XLI. BIOCHEMICAL STUDIES IN THE NITROGEN METABOLISM OF THE APPLE FRUIT.

II. THE COURSE FOLLOWED BY CERTAIN NITROGEN FRACTIONS DURING THE DEVELOPMENT OF THE FRUIT ON THE TREE.¹

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WHILST the carbohydrate, pectin and acid metabolism of the apple fruit during its growth on the tree, and of the detached fruit placed at various constant temperatures in darkness, have been closely studied, the nitrogen metabolism has received little detailed attention.

The results of the work on the metabolism of carbon make it clear that there are three physiologically important stages in the development of the apple. Firstly, meristematic cell-division ceases at an early stage in development—when the fruit weighs only 1 or 2 g. Secondly, there is a sharp maximum in the curves representing the concentration of starch during August, the exact date of this maximum varying slightly in different varieties of apples. Thirdly, the concentration of sugars, which is low in the very young fruits, rises to a maximum as the fruit matures.

It is obviously important to ascertain whether any such definite stages occur in the metabolism of nitrogen in the fruit.

The first recorded attempt to determine changes in the constituent groups of the total nitrogen is that of Archbold [1925], who confined her attention to mature detached fruits. This worker was unsuccessful in her efforts to isolate an amount of soluble nitrogen sufficient for the estimation of the fractions thereof and came to the conclusion that the nitrogenous substances present in the apple fruit are all of a protein nature, a small fraction of this protein being water-soluble. Pilling and Pearsall [1930], in work of a preliminary nature, were able to obtain appreciable amounts of non-protein-nitrogen soluble in 60–80 % alcohol from mature apple fruits.

EXPERIMENTAL.

1. *Material and methods of sampling.*

Preliminary work has indicated that it is essential to devote considerable attention to the sampling of the material and to its preparation for analysis, as well as to the methods of chemical analysis to be employed. Duplicate samples must be prepared and analysed at intervals during the course of an experiment so that a statistical treatment of results becomes possible.

¹ The major part of this paper has been abstracted from a thesis accepted for the degree of Ph.D., Cambridge University, 1935.

The material and the method of sampling employed in the present work were, briefly, as follows.

Series HY/33. Fruit from 10 Bramley's Seedling trees on Stirling Castle stocks (18 years old) at Messrs Chivers' farm, Histon. Date of petal-fall,¹ May 15th, 1933. Firstly, each tree and each main branch of each tree were numbered; each apple and each main branch were also given a number, the first apple on each branch being number 1, the second number 2 and so on. Before each gathering the total number of fruits per tree to be gathered was decided. Next, the branch and the apple on each branch to be gathered were determined by a random selection of numbers. Any fruit obviously blemished or dropped during gathering was discarded, and the next in order up its branch was taken in its place. When two samples were gathered at once, alternate fruits were placed in each of two tins to give the two samples. Each sample was composed of not less than 25 fruits. The fruit was transferred to the laboratory in tins provided with well-fitting lids to minimise evaporation.

Series MBY/34. Fruit from 18 Bramley's Seedling trees (15 years) on East Malling stocks I and II on an unmanured plot at East Malling Research Station. Date of petal-fall, May 20th, 1934. Method of sampling exactly as for series HY/33.

Immediately on reaching the laboratory the fruit was weighed and halved, and the seeds were removed and weighed into sealed boiling-tubes. Fruit and seeds were then transferred to a room maintained at -20 to -25° to await preparation for analysis.

2. Preparation of material for analysis.

Owing to the time taken for analysis *etc.*, it became obvious that a method of preparation of the material for analysis must be chosen which would allow of at least several weeks of storage prior to analysis. The method finally adopted was that described by Onslow *et al.* [1927].

The advantages of this method of preparation and storage of the material are that it allows of:

- (1) Reasonably constant separation into peel and pulp.
- (2) Complete disintegration of the tissue.
- (3) Thorough mixing of the resultant frozen powder.
- (4) No change in the nitrogenous fractions in the tissue over periods of at least 2 months [Hulme, 1932].

Since, as will be seen later, soluble nitrogen is to be extracted with 60–80% alcohol, this alcohol can be added to the frozen powder, so that no enzymic action is likely to occur during thawing of the tissue.

Separation of peel and pulp. The difficulty in any attempted separation of the peel and the pulp of the apple fruits is that while "true" peel-cells differ anatomically and chemically from "true" pulp-cells, the two tissues are contiguous, and merge into one another in the region of their juncture. The procedure found to give as good a separation as possible without special treatment (which might interfere with the distribution of nitrogen in the tissues) was to peel the apples while frozen hard with an ordinary domestic potato peeler set to a slightly smaller cutting angle than normal. Microscopic examination of the "peel" thus obtained, whilst showing, as would be expected, that a shallow layer of pulp was included, nevertheless indicated that a reasonably uniform separation could be achieved. It should be noted that the peel has a considerably higher

¹ "Petal-fall" refers to the stage when practically all the petals had shrivelled and fallen from the young apple fruits—the weight of the fruit at this stage was of the order of 0.2 g.

content of nitrogen than the pulp and differs considerably from it in the distribution of the various fractions of the nitrogen.

The values for the ratio pulp/peel for the two series of apples are shown in Table I. Care was taken to lose as little tissue as possible during peeling, and from the total weight of peel and of pulp for each sample (taken before grinding) the amount of each of these tissues per apple of the sample was calculated.

Table I.

Series	...	HY/33				MBY/34					
		Days' development		Loss	Ratio			Days' development		Loss	Ratio
Sample		(from petal fall)	Av. wt./ apple	on peeling* (%)	pulp/ peel	Sample		(from petal fall)	Av. wt./ apple	on peeling* (%)	pulp/ peel
V		22	8.0	<1	2.81	I		19	3.2	4.1	2.30
VI (1)		29	18.6	2.8	4.95	II (1)		26	6.5	5.2	3.84
VI (2)		29	18.9	2.8	4.77	II (2)		26	6.5	4.0	4.01
VII		43	50.3	2.3	6.67	III		33	16.7	2.3	5.22
VIII		58	84.8	1.8	6.50	IV a (1)		40	26.2	3.2	5.52
IX (1)		67	113.2	1.4	6.96	IV a (2)		40	25.6	3.2	5.43
IX (2)		67	111.1	1.6	6.06	V		51	41.1	2.6	8.24
X		77	124.9	1.9	8.79	VI a (1)		60	49.7	3.8	7.02
XI (1)		93	168.4	2.0	8.75	VI a (2)		60	52.7	3.2	7.22
XI (2)		93	164.6	1.3	7.76	VII		73	71.8	4.0	7.03
XII		106	187.9	1.1	10.0	VIII		92	102.4	1.2	7.78
XIII a (1)		124	219.0	1.1	11.1	IX a (1)		116	123.8	3.1	8.01
XIII a (2)		124	218.5	1.0	11.0	IX a (2)		116	126.9	2.7	8.19
†XIII b (1)		124	209.0	2.0	11.4	X		138	159.4	3.2	7.10
†XIII b (2)		124	214.0	2.3	10.8	XI a (1)		150	157.6	2.8	8.18
XIV		141	245.6	1.7	12.5	XI a (2)		150	164.3	2.5	8.38
XV (1)		170	243.7	1.8	12.0	XII a		163	172.7	1.8	8.14
XV (2)		170	240.0	2.0	11.8	XII b		163	178.1	2.6	8.53
Average value 1.8						Average value 3.1					

* The inevitable loss of a small amount of tissue during peeling (see Table I, columns 4 and 9) causes a slight error in the calculations, but it is clear that this error is very small and reasonably constant.

† Samples for experimental storage not described in this paper.

3. Methods of analysis.

Determination of the total nitrogen content of the tissue.

Total nitrogen was determined on aliquot portions of the frozen, ground tissue by the usual macro-Kjeldahl method. Normally 50 g. of the pulp and 30–40 g. of the peel were taken for each determination. Despite the tendency for the digestion mixture to froth over during the early stages of heating it was found preferable not to dry the tissue before digestion, since this latter process was slow and gave erratic results. In agreement with the results of Pilling and Pearsall [1930], nitrates were found normally to be absent from the material, so that modifications of the Kjeldahl method to include these compounds were not used in routine determinations. In later work selenium was used [Lauro, 1931] in addition to copper sulphate as catalyst, thereby considerably reducing the time of digestion. The agreement obtained in these estimations is illustrated by the following results for six 50 g. sub-samples of a sample of pulp: average nitrogen content 0.0345%; standard deviation 0.00045%, average "blank" value on reagents alone, 0.0002%.

Separation of protein- and non-protein-nitrogen. In the present instance it was not desired, necessarily, to determine the protein-nitrogen directly, but to be certain of its separation from the simpler non-protein-nitrogen in order that a detailed investigation of the latter might be made. The method of attaining this object was based on the following facts established after a lengthy survey of the problem, using Bramley's Seedling apple fruits.

1. Water in the cold (with or without neutralisation of the free acid of the tissue as extraction proceeded), Mothes's tannin solution [Mothes, 1926] and 60–80% alcohol (under the conditions of the "standard" alcohol extraction method described below) extract equal amounts of nitrogen from apple pulp tissue. In the case of peel tissue small differences occur in the total extractable nitrogen which are reflected in the "precipitated" nitrogen fraction (see below). Such differences appear to be connected with the difference in solubility in water and in alcohol of the chlorophyll and associated pigments of the tissue.

2. Excluding the seeds, proteins directly soluble in water¹ or in 60–80% alcohol appear definitely to be absent from the apple fruit. In view of the work of Finn [1932] on the effect of freezing in relation to denaturation of proteins it cannot be argued with any certainty that the method of preparation of the tissue for analysis, involving as it does rapid freezing, is likely to lead to denaturation of protein with consequent loss of solubility in water. The results of Lincoln and Mulay [1929] for plant material also tend against such a possibility.

3. Extraction of the tissue with boiling 80% alcohol at atmospheric pressure gave a significantly lower result for total non-protein-nitrogen as compared with that obtained by the three methods described above.

4. After hydrolysis of a sample of apple pulp tissue with 25% hydrochloric acid, removal of excess acid, adjustment of the p_H of the hydrolysate to 3.5 (within the region of the p_H of fresh juice from Bramley's Seedling apples) followed by extraction of this hydrolysate with 70% alcohol exactly as in the "standard" method described below, 99% of the total nitrogen of the tissue was recovered in the extract finally obtained.

5. Hiller and Van Slyke [1922], working with blood, found that whilst 84% alcohol precipitated intermediate protein hydrolysis compounds fairly completely and consistently, about 27% of the free amino-acids present were adsorbed on the protein precipitates. These workers indicated, however, that this fraction (adsorbed amino-acids) "is sufficiently constant to validate conclusions drawn from comparable results in physiological experiments". In the case of apple tissue the similarity in the amount of nitrogen extracted by water and by alcohol makes it clear that there can be no appreciable adsorption of amino-acids on protein precipitated within the tissue by alcohol when the latter is used as extractant.

6. It is extremely difficult to attain a satisfactory separation of water extracts from the residual apple tissue. Filtration, with or without suction, is impossibly slow for routine work and centrifuging is little better. On the other hand alcoholic extracts filter readily. This difference is due, apparently, to the large amount of pectin present in the tissue, which, during water extraction, forms a slimy gel around the surface of the tissue.

In view of these considerations the following "standard" alcohol extraction method was used to obtain the non-protein-nitrogen fraction of the fruit tissue.

A suitable amount of tissue (300–500 g. in the case of pulp and 100–250 g. in the case of peel, depending on the amount of nitrogen present) was allowed to stand overnight at 12° with sufficient 97% alcohol to make the final alcohol concentration about 65%, assuming the moisture content of the tissue to be 85% of its fresh weight. The alcoholic extract (1st extract) was then filtered through a Soxhlet thimble of sufficient size to retain all the residual tissue. The tissue in the thimble was then extracted in a continuous vacuum extractor until extraction of soluble nitrogenous compounds was complete (2nd extract). This required 6–8 hours depending on the nitrogen content of the tissue. No attempt was made to neutralise the acids present in the tissues before or during extraction but the extractor was of such a form that the tissue only came into contact with alcohol at room temperature and the temperature of the extract either during extraction or during the subsequent evaporation of the alcohol never rose above 30°. The actual strength of the alcohol coming into contact with the tissue in the extractor was 85–87%. The 1st and 2nd extracts were combined, the alcohol was removed and the extracts were concentrated to a small volume *in vacuo*. The final alcohol-free extract was transferred to a standard flask and brought to a definite volume (60–150 ml. depending on the amount of nitrogen present). After bringing to volume the extract was filtered by suction through an asbestos pad. The residue on the pad was composed of com-

¹ It is interesting to note that Karmarkar [1934] found practically no water-soluble protein in the bark, wood and leaves of the Newton Wonder apple.

pounds which are precipitated during the removal of the alcohol (alcohol-soluble, water-insoluble compounds) and which appear to be chiefly chlorophyll and associated pigments. Its bulk was very small in the case of pulp tissue but larger in the case of peel. The nitrogen of this residue—designated “precipitated” nitrogen—was low and relatively constant (pulp 1–1.5% of the total nitrogen of the tissue; peel 2–3% of the total nitrogen).

Owing to the small amount of nitrogen present it was necessary to use micro-methods for the investigation of the nitrogen content of the extracts. The following are the fractions determined and the methods used in their determination.

1. *Total soluble nitrogen.* This refers to the total nitrogen of the extracts after removal of the “precipitated” nitrogen. A modification of the micro-Kjeldahl method as described by Pregl [1924] was used in this determination. An investigation of several methods of decreasing the time of the acid digestion led to the employment of hydrogen peroxide in this connection. As found by Christiansen and Fulmer [1927] for macro-Kjeldahl determinations on yeast, the judicious use of this reagent, apart from greatly accelerating the digestion, also gave results as much as 10% higher than those obtained by the normal digestion method. 2 ml. of 100 vol. hydrogen peroxide (B.D.H., N.A.R.) were found to give optimum figures for the nitrogen content using 1 ml. of a whole range of pulp and peel extracts. The method adopted was to add to 1 ml. of extract 1 ml. of hydrogen peroxide together with 2 ml. of sulphuric acid and heat gently for about 15 min. After cooling slightly a further 1 ml. of hydrogen peroxide and 3 ml. of sulphuric acid were added together with the usual amount of potassium sulphate and copper sulphate.¹ The digestion and subsequent steam-distillation were proceeded with as usual using N/70 acid with methyl red indicator to absorb the ammonia evolved in the latter process.

2. *Free ammonia- and amide-nitrogen.* Free ammonia was estimated as follows: 10 ml. of apple extract were just neutralised with 40% NaOH, 5 ml. of a 10% suspension of pure magnesium oxide were added and the ammonia liberated was distilled under diminished pressure at 40° into N/70 acid. The special micro-distillation apparatus used was the same as that used in the amide-nitrogen estimations. Amide-nitrogen was determined by the method used by Chibnall and Westall [1932]. 5 ml. lots of the apple extracts were used in this estimation. On employing the method suggested by Chibnall and Westall for the differential estimation of asparagine and glutamine with several extracts from mature apples, it became clear that glutamine was absent. The assumption was made, therefore, that all the amide-nitrogen originated from asparagine. The possibility of the existence of glutamine in immature apples was not investigated, but this compound is unlikely to be stable in so acid a medium as that of the Bramley's Seedling apple. Hence the *asparagine-nitrogen* = *amide-nitrogen* × 2.

3. *Amino-nitrogen.*² This fraction was estimated on the residue from the ammonia-nitrogen estimation by the method already described [Hulme, 1935, 1]. Various modifications of the formaldehyde and alcohol titrations for the estimation of amino-acid groups were also investigated. It was found impossible to obtain satisfactory results with these methods chiefly owing to the large amount of colouring matter present. Richardson [1934] has also pointed out the advantages of the Van Slyke gasometric method for estimating amino-nitrogen in coloured biological liquids.

Amino-acid-nitrogen = *amino-N* – *amide-N*.

4. “*Rest*”-nitrogen = total soluble nitrogen – (free ammonia-N + asparagine-N + amino-acid-N).

5. “*Protein*”-nitrogen = total N – total soluble N.

Titrateable acid was also estimated in the apple extracts. 2–5 ml. of extract were titrated with 0.1N NaOH, after dilution to 500 ml., using chlorophenol red ($p_K=6$) as indicator according to the suggestion of Bennet-Clark [1933]. Since the colouring matter present interfered somewhat with the end-point in these estimations—even after the dilution of the extracts—the end-points were checked with the Morton glass electrode. The titrateable acid figures given in the present paper were calculated as malic acid. All p_H values quoted were obtained by means of the Morton glass electrode.

¹ Since the above method was evolved it has been noted that Pregl [1930] now recommends the use of hydrogen peroxide to accelerate the micro-Kjeldahl digestion.

² This fraction is, of course, derived entirely from aliphatic amino-groups.

Statistical treatment.

Standard deviations were calculated from the following equations [Fisher, 1928].

Variance = $\Sigma \partial^2 / (\text{degrees of freedom} - \text{no. of means})$, ∂ being the deviation of duplicates from their mean.

Standard deviation, $\sigma = \sqrt{\text{variance}}$.

The significant difference between two consecutive results in a series was obtained from the equation

$$\text{Sig. } D = \sigma \times \sqrt{2 \times t},$$

values of t ($P=0.05$) being obtained from Fisher's tables. In the many cases examined the standard deviation of sampling (for all the nitrogen fractions) was found to be 3 or 4 times as great as the standard deviation due to extraction alone (this latter of course also includes deviations due to experimental "error" of actual estimation), hence the former deviation only will be considered for each series. The vertical lines given to the right of the progress curves for each nitrogen fraction in the figures represent the magnitude of the significant differences calculated from this standard deviation.

RESULTS.

The results obtained are given in Figs. 1 to 9.

The following is a key to Figs. 2 to 8.

■	Total nitrogen	} Series MBY/34
●	"Protein"-nitrogen	
○	Total soluble nitrogen	
□	Amino-acid-nitrogen	
△	Asparagine-nitrogen	
▲	"Rest"-nitrogen	
×	Free ammonia-nitrogen	

The results for series HY/33 are expressed by the same symbols with a vertical line through them, a discontinuous line being used to join up the points. Total nitrogen is plotted as mg. per apple and as a percentage of the fresh

Fig. 1. Growth curves for the two series of apples.

Fig. 2. Total nitrogen as % fresh weight.

A in the figures refers to results for fruits attached to the tree and B to results for fruits which had fallen into small bags attached to their branches at the time of the penultimate gatherings. The chemical results for the pulp were practically identical in both cases and are not plotted separately.

Fig. 3. Total nitrogen, mg./apple
 Fig. 4. "Protein" total soluble nitrogen, mg./apple. I. Peel. II. Pulp.
 Fig. 5. Total soluble nitrogen. I. Peel. II. Pulp.
 Fig. 6. Pulp. Soluble N fractions, mg./apple. I. Peel. II. Pulp.

weight. The remaining nitrogen results are expressed in mg. per apple and as a percentage of the total nitrogen. Acid is expressed in g. per apple. Results for this fraction expressed as g. per 100 g. of water (the amount of water in the

Fig. 7.

Fig. 8.

Fig. 7. Pulp. Soluble N fractions as % total nitrogen.

Fig. 8. Peel. Soluble N fractions. I and II in mg./apple.
III and IV as % total nitrogen.

Fig. 9. Titratable acid (as malic).

tissue was obtained by evaporation at 55° *in vacuo* and determination of the weight of the dry residue) showed a steady fall throughout development in both series after about the 40th day.

The primary object of the present paper is to place on record the data expressed in the figures so that only the more obvious points arising from these data will be commented upon.

The different characters of the two sets of fruit are well illustrated in Fig. 1 from which it is seen that the maximum weight of the fruit in series HY/33 was approximately 245 g. and in series MBY/34 only about 165 g.

In the case of series HY/33 the amino-nitrogen was determined by a modification of the Van Slyke method giving only a partial correction for tannin (caseinogen precipitation method [Hulme, 1935, 1]). From estimations of the approximate tannin content of the fruit of this series at various stages during development [Hulme, 1935, 2] it was evident that from about the 80th day onwards, in the case of the pulp tissue, the amount of tannin present was insufficient seriously to affect the Van Slyke results obtained. Hence these latter results are given in the figures from gathering X onwards (see Table I) and may be considered accurate enough for purposes of comparison with the general drift in series MBY/34. No amino-acid results for the peel of series HY/33 are given, since throughout the series the amount of tannin present was relatively large.

The most striking difference between the results for the two series is the opposite directions of the trend in total soluble and asparagine-nitrogen, when considered as a percentage of the total nitrogen (Figs. 5, II and 7, II), during the first period of development. Although this difference emphasises the need for a further study of this region, it may not be so significant as appears at first sight. At this early stage in the development of the fruits the cells of the tissue are densely filled [Tetley, 1931] and metabolic changes are relatively great. Furthermore, results for earlier samples of series HY/33, obtained for whole fruits (in these samples the apples were too small for peeling), gave, at day 8, total soluble nitrogen 24.5% of the total nitrogen and at day 15, 27.0%. If the results for this fraction at days 22 and 29 are calculated for the whole fruit the values obtained are 36.6 and 29.5% respectively. The asparagine-nitrogen figures for these four dates show a similar relation to one another. Hence it would appear that the soluble and asparagine-nitrogen results for day 22 (series HY/33) are abnormally high and further work may show that during this early stage of development the results for series MBY/34 more truly represent the general course of events.

From about the 30th day onwards there is a distinct similarity between the two series in the general drift of the nitrogen fractions when due weight is given to the values of the significant differences. This is especially striking in view of the different type of the fruit in each series. It would appear, therefore, that the processes underlying the recorded changes are fundamental in the development of apple fruits—at least of the Bramley's Seedling variety. Taking the results for the pulp as a whole three phases in the nitrogen cycle are apparent. An early stage of rapid increase in soluble nitrogen compounds, especially asparagine, is followed by a stage of relative equilibrium between protein and non-protein compounds. Finally when the fruit is mature a state of net synthesis of protein ensues. This last phase is striking and quite unexpected in a senescent organ. It is also a feature of mature apples when detached from the tree and will be considered more fully in later papers.

In the case of the peel, as might be expected, changes in the nitrogen fractions are small but, in general, run parallel with changes in the pulp. Again, the most noteworthy fact is the apparent synthesis of protein when the fruit has reached maturity.

Finally the present data may be considered in relation to the suggestions of Ruhland and Wetzel [1926; 1927] that acid formation in plants (other than members of the Crassulaceae [Wolf, 1931]) is intimately connected with nitrogen metabolism.

The low p_H of the sap of the Bramley's Seedling apple would place it in the class of plants called by Ruhland and Wetzel "ammonia" or "acid" plants, in which case ammonium salts should predominate over amides. Actually the reverse proves to be the case. From Fig. 6, II, it is clear that asparagine is greatly in excess of ammonium salts in the tissues of the apple fruit throughout the whole growth period examined. In fact only at one point is the latter fraction present in appreciable amounts. The ammonia-nitrogen results for series MBY/34 only are given since the results for series HY/33 are substantially identical with them with the exception that the maximum near the end of the growth period is considerably lower. Further, in considering Ruhland and Wetzel's contention that the plant acids arise chiefly in the course of protein metabolism the present data show that this is unlikely to be the case in the apple, for the amount of acid present (Fig. 9) is always greatly in excess of that of any of the nitrogen compounds. The evidence is not, however, entirely conclusive since both the amount of acid entering the fruit from the tree and the amount (if any) of the nitrogen compounds passing from the pulp tissue to the seeds are unknown.

SUMMARY.

1. Details are given of the methods of sampling and preparation of the fruit for analysis.

2. The separation of the total nitrogen into the two fractions protein- and non-protein-nitrogen is briefly discussed and reasons are given for the use of alcohol extraction to attain this end in the present instance.

3. Methods are given for the estimation of total nitrogen and of the various constituent fractions of the soluble nitrogen.

4. Graphs are presented showing changes in size of fruit, total nitrogen, total soluble nitrogen, ammonia-nitrogen, asparagine-nitrogen, amino-acid-nitrogen, "rest"-nitrogen and titratable acidity during the course of development, from petal fall to maturity, of fruit from two series of Bramley's Seedling apple trees. The nitrogen fractions are given for both peel and pulp of the fruits.

5. Certain points arising out of these results are briefly discussed and it is concluded that there are three stages in the nitrogen cycle of the developing apple fruit.

6. The results for free ammonia and for amino-acid-nitrogen and titratable acid provide evidence which appears to be inconsistent with the hypothesis of Ruhland and Wetzel that acid formation in plants is intimately connected with the nitrogen metabolism.

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