REFERENCES

- Davies, D. S. & Hinshelwood, C. N. (1943). *Trans. Faraday Soc.* **39**, 431.
- Dorfman, A., Berkman, S. & Koser, S. A. (1942). *J. biol. Chem.* **144**, 393.
- Gaddum, J. H. (1937). *J. Physiol.* **89**, 7P.
- (1943). *Trans. Faraday Soc.* **39**, 323.
- Greig, M. E. & Hoogerheide, J. C. (1941). *J. Bact.* **41**, 549.
- Hewitt, L. F. (1932). *Biochem. J.* **26**, 208.
- Hills, G. M. (1943). *Biochem. J.* **37**, 418.
- Hirsch, J. (1942). *Studien über die mikrobiologischen Grundlagen der Sulphanilamid-Therapie*. Istanbul: Kenan Basimevi.
- Huntingdon, E. & Winslow, C.-E. A. (1937). *J. Bact.* **33**, 123.
- Lodge, R. M. & Hinshelwood, C. N. (1939). *J. chem. Soc.* p. 1692.
- (1943). *J. chem. Soc.* p. 213.
- McIlwain, H. (1939). *Brit. J. exp. Path.* **20**, 330.
- (1942a). *Brit. J. exp. Path.* **23**, 95.
- (1942b). *Biochem. J.* **36**, 417.
- (1943). *Trans. Faraday Soc.* **39**, 359.
- & Hawking, F. (1943). *Lancet*, **1**, 449.
- Pappenheimer, A. M., Jr. & Hottle, G. A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **44**, 645.
- Sevag, M. G. & Shelburne, M. (1942). *J. Bact.* **43**, 411.
- Snell, E. E. (1941a). *J. biol. Chem.* **139**, 975.
- (1941b). *J. biol. Chem.* **141**, 121.
- Strauss, E., Lowell, F. C. & Finland, M. (1941). *J. clin. Invest.* **20**, 189.
- Teague, P. C. & Williams, R. J. (1942). *J. gen. Physiol.* **25**, 777.
- Wyss, O. (1941). *Proc. Soc. exp. Biol., N.Y.*, **48**, 122.
- Wilson, P. W. (1939). *Respiratory Enzymes*. (Edited by Elvehjem, C. A. & Wilson, P. W.). Minneapolis: Burgess Publishing Co.

Synthesis of Vitamin C in Stored Apples

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Previously (Zilva, Kidd & West, 1938) we demonstrated that there was a change in the relative proportions of *l*-ascorbic and dehydroascorbic acids in Bramley's Seedling apples as the fruit developed, the equilibrium shifting in favour of *l*-ascorbic acid as the apple approached maturity. The general evidence made it reasonably probable that this change took place actually in the living tissue of the apple and not during analytical manipulation. In continuing this inquiry we discovered further that vitamin C was synthesized when apples were stored at 3° and that the capacity for synthesis was, as in the case of dehydroascorbic acid, high in the young but almost non-existent in the mature fruit. This was first observed in 1938 and the observation was confirmed every year subsequently, leaving little doubt that it is reproducible under our experimental conditions. In this communication an account of a characteristic experiment performed in 1942-3 is given.

METHODS

As already mentioned, in each of the four years preceding 1942 it was definitely established that there was a formation of vitamin C in young apples during storage. This was the case whether the comparison was made between the vitamin C concentration in the tissues of the fruits or between the contents of the entire apples before and after storage. In planning the final experiment not only were these two points borne in mind but an attempt was also made to

ascertain whether the weight of the fruit at the time of picking, and the tree of origin, influenced this formation of the vitamin. In devising the experiment we were fortunate in having, on the statistical aspect of the problem, the valuable advice of Mr T. N. Hoblyn of East Malling Research Station, and the following scheme was adopted.

Sampling. The apples came off two 25-year-old three-quarter standard Bramley's Seedling trees on No. VI root-stock, grown at East Malling. The fruits were picked on 2 July, 20 July, 10 August and 12 October 1942, and were stored until 11 February, 25 February, 10 March and 6 April 1943, respectively.

The fruits were on each occasion picked from the two trees between 8 and 10 a.m. and were immediately sorted out into the following approximate weight groups: first picking—tree A, 18-19, 21-22, 24-25 g.; tree B, 18-19, 21-22, 24-25 g.; second picking—tree A, 39-41, 42-42, 44-45 g.; tree B, 43-45, 45-47, 48-49 g.; third picking—tree A, 65-68, 68-69, 70-71 g.; tree B, 62-62, 68-69, 70-71 g.; fourth picking—tree A, 119-122, 122-128, 128-132 g.; tree B, 122-124, 124-133, 134-138 g. Three apples from each weight group and each tree, i.e. 18 fruits of each picking, were analyzed immediately.

Storage. Similar samples, the individual weights being marked on each fruit, were simultaneously placed in store at a constant temperature of 3° and a humidity of 83% saturation. Three fruits from each weight group and each tree were removed from store at the times mentioned above and were analyzed immediately.

Analysis. The dry weight was determined as follows. The apples were cut into small pieces which were then dried in an oven at 50° in a vacuum. The drying was continued

until two consecutive weighings agreed. The process usually took about 4 days. The apples used for the dry and fresh weight determinations were stored under the same conditions as those used for the vitamin C determinations.

The total vitamin C and the *l*-ascorbic acid were determined by titration with indophenol. The apples were ground with sand under an equal weight of 5% trichloroacetic acid and filtered through muslin. This process was repeated by regrounding the residue with the filtrate. The final extract

the total vitamin C in these apples has approximately doubled after 7 months of storage. In 1938 a similar increase was established by biological assessment.

Statistical analysis of results

The results obtained in 1942-3 were kindly analyzed by Mr T. N. Hoblyn and Mr S. C. Pearce of East Malling Research Station, who write:

Original apples picked 26 June 1939

After 7 months' storage

Fig. 1. Growth curves of groups of guinea-pigs fed fresh and stored apples, and of control groups fed pure ascorbic acid. ↓ = onset of clinical symptoms of scurvy; C = killed by chloroform; + = died; N = normal; VSS = very slight scurvy; SS = slight scurvy; S = scurvy; I = intercurrent disease.

was titrated without further treatment with indophenol before and after reduction with H_2S at pH 4.0-4.4. The titration value obtained before reduction was assumed to be due to *l*-ascorbic acid; that after reduction indicated the total vitamin C content. The fresh and dry weights of the fruit at the time of removal from store were used for calculating the results. In order to make sure that the increase in the indophenol reduction was due to vitamin C, biological tests were carried out in 1938 and 1939. Fig. 1 represents the results obtained in 1939, which leave little doubt that the indophenol-reducing substance formed on storage was mainly vitamin C. The simultaneous biological and titration tests show further that the concentration of

'Attention was concentrated on four sets of measurements, as follows:

- 'I. mg. of vitamin C/100 g. fresh weight.
- 'II. mg. of vitamin C/100 g. dry weight.
- 'III. mg. of vitamin C/apple.
- 'IV. Percentage of vitamin C present as *l*-ascorbic acid.

'For I-IV a statistical analysis was made for each picking separately; and for I-III additional analyses were made also for all the unstored fruit, using all four pickings.

'In dealing with the weight of vitamin C per apple (III) we employed the statistical device of using the square roots of the data. These figures are italicized in Table 1. This is not meant to suggest that these square roots are of interest

in themselves, but they are of use because significant differences can be applied to them and not to the original data.

The proportions of vitamin C in the fresh and dry materials (Table 1, I and II) are obviously closely related, and both were analyzed because of the difficulty of choosing between them. On the whole the data based on dry weight proved more sensitive, but both indicated very similar results.

RESULTS

The effect of storage. There is a marked effect of storage, which tends to increase the total vitamin C content (Table 1, I, II and III), especially with the early picked fruit. Not only is there this general increase, but the proportion present as *l*-ascorbic acid also increases on storage (Table 1).

Table 1. *The effect of storage at 3° on the vitamin C content of Bramley's Seedling apples*

Picking	Stored	Unstored	Significant difference ($P = 0.05$)
I. mg./100 g. fresh weight			
1	33.7	21.4	2.0
2	29.3	18.7	1.5
3	24.5	19.3	1.9
4	20.8	18.8	Not significant
II. mg./100 g. dry weight			
1	303	229	13
2	267	192	14
3	198	192	Not significant
4	160	176	Not significant
III. mg./apple (actual wt. in mg. above, square root in italic, below)			
1	6.47	4.59	
	2.53	2.14	0.08
2	11.95	8.09	
	3.46	2.84	0.09
3	15.72	12.96	
	3.96	3.59	0.17
4	26.83	23.89	
	5.13	4.88	0.25
IV. Percentage of total vitamin content present as <i>l</i> -ascorbic acid			
1	74.6	42.9	6.1
2	84.1	67.5	3.3
3	87.8	79.5	3.1
4	95.8	90.2	3.1

The effect of time of picking. Comparison of the unstored fruit from the different pickings shows that there is a marked tendency for the total vitamin C in an apple (Table 1, III) to increase with age though the proportion of vitamin C (II) drops slightly. In other words, the increase of vitamin does not keep up with the increase in apple tissue. Meanwhile, the proportion present as *l*-ascorbic acid (IV) is increasing rapidly. This effect is shown in Table 2.

No statistical analysis was performed on the percentage of vitamin C present as *l*-ascorbic acid (Table 2, IV), but the general trend is obvious.

Table 2. *Effect of time of picking on vitamin C content of Bramley's Seedlings*

Data concerned (see text)	1	2	3	4	Significant difference ($P = 0.05$)
Picking ...					
I	21.4	18.7	19.3	18.8	Not significant
II	229	192	192	160	17
III { Actual	4.59	8.09	12.96	23.89	
Square	2.14	2.84	3.59	4.88	0.50
root					
IV	42.9	67.5	79.5	90.2	Not analyzed

Differences between trees. For the second and fourth pickings tree A had more vitamin C than tree B, as measured by the proportions of total vitamin C to fresh and to dry weight; also in tree A a greater proportion was present as *l*-ascorbic acid. For the fourth picking only, the total vitamin C per apple was greater for tree A. These effects mostly evened up on storage.

Differences in size of apples. For the first three pickings no differences connected with fruit size were observed. In the fruit picked last there did appear to be a larger proportion of vitamin C in the large apples, but only in the stored fruit. This apparent result must be regarded with some suspicion. The correct interpretation is difficult in view of the apparent contradiction of the results of earlier pickings.

The fact that differences were shown, however small, between trees which were closely similar horticulturally, and between any of the different-sized fruits, emphasizes the importance of taking such factors into consideration in the design of these experiments.

DISCUSSION

The analysis of the results of this final experiment supplies the information which we set out to obtain. It confirms beyond doubt our earlier observations that synthesis of vitamin C in apples takes place on storage and that the capacity for this synthesis diminishes with the age of the fruit. It thus seems to run the same course as the decrease in ratio of dehydroascorbic acid to *l*-ascorbic acid in the total vitamin C content as the apple matures. There are differences between the total vitamin C content of fruits from different individual trees of similar type, but these disappear on storage. There is no real evidence that the capacity for synthesis on storage is influenced by the weight of any of the fruits in any single picking, at least not in the apples of the first three pickings in which this capacity for synthesis is most marked. In spite of the fact that the proportion of *l*-ascorbic acid to dehydroascorbic

acid also increases on storage it is not yet possible to say in which form the vitamin is primarily synthesized.

The nature of the changes underlying the phenomenon is still obscure. The evidence strongly suggests, but does not absolutely prove, that the vitamin is formed from a chemically related precursor during storage. The increase of vitamin C in an apple of about 22 g. during 7 months of storage was found to be equivalent to about 2 mg. of ascorbic acid, the total content reaching a level of about 7 mg. per fruit. Some years the increase is somewhat greater (cf. Fig. 1). On the other hand, the total vitamin content per apple picked normally in October of the same year was about 24 mg. The total quantity of the vitamin present in these immature fruits was therefore very much less than that found in a Bramley's Seedling apple, picked at the usual time. If it be assumed that the precursor in young apples is fully utilized during storage, the extra vitamin found in the apple matured on the tree must in consequence have either been synthesized in the fruit from a precursor supplied from other parts of the plant as the apple

developed or, more probably, it must have reached the fruit in a preformed state. Even if the latter were the case, the process of formation of the vitamin may be similar to that occurring during the storage of the young apple. These and other points which arise cannot be settled on the basis of existing knowledge and the information so far obtained is insufficient to shape a comprehensive hypothesis. Nevertheless the data presented here are consistent enough to serve as a basis for a more detailed inquiry, the results of which might eventually throw some light on the physiological function of *l*-ascorbic acid not only in the plant but also indirectly in the animal organism.

SUMMARY

Vitamin C is synthesized in Bramley's Seedling apples during storage at 3°. The capacity for this synthesis diminishes with the age of the fruit.

We should like to express our thanks to Dr R. G. Hatton, Director of East Malling Research Station, for facilities placed at our disposal during the years 1939-42 and to Mr T. N. Hoblyn for statistical guidance in the work.

REFERENCE

Zilva, S. S., Kidd, F. & West, C. (1938). *New Phytol.* **37**, 345.

Metabolism of Steroids

4. KETONIC ACIDS DERIVED FROM CHOLIC ACID

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It was shown by Schmidt, Hughes, Green & Cooper (1942) that cholic acid is converted by *Alcaligenes faecalis* into a mixture of ketonic acids, one of which is certainly dehydrocholic acid. Moreover, the many experiments of Japanese workers on the administration of bile acids and their derivatives to several species of animal suggest that ketonic bile acids may be of importance in animal metabolism. It has therefore seemed of interest to complete the series of compounds derived by oxidation of the secondary hydroxyl groups of cholic acid to carbonyl.

With the steric configuration of cholic acid, six hydroxy-keto acids are theoretically possible. Of these, three are listed by Sobotka (1938), and the remaining three are 7:12-dihydroxy-3-keto- (Sihn, 1938), 3:12-dihydroxy-7-keto- (Gallagher & Long, 1943; Haslewood, 1943), and 12-hydroxy-3:7-diketo-cholanic acid, now described. The preparation of

this last acid depended on a method used by Reichstein and his collaborators (e.g. Lardon & Reichstein, 1943), by which C_3 steroid esters are 'alcoholized' by acidified alcohols to give the corresponding C_3 hydroxy compounds. Thus, methyl triacetyl cholate (I) was converted by methanol-HCl, followed by chromic acid oxidation, to methyl 7:12-diacetoxy-3-keto-cholanoate (II), which was identified by conversion to 7:12-dihydroxy-cholanic acid (III).