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

III. PRELIMINARY EXPERIMENTS ON THE EFFECT OF INJECTING NITROGENOUS COMPOUNDS INTO APPLE TREES ON THE COMPOSITION OF THE FRUIT. (WITH AN APPENDIX DESCRIBING A NEW VACUUM EXTRACTOR.)

BY ALFRED CRESSWELL HULME AND
WILLIAM ARTHUR ROACH.

*From the Ditton Laboratory, and the East Malling Research
Station, East Malling, Kent.*

(Received 15 June 1936.)

THE nitrogen content of the mature¹ apple varies markedly both in total amount and in the manner in which it is distributed amongst the compounds in which it occurs [Hulme, 1932; 1934; and unpublished data]. The possible connexion between these initial differences of composition and the behaviour of the fractions of the nitrogen and of the fruit in general, when detached from the tree and kept under various conditions of environment, is being studied. The purpose of the present investigation was to determine whether the injection of nitrogenous compounds into the tree is followed by a modification of the nitrogenous composition of the fruit. In this event injection would be a valuable method for modifying at will the nitrogenous composition of the fruit as a preliminary to the above study. Methods have been developed for injecting whole trees so that each branch received its proper share; further, separate branches, twigs, leaves or portions of leaves may each be injected with a different solution without risk of the injected substance reaching any part of the tree other than the one for which it is intended.

Preliminary papers have already appeared [Roach, 1933; 1934, 1, 2, 3; 1935; Hearman *et al.* 1935] and the details of the methods are at present being prepared for publication. Such methods would make it possible to treat fruit at any stage in its development without affecting the relations between the tree and the soil and without undue interference with the physiology of the tree.

EXPERIMENTAL.

(1) *Material and details of tree-injection.*

Six bush trees of variety King Edward VII budded on Malling Type IX rootstock and four trees of variety Bascombe Mystery budded on Malling Type V rootstock were used for the experiment. The trees were planted as maidens in

¹ "Maturity" is here used in the somewhat loose sense of the stage at which the fruit is normally gathered for commercial purposes. It is not yet clear to what definite stage (if any) in the physiology of the fruit this approximates.

December 1921. The King Edward VII trees varied in branch spread from 2.1 to 2.8 m. and the Bascombe Mystery ones from 3.2 to 3.7 m. These trees varied in size and in other respects but they were amongst the few trees which escaped the disastrous frost of the night of 16–17 May 1935, and were used in the absence of any better material; they proved sufficiently uniform for the present purpose.

Injection. The injection of each tree was carried out as follows: A hole of $\frac{1}{4}$ in. diameter was bored through the main stem just below the crotch and so placed that each end came below the junction between two branches and so that the parts of the top on either side of the hole were as nearly equal as possible. One end of this hole was connected with rubber tubing to the reservoir containing the liquid which was supported a few inches above the level of the hole, and into the other end of the hole a short length of rubber tubing was inserted (see Fig. 1). Both pieces of tubing were inserted only to the depth of the bark. The

Fig. 1.

joints between the tubing and the bark were made watertight by painting with a molten solution of rubber and paraffin-wax. The siphon was started by sucking the short rubber tube, which was plugged when the liquid came through. The liquid was absorbed in from 1 to 3 days. Two Bascombe Mystery trees were injected a second time through holes at right angles to the first ones (see Table II).

Substances injected. The substances injected will be seen from Tables I and II. Each solution contained a standard amount of nitrogen per unit volume.

Dosage. A system for deciding the correct dosage for trees according to their size has not yet been worked out; the volumes of solution given were decided on by inspecting the trees and comparing them with others injected in the past, the aim being to give each tree an amount of nitrogen proportional to its size.

Leaf damage. Traces of damage appeared on the leaves of most of the trees the day after the injection was finished but this was of no importance; the foliage of the Bascombe Mystery tree injected with ammonium nitrate was, however, heavily damaged. This damage has been described elsewhere [Hearman *et al.* 1935]. Some leaves fell but later fresh leaves expanded and the tree again had a normal amount of foliage. Reference has already been made to the high toxicity of nitrate-nitrogen as compared with urea-nitrogen [Thomas & Roach, 1934].

(2) *Sampling of the material and its preparation for analysis.*

At the time of gathering the fruits were greatly reduced in number owing to falls, chiefly as a result of codling moth attack. All the fruits were gathered from each tree, any seriously blemished fruit being discarded. It was not necessary to discard any complete apples in the case of the King Edward VII variety, but

one or two fruits per tree had to be eliminated in the case of the Bascombe Mystery trees. Where only a small amount of the apple was damaged the damaged portion was cut out immediately before freezing and allowance made in calculating the weight of pulp and peel.

For the King Edward VII trees, details of the fruit as gathered are given in Table I which also includes details of the solutions injected into the tree.

Table I.

Sample “(K.E.)”	Tree	Injection 15. vii. 35	No. of fruits	Av. wt. of apple g.	Total wt. of pulp* g.	Total wt. of peel g.	Ratio pulp/peel
C (1)	6 + 12	None	32	124.4	3368	420	8.02
C (2)	12	None	32	130.0	3508	457	7.68
N	2	10 l. 0.33 % NH_4NO_3	30	124.3	3163	409	7.73
A	4	7 l. 0.55 % asparagine	30	126.6	3247	410	7.92
U (1)	10	10 l. 0.25 % urea	30	134.0	3400	430	7.91
U (2)	10	10 l. 0.25 % urea	2	155.7	1599	201	7.91
	8	10 l. 0.25 % urea	10				

* Corrected for seeds and bad areas removed. The amount of the latter was between 5 and 8 % of the whole of each sample. It was fairly uniformly distributed between the fruits of all the trees. The amount of peel tissue concerned was negligible.

In the case of the Bascombe Mystery trees (see Table II) the number of fruits per tree was larger although considerably less than at the time of injection. The larger number of fruits enabled more than one sample to be made up from each tree (except tree 3, urea treatment) and an additional sample was placed in the dark for 54 days at 9.5°, so that the change in the nitrogenous fractions after detachment from the tree could be studied. The concentrations of the various solutions injected were the same as for the King Edward trees (Table I).

Table II.

Sample	Tree	Injection		No. of fruits	Av. wt. of apple g.	Total wt. pulp g.	Total wt. peel g.	Ratio Pulp/ Peel
		15. vii. 35	1. x. 35					
CO (1)	1	None (control)		26	62.6	1335	207	6.45
CO (2)	1			30	69.0	1730	268	6.46
CI*	1			30	69.1	1607	270	5.95
NO	2	15 l. NH_4NO_3		30	52.9	1298	223	5.82
NI*	2			30	52.6	1205	226	5.58
UO	3	15 l. urea	10 l. urea	34	73.6	2064	330	6.26
AO	4	13 l. asparagine	11 l. asparagine	29	61.8	1470	236	6.23
AI*	4			28	62.7	1340	234	5.73

* Samples placed at 9.5° for 54 days. During this period there was, of course, some loss of weight as moisture and CO_2 . This loss of weight was measured and allowed for in the calculation of results.

From the values of the ratios of pulp/peel given in Table II it would appear, as might be expected, that the pulp tissue undergoes a greater loss in weight during storage than does the peel.

The material was frozen to -20°, peeled and prepared for analysis by the method previously described [Hulme, 1936].

(3) *Methods of analysis.*

The various fractions of the nitrogen and the titratable acidity were estimated by the methods already described [Hulme, 1936], with the exception that in the extraction of the soluble nitrogen an improved form of continuous vacuum extractor was used. This extractor, when used in conjunction with Naumann evaporators [Naumann, 1935] to remove the excess alcohol from the extracts, greatly reduced the time required to obtain the final extracts. A brief description of this new extractor is given in an appendix (p. 1402).

Urea was estimated in several samples—chiefly those from trees injected with urea—by a micro-method based on the urease method of Yee & Davis [1935].

It is, perhaps, improbable that urea injected into the tree would reach the fruits as such, unless it was in excess of that with which the leaves could deal. Nevertheless, it is important to make certain of this point, since, apart from the direct interest of the presence of urea in the fruits, appreciable amounts of urea in the extracts would seriously interfere with the estimations of amide and amino-nitrogen [Chibnall & Westall, 1932]. In no case were amounts of urea found greater than 0.3 % of the total nitrogen, and the fruit from trees injected with urea gave values similar to those injected with asparagine.

Glutamine determinations were made on the extract from sample K.E. C (1) by the method of Vickery *et al.* [1935] but this amide was found to be absent.

RESULTS.

The results obtained for the apples as gathered from the trees are given, for both varieties, in Table III, A, B, and for the stored fruits of the Bascombe Mystery variety in Table III c.

It is obvious from Table III that the injections have had a considerable effect on the total nitrogen content of the fruits. In all cases the total nitrogen of the fruits from injected trees is greater than in those from the uninjected ones. On the whole the figures for the pulp and the peel show the same general trend. The most striking fact is that, whilst the total nitrogen has increased considerably, the increase is mainly accounted for by an increase in the non-protein fraction. Whilst in the case of the King Edward variety the absolute amount of protein (even allowing for the difference in size of the fruits) is slightly higher in all the injected trees, the relative amount of protein (i.e. protein as % of the total nitrogen) is considerably decreased. The results for sample U (2) are probably "abnormal" since the sample was mainly composed of fruits from tree 8 which bore, at the time of gathering, only 10 fruits and these were much larger in size than those from any of the other trees of this variety.

The increase in soluble nitrogen is chiefly in the form of asparagine; the fraction of the soluble nitrogen in the form of amino-acids shows relatively little variation throughout the series. The same is even more strikingly true for the Bascombe Mystery series. This is in agreement with the view of Prianishnikow and many modern authors that asparagine may function as a means of storing excess nitrogen in a form innocuous to the plant.

In both series injection of urea appears to result in a small increase in the proportion of amino-acids and a lowering of the relative amount of protein. The two effects are not, however, equal quantitatively. The Bascombe Mystery fruits injected with ammonium nitrate are, as stated above, probably abnormal, and the very low proportion of protein which they contain is possibly due to the temporary damage to the tree following injection.

Table III.

Sample	Total N		Protein-N		Total soluble N		Free NH ₃ -N		Asparagine-N		Amino-acid-N		“Rest”		Titratable acid (as malic) mg./apple
	mg./apple	% fresh weight	mg./apple	% total	mg./apple	% total	mg./apple	% total	mg./apple	% total	mg./apple	% total	% total N	pH of extracts	
A. King Edward VII apples as gathered.															
Pulp K.E. C (1)	45.8	0.0435	24.5	53.4	21.3	46.6	0.19	0.19	10.3	22.5	5.8	12.7	11.1	2.79	1297
Peel K.E. C (1)	12.4	0.0943	10.5	84.8	1.9	15.2	0.19	0.19	0.7	5.4	0.6	4.9	4.7	—	147
Pulp K.E. C (2)	46.8	0.0428	25.0	53.4	21.8	46.6	0.25	0.25	11.7	24.9	6.2	13.2	8.2	2.83	1233
Peel K.E. C (2)	12.9	0.0904	10.9	84.4	2.0	15.6	0.20	0.20	0.8	6.0	0.7	5.1	4.3	—	149
Pulp K.E. N	56.3	0.0534	27.8	49.4	28.5	50.7	0.26	0.26	16.4	29.1	8.9	15.8	5.5	2.78	1340
Peel K.E. N	13.4	0.0983	11.0	82.5	2.3	17.5	0.35	0.35	0.9	6.8	0.8	5.7	4.7	—	165
Pulp K.E. U (1)	64.6	0.0570	28.7	44.4	36.0	55.7	0.18	0.18	23.2	35.9	10.0	15.5	4.1	—	1348
Peel K.E. U (1)	14.6	0.1013	11.7	80.3	2.9	19.7	0.22	0.22	1.2	8.2	1.1	7.4	3.9	—	170
Pulp K.E. U (2)	89.4	0.0672	28.7	32.1	60.7	67.9	0.17	0.17	37.4	41.8	13.6	15.2	10.7	2.79	1804
Pulp K.E. A	53.8	0.0497	25.8	48.1	28.0	51.9	0.24	0.24	16.1	29.9	7.4	13.8	8.0	2.76	1340
Peel K.E. A	13.2	0.0963	10.8	82.2	2.4	17.9	0.25	0.25	0.9	6.8	1.0	7.2	3.5	2.98	161
B. Bascombe Mystery apples as gathered.															
Pulp B.M. C (1)	20.4	0.0397	11.4	55.7	9.0	44.3	0.34	0.34	4.7	23.1	2.4	11.8	9.1	2.95	402
Peel B.M. C (1)	7.6	0.0955	6.6	87.2	1.0	12.8	0.33	0.33	0.3	4.5	0.3	4.3	3.7	—	54
Pulp B.M. C (2)	22.6	0.0393	12.5	55.3	10.1	44.7	0.35	0.35	5.3	23.4	2.7	11.9	9.0	2.96	414
Peel B.M. C (2)	8.4	0.0943	7.3	87.0	1.1	13.0	0.21	0.21	0.4	4.4	0.4	5.0	3.4	—	59
Pulp B.M. N	30.9	0.0713	10.4	33.7	20.5	66.3	0.16	0.16	14.4	46.6	3.3	10.8	8.7	2.90	310
Peel B.M. N	8.4	0.1125	6.1	73.5	2.2	26.5	0.32	0.32	1.3	15.7	0.4	4.8	5.7	—	51
Pulp B.M. U	32.0	0.0527	15.1	47.2	16.9	52.8	0.29	0.29	10.1	31.5	4.1	12.7	8.3	—	529
Peel B.M. U	9.5	0.0978	7.7	80.7	1.8	19.4	0.35	0.35	0.8	8.3	0.6	6.0	4.7	—	78
Pulp B.M. A	29.2	0.0576	13.4	45.8	15.8	54.2	0.14	0.14	9.7	33.2	3.2	10.8	10.1	2.89	487
Peel B.M. A	7.9	0.0970	6.3	79.7	1.6	20.3	0.27	0.27	0.7	8.8	0.4	5.0	6.2	—	68
C. Bascombe Mystery apples after storage.															
Pulp B.M. C (1)	21.6	0.0378	14.4	66.5	7.2	33.5	0.83	0.83	4.1	19.0	1.8	8.5	5.2	2.96	357
Peel B.M. C (1)	9.1	0.0949	8.0	87.1	1.2	12.9	0.46	0.46	0.4	4.3	0.2	2.2	5.9	—	48
Pulp B.M. N (1)	28.7	0.0669	9.6	33.6	19.1	66.4	0.80	0.80	14.0	48.8	3.0	10.4	6.4	3.11	220
Peel B.M. N (1)	9.6	0.1246	7.2	75.0	2.4	25.0	0.49	0.49	1.6	16.4	0.3	3.4	4.7	—	30
Pulp B.M. A (1)	30.3	0.0596	12.9	42.5	17.4	57.5	0.89	0.89	11.5	37.9	2.7	9.0	9.7	3.01	360
Peel B.M. A (1)	9.9	0.1110	7.9	79.6	2.0	20.4	0.50	0.50	1.1	11.1	0.3	3.1	5.7	—	45

We may next turn to a brief consideration of the observed changes in the nitrogen fractions of the Bascombe Mystery fruits during their short period of storage (Table III c). It has been found [Hulme, unpublished data] that in young fruits (Bramley's Seedling variety), when detached from the tree, the soluble nitrogen rises but when "mature" fruits are stored a net protein synthesis takes place. It will be seen that in the fruits from uninjected trees (B.M. C (1)) the % of the total nitrogen in the form of protein has increased, i.e. a considerable net synthesis of protein has occurred in storage. On the other hand in the fruits from trees injected with ammonium nitrate the % protein-nitrogen has remained stationary, and there has been a slight decrease in this fraction in the fruit from the trees injected with asparagine. It remains for further work to decide whether this difference between the injected and control fruits is a direct one or whether the effect of the injection has been to retard the ripening of the fruits and so cause them to exhibit the characteristic behaviour of fruits not yet "mature". In this connexion it is important to learn the effect of the injection on the carbohydrate metabolism of the fruits. This is especially important in view of the recent work of Paech [1935] which suggests that in presence of excess soluble nitrogen compounds (ammonia and amides) protein synthesis may be limited by the amount of carbohydrate residues available.

SUMMARY.

1. Two varieties of apple trees were injected in the summer of 1935 with solutions of ammonium nitrate, urea and asparagine.
2. The fruit, gathered in the autumn, from the injected trees contained greater amounts of total nitrogen than from the uninjected trees; the distribution within the total nitrogen was also different.
3. When detached from the tree and placed at 9.5°, fruit from trees injected with ammonium nitrate and with asparagine did not synthesize protein, whereas that from untreated trees synthesized considerable amounts of protein.
4. Further experiments are necessary to determine whether this difference in behaviour is a direct result of the injection or whether it is due to delayed ripening of the fruits from the injected trees.

APPENDIX

A rapid continuous vacuum extractor.

The apparatus is shown in Fig. 2 which is drawn to scale. It is based on the Soxhlet principle operating under reduced pressure and in size is designed to handle up to 500 g. of frozen apple powder.

The flask, *A*, in which the solvent (600 ml. 85 % alcohol) is placed is connected to the extraction vessel, *B*, by means of a rubber sleeve. The side-tube, *S*, of the vessel *B* is wide (3 cm. diam.) and the bends in it are as "smooth" as possible to increase evaporation from the liquid in *A* by reducing frictional resistance to and turbulence in the vapour. The condenser, *C*, is a seven tube brass condenser¹ (seven $\frac{5}{8}$ in. $\times$ 24 G. tubes 10 in. long in a jacket $2\frac{1}{2}$ in. $\times$ 24 G.) at the top of which suction is applied by means of a water-jet pump. It is advisable to insert a calcium chloride trap between the pump and

¹ This condenser was designed and made by Mr F. G. Forsyth of Ditton Laboratory.

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for water-jet pump *read* rotary oil air pump (or a water-jet pump)

the condenser. For safety the author also inserts between the calcium chloride tower and the condenser a mushroom non-return valve actuated by gravity. The condenser is cooled by water which has passed through a large rectangular (30 ft. $\frac{3}{16}$ in. bore) copper coil immersed in an insulated ice-box.

Fig. 2.

The thermometer, *T*, enables the rate of flow of the water through the condenser to be regulated to give maximum condensation. The bottom tube, *F*, of the condenser must be wide (3 cm.) to prevent bubbles forming where the rising vapour meets the falling condensate. Bubbler tube, *D*, is a Naumann stainless steel tube with a stiletto wire down the centre to allow of regulation of the rate of bubbling.

Water-bath, *E*, is thermostatically controlled to 45–50°. Table IV illustrates the “characteristics” of the extractor with 400 g. of apple pulp tissue in a thimble in vessel *B*. The figures are for one of two extractors running side by side in the same water-bath with the same suction line and condenser water supply.

Table IV.

Temperature of laboratory ° C.	Temperature of bath, <i>E</i> ° C.	Temperature of alcohol extract <i>B</i> ° C.	Temperature of thermometer <i>T</i> ° C.	Time for <i>B</i> to fill and siphon (min.)
16.9	45	20	10.3	6.5
18.4	49	20	9.5	4.5
18.6	47	21	10.0	5.0
19.0	47	21	10.0	5.5
19.2	48	22	9.4	5.0
19.5	45	22	9.8	6.0
20.0	50	22	10.0	4.5
21.0	48	22	9.7	4.8

The temperature of the liquid in the flask *A* never rises above 20° owing to the rapid evaporation from its surface. The concentration of the alcohol falling from the condenser into *B* is about 87 %.

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