the other amino acids in the course of each season, but was never a major constituent. Phenylalanine reached its greatest prominence at 96 days, and decreased later. β-Alanine was present always as a minor constituent, as were unknowns *B* and *C*. The yellow spots of proline and unknown *A* made it hard to compare their concentrations with those of the other amino acids; both were always present.

(*d*) *Amino Acids in Leaves*

Leaves differed from the fruit mainly in having less asparagine and more glutamine. On a wet-weight basis the amino acids were always less concentrated in leaves than in fruit. Asparagine crystallized from extracts of fruit but not of leaves. All the amino acids found in the fruits were found in the leaves also, though unknown *C* was found only in the earlier season. The most prominent were aspartic acid, asparagine, glutamic acid, glutamine, serine, alanine, threonine, valine, "leucine," γ-aminobutyric acid, and unknown *A*, which was more prominent compared with proline in the leaves than in the fruit. The concentration of all amino acids was less at 216 days in the 1950-51 season than in earlier picks and less still at 244 days, when aspartic acid was missing. In fallen leaves picked up at 257 days beneath the bare trees no amino acids could be identified, though the extract gave a faint colour with ninhydrin. A similar decrease had started by 215 days in the 1951-52 season; alanine gave a prominent spot, glutamic acid and asparagine small spots; but no other amino acids showed more than a trace. The decrease in amino acids in the leaves may, as suggested by Pearson and Robertson (1953), be related to the increase in amino acids in the fruit.

(*e*) *Amino Acids in Branches*

All the amino acids recorded for the fruit were found also in tthe branches, asparagine, alanine, glutamine, and sometimes unknowns *A* and *C* being particularly prominent.

(*f*) *Amino Acids in Protein Hydrolysates*

Acid hydrolysates of fruit protein at 188 and 216 days (1950-51) and 257 days (1951-52) showed many of the amino acids found free in the fruits, leaves, and stems. The most notable differences were the presence in the protein of glycine and hydroxyproline, and the absence of pipecolinic acid, β-alanine, γ-aminobutyric acid, and all the unknowns. Tyrosine was more prominent in the protein than in the free amino acids. Asparagine, glutamine, and tryptophane, if present in the protein, would be destroyed by acid hydrolysis.

(*g*) *Changes in Amide Nitrogen*

Amide nitrogen was estimated on dried samples of the last four picks of the 1950-51 season. The results are shown in Table 5. The last five columns in this table are derived from the data of Pearson and Robertson (1953).

IV. Discussion

The amino acids of apple fruits have been studied also by Joslyn and Stepka (1949), Hulme and Arthington (1950*a*, 1950*b*, 1952), and Hulme (1951). Joslyn and Stepka (1949) used dilute extracts from which sugars were not removed; they recorded only asparagine, aspartic acid, serine, γ-aminobutyric acid, valine, and leucine, but the failure to detect other amino acids cannot establish their absence. The amino acids found by Hulme and Arthington (1950*a*) in Bramley's Seedling apples included glycine and tryptophane, neither found as yet in Granny Smith. The amino acids found in Granny Smith but not in Bramley's Seedling are threonine, tyrosine, and the unknowns mentioned earlier. Hulme and Arthington (1950*b*) report glycine also in the varieties Worcester Pearmain and Cox's Orange Pippin, which both contain hydroxyproline, not yet found in Granny Smith. Hulme (1951) reports in the acid hydrolysate of the protein of Bramley's Seedling fruits all the amino acids, except hydroxyproline, recorded here for a similar preparation from Granny Smith; he found also arginine and lysine. Pipecolinic acid, already known from the apple (Hulme and Arthington 1952), is a recently recognized plant constituent which may be generally distributed. It has been recorded in several species by Morrison (1952) and by Zacharius, Thompson, and Steward (1952) and has since been found (Morrison, personal communication) in 25 out of 33 angiosperms examined.

Table 5

AMIDE NITROGEN IN RELATION TO OTHER NITROGEN FRACTIONS (1950-51 SEASON)

Days After Full Blossom	Amides as % Dry Weight	Amides as % Total Nitrogen	Amides as % Soluble Nitrogen	Amide Nitrogen ($g \times 10^{-10}$/cell)	Soluble Nitrogen ($g \times 10^{-10}$/cell)	Soluble Nitrogen Other Than Amide ($g \times 10^{-10}$/cell)
160	0·11	35	80	8	10	2
188	0·10	40	80	8	10	2
216	0·10	35	67	10	15	5
244	0·13	33	58	14	24	11

The present work has failed to detect glycine or the sulphur-containing amino acids, methionine, cystine, and cysteine, in alcohol extracts of Granny Smith apples. Of their oxidation products methionine sulphoxide occurs regularly and methionine sulphone sporadically. Methionine sulphoxide is found both free and in protein hydrolysates. It may be formed by the oxidation of methionine during analysis.

Pearson and Robertson (1953) found large increases in soluble nitrogen per cell at 216 and 244 days in the 1950-51 season, both samples being taken after the normal picking date for commercial fruit. Table 5, based on their results, shows that, though part of this increase is due to amides, there is also a fivefold

increase in non-amide soluble nitrogen per cell. The material analysed may have lost some amide in drying, but since extracts of dried material contain glutamine, losses of the more stable asparagine are probably small. Since asparagine is the main amide present, losses of amide in drying are unlikely to be important. Moreover, at 160 and 188 days (Table 5) most of the soluble nitrogen is amide nitrogen. This is incompatible with extensive amide breakdown, as half of the amide nitrogen lost would appear in an amino acid, and much of the other half would probably be retained as ammonium salts of organic acids.

The compounds involved in the increase of soluble nitrogen in the later samples are not specified by the present work, which does, however, confirm qualitatively the earlier quantitative observations of Pearson and Robertson (1953). There is a general increase in both amides and non-amide amino acids but nitrogenous compounds not reacting with ninhydrin may also be involved.

Glutamine disappears about the same time as starch accumulation (Pearson and Robertson 1953) reaches its maximum, suggesting a possible connection between the metabolism of protein and of carbohydrate; its clarification, however, must await further work. The disappearance of glutamine is not due to a shortage of the glutamic acid used in its synthesis. If views expressed earlier (Robertson and Turner 1951) on the relations between phosphorylations and the synthesis of starch and other substances in the apple are correct, the reduced glutamine content may reflect a shortage of adenosinetriphosphate, known (Elliott 1948; Speck 1949) to be required for its synthesis *in vitro*.

The almost complete constancy of the amino acid pattern during the development of the apple fruit is a striking and rather surprising feature of the results. It appears that, even when intense protein synthesis occurs, the amino acids (except glutamine) likely to occur as residues in protein molecules are always in excess.

V. Acknowledgments

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