Apparent Vitamin C in the Walnut (*Juglans regia*)

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A previous communication (Wokes & Melville, 1948) described studies carried out over several years on the seasonal distribution of vitamin C in different tissues of the fruit and other parts of the walnut tree. The results showed that the translocation of vitamin C as such from the epicarp, leaf mesophyll and other photosynthetic centres, where the vitamin is probably synthesized, to the endocarp, where it occurs in such remarkably high concentration, seems to involve its passage up a steep concentration gradient presenting unusual difficulties as compared with the leaf to fruit gradients in most other species which have been investigated. In our studies on the walnut, apparent vitamin C was necessarily determined in all the samples, since the results for true vitamin C were determined generally by subtracting the figure for apparent vitamin C from that for total vitamin C. However, the data for apparent vitamin C were omitted from our previous paper in order to demonstrate the type of picture which is obtained if the presence and possible functions of the apparent vitamin C are ignored. The present paper supplies the missing data, enlarging and clarifying the picture.

Gerghelezhii (1937), who discovered the high concentration of vitamin C in unripe walnut fruits, did not seem to realize that his raw materials could contain considerable amounts of dye reductants which were not ascorbic acid, but this may have been due to his use of sulphur dioxide in the extracting fluid which, as will be shown later, can prevent the apparent vitamin C from reacting with the dye. His findings were confirmed by Pyke, Melville & Sarson (1942) and by Ranganathan (1942), but neither paper reported the presence of an appreciable amount of apparent vitamin C in the unripe walnuts examined, perhaps because these were too mature. Ranganathan (1942) stated that tests for interfering substances showed these to form 4–6% of the total vitamin C. Taylor (1943) reported the presence of about 13% of interfering substances in the total vitamin C in some unripe walnuts which he had examined. Melville, Wokes & Organ (1943) found the total vitamin C in six unripe fruits of *Juglans regia* and fifteen of allied species, to contain 12–73% of interfering substances, for which they suggested the provisional term 'apparent vitamin C'. They used a modification of Lugg's (1942) formaldehyde

method (Wokes, Organ & Jacoby, 1943) in which the residual apparent vitamin C, after destruction of the true vitamin C by formaldehyde at pH 4.5, was titrated with the dye at a pH below 1.

Mapson (1943) found that some 14% of the total dye titration for walnuts preserved in syrup was due to non-specific reductants to which he applied the term 'reductones'. In his modification of Lugg's (1942) method, the 'reductones' were titrated at pH 0.6 and total vitamin C at pH about 1.2 (except when the presence of sulphides, sulphites or thiol compounds was suspected). His assumption that the apparent vitamin C in walnuts consisted of reductones was understandable in view of the finding of Wokes, Organ, Duncan & Jacoby (1943) that the apparent vitamin C in molasses, malt extract and certain fruit juices after prolonged storage closely resembled reductone or reductic acid in chemical and physical properties. Nevertheless, the apparent vitamin C in walnuts cannot be reductone, since its dye titration value is very low at pH 2 and increases markedly as the titration pH is lowered (Wokes, 1946*a*), in contrast with reductone, the dye titration value of which decreases as the titration pH is lowered (Martius & von Euler, 1934; Wokes, 1946*a*). The use of too high a titration pH probably explains the failure of Lugg & Weller (1943) and of Klose, Peat & Fevold (1948) to discover much apparent vitamin C in unripe walnuts, though it should be added that the latter workers complicated their results by using sulphur dioxide in the extracting fluid. On the other hand, when Tuba, Hunter & Osborne (1947) applied to walnuts Levy's (1943) method of estimating ascorbic acid in the presence of reductones and other interfering substances they found 24–38% of the total vitamin C to be non-specific dye reductants.

METHODS

Collection and dissection of material was as described previously (Wokes & Melville, 1948).

Estimation of apparent vitamin C

(a) *Formaldehyde method.* The visual method described by Wokes, Organ & Jacoby (1943) was used, taking care to add after the formaldehyde treatment sufficient 25% (w/v) HPO_3 to ensure that the pH was brought down to 0.5–0.7

before titrating. (This is the pH of the reaction mixture when determining total vitamin C.) The pH of the titration mixture was determined and the result corrected to pH 0.7 by means of the curve previously published (Wokes, 1946a). When calculating from the dye readings the concentration of apparent vitamin C, its dye factor was assumed to be the same as that of ascorbic acid. This procedure, the most convenient when estimating true vitamin C by difference, leaves the results for apparent vitamin C open to correction when its chemical nature is established. It does not, however, affect their relative values. When sufficient material was available, estimations of total and apparent vitamin C were occasionally made by the potentiometric method (Wokes, Organ & Jacoby, 1943). This always gave results in good agreement with the visual results, and also provided information on the effect of the titration time on the dye titration value (see p. 350). The fact, repeatedly confirmed, that the dye titration value increased as the pH was reduced below 1.5 gave strong reason for believing that the apparent vitamin C was not reductone.

(b) *By using ascorbic acid oxidase.* This enzyme, derived from cucumber juice, was found to destroy the whole of the ascorbic acid in walnut extracts without exerting any appreciable effect on the apparent vitamin C. To ensure that the destruction of the ascorbic acid was complete, the mixture was incubated with the enzyme at pH 5 and at 38–40° until dye readings on samples became constant. Recovery experiments carried out with different levels of added ascorbic acid gave similar results. The data obtained in a typical experiment are plotted in Fig. 1. From the

Fig. 1. Use of ascorbic acid oxidase to differentiate apparent from true vitamin C in walnut leaves. Results obtained on extract of leaves, $\times$ — $\times$; results obtained on extract plus added ascorbic acid, $\circ$ — $\circ$.

general shape of the curves, it was clear that the enzyme had ceased to act on any dye reductant after about 20 min., either in the recovery or in the test experiment. The amount of apparent vitamin C then left in the two reaction mixtures was practically the same, indicating 349 and 344 mg./100 g. respectively in the original leaves, as compared with about 320 mg./100 g. by the formaldehyde method. Hence it was assumed that the enzyme had exerted no appreciable effect on the apparent vitamin C. Since ascorbic acid oxidase acts

slowly on reductone, further evidence was thus provided that the apparent vitamin C was not reductone. The method was not applied to many of the samples because it was tedious and required large amounts of material.

(c) *By differential titration.* Since the dye titration value of the apparent vitamin C in walnuts falls to zero as the titration pH is raised above 2, it is possible to titrate the true vitamin C as such at a pH of about 3, then to add excess of HPO_3 to bring down the pH to 0.6 or 0.7 and continue the titration to determine the apparent vitamin C. In practice this method was found less convenient and accurate than the formaldehyde method, and was applied only on a few occasions, when it gave fair agreement with other methods.

Fig. 2. Absorption curve of the yellow colour obtained when titrating walnut extracts with indophenol dye.

Fig. 3. Correlation of concentration of apparent vitamin C in walnut tissues with increase in yellow colour (measured spectrophotometrically at 420 mμ.) when extracts of the tissues were titrated with indophenol dye. Results on fruit tissues indicated thus $\circ$; results on stem and leaf indicated thus $\times$. Regression line calculated from the equation $y = 0.9618 + 0.002047(x - 333)$.

(d) *By increase in yellow colour.* During titration of walnut extracts containing apparent vitamin C a yellow colour develops. Spectroscopic examination of this yellow colour revealed a broad absorption band in the visible spectrum with a maximum at about 420 mμ., and a narrow band of four or five times the intensity with a maximum at about 250 mμ. (see Fig. 2). Using the Hilger-Nutting spectrophotometer, we determined the increase in yellow colour at

420 $\mu\mu$., caused by the dye titration, employing the cross-over technique (Wokes & Still, 1942). The results, expressed as increase in $E_{1\text{cm}}^{1\%}$, plotted against the concentration of apparent vitamin C (in mg./100 g.), determined by the formaldehyde method, are shown in Fig. 3, together with the regression line. The correlation coefficient for these data, $r=0.83$, was highly significant ($t=14.14$, $P<0.01$) and is strong evidence that apparent vitamin C usually consists of a single compound which is converted into a yellow pigment on oxidation. Nevertheless, occasional aberrant results preclude the use of this yellow colour for a routine estimation.

Loss of apparent vitamin C during dissection. In a number of experiments, the weights of apparent vitamin C in the dissected parts of leaflets and nuts were compared with the average yield from whole leaflets and nuts from the same spray. Losses due to dissection were found to be no greater than for true vitamin C in the same material (cf. Wokes & Melville, 1948). For example, in samples of leaves collected on 10 different days between May and September the weighted mean concentration of apparent vitamin C in dissected leaflets ranged from 85 to 136 and averaged 107% of the concentration found in whole leaflets ground without dissection; but in samples collected on 3 different days in October the corresponding percentages were 82, 71 and 44. Nuts were more variable, owing to a greater sampling error. For example, two dissected nuts gave 177 and 287 mg./100 g., averaging 222 mg./100 g., which was in reasonable agreement with the mean of 214 ± 23 mg./100 g. obtained for ten whole nuts from the same sample, but the results on the individual nuts ranged from 131 to 382 mg./100 g. It is

probable that oxidizing enzymes capable of destroying apparent vitamin C are present in walnut tissues, since, on grinding with water and quartz sand, a yellow colour developed similar to that formed during titration. At the same time there was a rapid drop in the dye titration value. On the other hand, the apparent vitamin C in HPO_3 extracts of these tissues was fairly stable during storage in brown bottles under N_2 in the refrigerator, losses as low as 1-2%/month being frequently found after more than a year's storage, although considerably greater losses could occur if there was much exposure to air.

RESULTS

Whole fruits (nuts)

A seasonal trend in the concentration of apparent vitamin C in entire fruits is fully established, although the results are more variable than those previously reported for true vitamin C. Data on nearly 100 fruits from samples collected June-August 1944, May-August 1945 and June 1946, are summarized in Table 1. The highest concentration of apparent vitamin C was found in the youngest fruits weighing not more than 1 g., and was usually between 800 and 1000 mg./100 g. As the fruits grew, the concentration fell until at maturity it was only 0.2-0.1 of the initial value. The concentration of apparent vitamin C was therefore already at its height when the earliest samples were taken, whereas

Table 1. *Seasonal changes in concentration of apparent vitamin C in walnut fruits*

Sample no.	Date gathered	Average wt. of fruits (g.)	Apparent vitamin C (mg./100 g.)		Apparent vitamin C	
			Individual fruits	Mean	(mg./fruit, mean)	(As % of total vitamin C)
From main tree						
1	7 June 1944	1	Pooled	800	8	62
2	13	1	Pooled	847	9	47
3	21	3	760, 715, 658	711	21	26
4	26	3.6	540, 520, 440, 430	483	17	19
5	4 July 1944	10	405, 325, 315	345	35	11
6	12	20.3	220, 275, 225, 320	260	51	14
7	18	17.5	185, 215, 120, 210	180	32	11
8	25	22.6	235, 195, 160, 248	210	48	13
9	1 Aug. 1944	24.9	155, 150, 120, 115	135	36*	11
10	9	32.1	135, 148	141	45*	14
11	22	21.7	190, 140	165	36	12
25	17 May 1945	0.3	Pooled	789	2.4	46
26	30	0.5	716, 475, 539	577	3	35
27	6 June 1945	1.75	568, 501	535	9	23
28	12	2.8	401, 323, 380	368	10	16
29	19	4.1	429, 308, 384	348	14	17
30	26	14.0	243, 205, 234	227	32	14
31	10 July 1945	25.2	197, 168, 139	168	42	15
32	23	29.7	158, 174, 172	168	50	14
33	8 Aug. 1945	32.0	87, 100, 103	97	31	9
45	18 June 1946	0.3	Pooled	525	1.5	43
From other trees						
2k2	29 June 1944	10.6	455, 405, 390	417	44	17
2k3	25 June 1945	18.5	223, 140, 200 214, 194, 167	190	35	10

* Excluding woody portion.

the peak value was not reached for true vitamin C until about a fortnight later and then fell gradually as lignification of the endocarp proceeded. The occurrence of peak values for these substances at different times at first suggested that apparent vitamin C might be a precursor of ascorbic acid in the plant. This idea had to be abandoned later when evidence was found that apparent vitamin C is related to juglone. The total amount of apparent vitamin C in a single fruit increases during growth from about 2 mg. to 50 mg., whereas true vitamin C increases much more rapidly from about 3 mg. to over 300 mg. per fruit. Thus, there is finally only about one-sixth as much apparent as true vitamin C. The change in proportion does not suggest any close physiological link between these substances in the plant, and no correlation was found between the observations (1944) of the true and apparent vitamin C in entire fruits. There was, however, a high correlation ($r=0.99$) between apparent vitamin C and true vitamin C a fortnight later ($t=28.37$, $P<0.01$). This result may be due to parallel trends in unrelated processes rather than to a causal relation.

Separate tissues of the fruits

Fruits were dissected as previously described and the separated epicarp, mesocarp, endocarp and kernel were assayed for both true and apparent vitamin C, the same fruits providing data for the whole fruit and separate tissues.

Initial high concentrations (well over 1 g./100 g.) of apparent vitamin C were found in the epicarp and mesocarp (Table 2). These values declined as growth proceeded, but there was a final small rise in the full-grown fruits. The concentrations in the endocarp were much lower, but in general showed similar trends. A definite concentration gradient was generally demonstrable from the epicarp and mesocarp towards the endocarp, and there was always a clear gradient into the testa and its contained endosperm or embryo. Often no positive gradient could be shown from the entire fruit stalk into the endocarp, but, in contrast with true vitamin C, a gradient through the phloem could usually be established as the concentration of apparent vitamin C was higher than in the other tissues. The final rise in concentration in the endocarp was greater if the non-lignified portion alone was considered. This could be explained by a differential partition of the apparent vitamin C at a stage when there was little difference in concentration between epicarp, mesocarp and endocarp. Correlations between true and apparent vitamin C in the fruit tissues were investigated, but the only significant correlation found was negative, between true and apparent in the epicarp ($r=-0.93$, $t=5.02$, $P<0.01$). At first this was interpreted as evidence that apparent vitamin C was a precursor of ascorbic acid, but later work, indicating a relationship to juglone, appears to rule out that view. The same difficulty arises with

Table 2. *Seasonal distribution of apparent vitamin C in different tissues of walnut fruits*

(Dates of collection of samples are given in Tables 4 and 7.)

Sample no.	Fruit stalk	Apparent vitamin C (mg./100 g.)				
		Epicarp	Mesocarp	Endocarp	Testa	Endosperm
1	229	1616	—	92	—	—
2	—	1497	—	203	—	—
3	—	712	924	102	—	—
4	120	595	1368	185	—	—
5	145	405	610	65	20	—
6	—	195	265	150	5	1
7	—	125	275	125	5	1
8	—	150	240	110	5	1
9	200	120	200	100*	9	1
10	120	130	145	160*	27	1
11	125	240	320	210*	12	1
26	106	1029	623	112	—	—
27	95	716	1094	117	—	—
28	106	383	802	111	—	—
29	81	428	919	134	—	—
30	63	145	411	82	—	—
31	134	102	235	139	—	—
32	108	185	122	129	3†	
33	88	69	112	204	—	—
34	30	167	229	—	—	—
2k1	—	450	780	78	—	—
2k2	—	265	715	30	10†	—
2k3	—	215	361	66	8†	—

* Excluding woody portion of endocarp.

† Result on whole kernel.

the correlation ($r=0.87$) found between apparent vitamin C in the epicarp and true in the endocarp a fortnight later ($t=4.85$, $P<0.01$).

Leaves

Data on leaves are given in Table 3. Results on twenty-one samples show that the concentration of apparent vitamin C was always highest in the mesophyll, where there was no definite seasonal change, whereas the concentration of true vitamin C fell gradually with age. While the fruits were growing, there was a clear concentration gradient of apparent vitamin C from mesophyll through the leaf vascular tissue and rachis as far as the leaf base. Hence a gradient into the main stem was more difficult to establish, especially from older leaves, despite attempts to allow for the proportion of phloem in the tissue examined.

apparent vitamin C in the vegetative buds usually increased in the early summer, shortly before they opened, but fell again rapidly as the tiny leaves grew. When the male catkin buds were just distinguishable in late summer, the concentration of 2-3 g./100 g. was higher than in any other plant tissue, but fell slowly during the autumn. Simultaneously, their average weight increased from about 50 to 80 mg., so that the amount of apparent vitamin C in a catkin bud remained at about 1 mg. In the spring, the male catkins grew rapidly till they weighed more than 1 g. each. The concentration of apparent vitamin C fell steadily to less than 500 mg./100 g. The amount in an average catkin fell slightly at the beginning of the spring growth, but then increased to 3 or 4 mg. As the catkins now contained a good deal of fresh green tissue, it is possible that some of this apparent vitamin C was formed by

Table 3. *Apparent vitamin C in walnut leaves*

(Dates of collection of samples are given in Tables 4 and 7.)

Sample no.	Apparent vitamin C (mg./100 g.)					
	Whole leaflets	Mesophyll	Vascular tissue	Rachis	Petiole	Main stem, cortex and phloem
9	250	—	—	—	120	—
11	—	340	160	—	120	—
25	243	299	233	260	119	91
26	242	231	150	141	94	132
27	215	206	182	116	87	173
28	243	277	171	95	95	151
29	271	411	182	109	106	259
30	144	169	95	91	48	304
31	204	349	116	83	81	245
32	192	226	94	94	72	459
33	224	325	118	106	87	536
34	427	410	310	158	51	546
35	243	—	—	37	36	97
36	360	298	291	52	76	179
37	234	68	116	116	68	156
38	202	235	117	64	36	500
43	306	—	—	—	—	412
44	270	542	363	235	—	232
45	195	203	199	162	136	123
46	192	211	170	111	131	169

Buds, catkins and main stem

Data obtained from August 1944 to October 1946 are given in Table 4. The concentration of apparent vitamin C in the buds often exceeded 1 g./100 g., but varied widely from time to time. This was not entirely due to the sampling error, since the results on vegetative buds were usually similar to those on reproductive buds in the same sample. In the early summer, when the reproductive buds began to develop into female catkins, there was a considerable increase in their size so that, although the total amount of apparent vitamin C in an average bud had increased, its concentration remained steady or even fell. On the other hand, the concentration of

photosynthetic activity in the catkins, and was not translocated from other parts of the tree. Since the concentration in the adjacent stem, cortex and phloem at this time was 300-500 mg./100 g., and about 5% of this consisted of sieve tissue, a comparatively small differential concentration in the phloem would have ensured a positive translocation gradient into the catkins. At other times of the year, the observed concentrations were less favourable to direct translocation into any type of bud, though it is not impossible that a positive gradient always existed in the phloem serving these buds. The data on the cortex and phloem from different parts of the stem showed that from October to April the concentration gradient of apparent vitamin C was con-

Table 4. *Seasonal distribution of apparent vitamin C in buds, catkins and stem*

(C.Y.G. = Current year's growth. P.Y.G. = Previous year's growth.)

Apparent vitamin C (mg./100 g.)								
Sample no.	Date gathered	Stem (cortex and phloem)			Buds		Male catkins	Apparent vitamin C (mg./catkin)
		C.Y.G.	P.Y.G.	Older	Vegetative	Reproductive		
12	29 Aug. 1944	215	—	—	—	995	2530	1.3
13	4 Sept. 1944	—	—	—	988	1040	2430	1.3
14	11	—	—	—	970	813	—	—
15	10 Oct. 1944	157	—	—	728	785	1485	1.1
16	14 Nov. 1944	795	—	—	1200	1040	2690	1.6
17	20	580	—	—	1220	—	2310	1.5
18	27	335	—	—	1080	1180	2130	1.3
19	8 Jan. 1945	340	—	418, 369	711	—	1630	0.8
20	21	297	195	119, 156	472	604	1467	0.9
21	28 Feb. 1945	310	264	174, 61	435	273	1146	0.9
22	13 Mar. 1945	444	202	201	627	575	762	0.9
23	4 Apr. 1945	306	—	—	371	568	481	3.4
24	13	—	—	—	—	—	488	4.3
25	17 May 1945	91	369	—	1022	776*	—	—
26	30	132	143	—	1190	577*	—	—
27	6 June 1945	173	409	523, 502	1132	—	—	—
28	12	151	360	—	935	615	—	—
29	19	259	287	434	1033	—	—	—
30	26	304	424	—	1033	—	—	—
31	10 July 1945	245	309	—	936	—	—	—
32	23	459	481	—	951	—	—	—
33	8 Aug. 1945	536	576	—	1051	—	—	—
34	11 Sept. 1945	546	446	346, 382	1214	—	2592	—
35	2 Oct. 1945	97	187	130, 87, 50, 23	964	1045	1919	—
36	9	179	180	183	292	—	1041	0.7
37	15	156	149	—	454	—	1303	0.9
38	22	500	156	—	695	—	1878	1.4
39	31	323	176	—	616	—	1674	1.2
40	13 Nov. 1945	269	201	—	817	—	1617	1.1
41	15 Jan. 1946	291	212	—	769	—	1535	0.9
B	21 Mar. 1946	252	—	—	—	—	—	—
42	2 Apr. 1946	189	119	—	499	500	699	1.4
43	15	412	223	—	412	—	354	2.9
44	29	235	259	—	581	—	198	—
45	18 June 1946	123	388	—	—	—	—	—
46	1 July 1946	169	303	—	1043	—	—	—
47	23 Sept. 1946	—	—	—	—	—	2200	0.6
48	24 Oct. 1946	100	179	248	365	—	1200	0.9

* Excluding woody portion.

sistently from new to older tissue and could be traced some considerable distance down the stem. From May to September the observed concentration gradient was consistently in the reverse direction, from older to newer tissue. In March 1945 we estimated the apparent vitamin C in all parts of a young walnut sapling. Our results (see Table 5) showed a concentration gradient from the cortex plus phloem of the previous year's growth down into the fine roots, where, however, there was still about 300 mg./100 g., several times the concentration of true vitamin C. The concentration in the cortex plus phloem of the current year's growth was lower than in the previous year's growth, but this may have been due to a higher proportion of cortex in the former. The value for the previous year's growth was

Table 5. *Apparent vitamin C in walnut sapling*

	Apparent vitamin C	
	(mg./100 g.)	(As % of total)
Buds	596	70
Stem (cortex plus phloem):		
Current year's growth	298	54
Previous year's growth	415	69
Roots	327	71
Fine roots	302	76

checked by a control experiment with added ascorbic acid on a second sample. The result (386 mg./100 g.) was in fair agreement with the previous result of 415 mg./100 g. and showed that the whole of the true vitamin C was being destroyed by the formaldehyde under the experimental conditions.

Storage experiments

From the evidence of concentration gradients apparent vitamin C seemed much more likely than true vitamin C to be translocated from leaf to fruit during the growth of the latter. This led to the hypothesis that the apparent vitamin C, after its arrival in the endocarp, may yield true vitamin C

vitamin C in the fruits dissected was about 20% lower than that in whole fruits. In 1945, a further storage experiment was made, extending over a period of 2 months (Table 7). The results, corrected for changes in weight during storage, showed no significant changes in the concentrations of either true or apparent vitamin C during the period, thus corroborating the experiment of 1944.

Table 6. *Changes in average concentration of true and of apparent vitamin C in walnut fruits during storage*

(Storage for 16 days at 4–5° in July 1944; all results given as mg./100 g.)

	Whole fruit		Epicarp		Mesocarp		Endocarp		Kernel	
	True	Apparent	True	Apparent	True	Apparent	True	Apparent	True	Apparent
After storage	2337	379	1025	233	963	704	3046	58	35	2
Before storage	2035	417	1195	265	1085	715	3140	30	140	10
Increase or decrease during storage	+302	-38	-170	-32	-122	-11	-94	+28	-105	-8
S.D. of mean (after storage)	171	37	17	29	31	61	4	10	—	—
S.D. of mean (before storage)	11	20	—	—	—	—	—	—	—	—

for which it has served either as a precursor or as a carrier. In order to test this hypothesis, we carried out in 1944 a storage experiment with some immature walnuts which, after sampling for assay, were stored in the refrigerator at 4–5° in a box with access to air. The results (Table 6) showed, after 16 days' storage, an increase of 302 mg./100 g. in the concentration of true vitamin C and a decrease of 36 mg./100 g. in the concentration of apparent vitamin C. (Allowance is made in these figures for the gradual loss in weight of the fruits during

Extraction experiments

Apparent vitamin C was found to be readily soluble in ethanol and aqueous solvents, but much less soluble in solvents immiscible with water, such as benzene, light petroleum, methyl or ethyl ether, chloroform, isobutanol, carbon tetrachloride, ethyl acetate and carbon disulphide. Thus, purification by phase separation seemed unpromising. Whilst it could be extracted from fresh walnut tissues by grinding with ethanol and quartz sand, this often led to heavy losses which could be avoided by adding a small amount of hydrochloric acid to the solvent (2–5% of concentrated acid by volume). The extracts contained much chlorophyll, which rendered visual dye titrations difficult. The chlorophyll could be removed by shaking with light petroleum, leaving a pinkish yellow solution of apparent vitamin C in the acid ethanol. The acid could be removed by evaporation *in vacuo*, yielding very unstable residues with initial concentrations of apparent vitamin C as high as 7%.

Table 7. *Average concentrations of true and of apparent vitamin C in whole walnut fruits at intervals during storage*

(Storage 4–5° in 1945; all results given as mean $\pm$ S.D.)

Date examined	Period of storage (days)	No. of fruits examined	Vitamin C (mg./100 g.)	
			True	Apparent
29 June	0	6	1849 $\pm$ 57	193 $\pm$ 15
5 July	6	4	1910 $\pm$ 138	243 $\pm$ 51
13	14	4	1363 $\pm$ 159	196 $\pm$ 28
19	20	4	1869 $\pm$ 37	238 $\pm$ 15
7 Aug.	39	4	1798 $\pm$ 90	198 $\pm$ 33
27	59	4	1815 $\pm$ 38	180 $\pm$ 30

storage.) Unfortunately, owing to the large sampling error, these differences did not reach significance. Moreover, the loss of apparent vitamin C was very much smaller than the gain of true vitamin C. Results on dissected tissues were also inconclusive, the data indicating a greater loss of true than of apparent vitamin C throughout. Ascorbic acid oxidase liberated during dissection may have caused this loss as the tissues had shrunk and become more brittle during storage. This view was supported by the observation that the mean concentration of true

Rate of reaction with indophenol dye

One of the most characteristic properties of apparent vitamin C is its slower rate of reaction with the dye, compared with the almost instantaneous reaction of the latter with ascorbic acid. The slower rate can be detected in visual titrations, but is particularly well marked in potentiometric estimations. Wokes, Organ & Jacoby (1943), using a modification of the potentiometric method of Harris, Mapson & Wang (1942), showed that the amount of apparent vitamin C found in a stored sample of dehydrated carrot could vary from 20 to 90 mg./100 g. as the time taken to carry out a titration was

increased from about 2 to 30 min. This observation justifies the importance we have attached to the control of the titration time. Attempts have been made to estimate true vitamin C in the presence of these interfering substances by adding a known excess of dye and measuring photoelectrically at short intervals (e.g. 15, 30, 60 sec. after the dye addition) the amount of unreduced dye. The readings are plotted against time and extrapolated back to zero time to estimate the true vitamin C. The interfering substance could then, if desired, be estimated by difference, assuming that the reaction

spectively about half and a fiftieth of that for ascorbic acid, and utilized these in a method of estimating ascorbic acid in the presence of reductones which gave good agreement with the formaldehyde method. Their method would not, however, distinguish between ascorbic acid and *iso*ascorbic acid or hydroxytetronic acid, because the velocity constants of these acids with the dye at pH 1.4 and at 3.5 were too close to one another. Such differentiation could be effected by the infrared absorption method of Trotter, Thompson & Wokes (1948), but this method was not available for the work described

Fig. 4. Effect of titration time on dye titration value (D.T.V.) of ascorbic acid and allied compounds. Ascorbic acid, $\times$ — $\times$; hydroxytetronic acid, $\blacktriangle$ — $\blacktriangle$; dihydroxy maleic acid, $*$. . . $*$; reductic acid, $\diamond$ — $\diamond$; glucoreductone, $\circ$ — $\circ$; diketogulonic acid, $\odot$ — $\odot$; *iso*ascorbic acid, $\bullet$ |||| $\bullet$. Results obtained by Harris & Mapson (1947) on left-hand side (A), by Wokes (1946b) on right-hand side of diagram (B). Results of present work in centre (C). Results on walnut extracts indicated thus ■.

time was adequate. This method certainly corrects for some of the interfering substance, but the correction is not necessarily complete as, in the short but definite time required for the dye to react with the whole of the ascorbic acid, it may also have acted with some of the interfering substance. This was clearly shown by Harris & Mapson (1944, 1947) using the continuous flow method, with which they used reaction times down to 0.28 sec. They found that at pH 1.4 about 12% of ascorbic acid had not yet reacted with the dye 1.1 sec. after mixing, whereas about 40% of reductone had already reacted in this time under the same experimental conditions. They obtained velocity constants for reductic acid and for reductone which were re-

in the present paper. However, no indication has been obtained of the presence of *iso*ascorbic acid or of hydroxytetronic acid in walnut tissues. Wokes (1946b) described a modified potentiometric method with which reaction times were obtained to less than 10 sec. Using a titration pH of 0.6–0.7, he confirmed that *iso*ascorbic and hydroxytetronic acids reacted as quickly as ascorbic acid with the dye, and that dihydroxymaleic acid reacted still more rapidly. He also found that under the given conditions diketogulonic acid reacted as rapidly as ascorbic acid, reductic acid rather more slowly, and reductone much more slowly (see Fig. 4). Apparent vitamin C from different walnut tissues resembled reductone in reacting more slowly.

Spectroscopic studies

For these we used purified acid ethanolic extracts prepared, as described above, from different walnut tissues, but especially the buds and catkins, in which the dye titrations had shown the apparent vitamin C to represent practically the whole of the dye reductant. The extracts were examined using the

Fig. 5. Absorption curves of extracts of walnut buds and catkins, all in HPO_3 , except the dotted curve which was obtained on an extract the pH of which had been adjusted to 4.5.

Hilger E 3 quartz spectrograph, and showed a broad absorption band with a main peak at 280–295 and indications of subsidiary peaks at 325–330 and 335–343 $\text{m}\mu$. (Fig. 5). Except in one rather doubtful case, we were unable to obtain any evidence of the presence of reductone or of reductic acid, two of the possible constituents of apparent vitamin C which have been found in other plants.

DISCUSSION

The discovery in walnut tissues of extraordinarily high concentrations of apparent vitamin C raised interesting questions regarding its nature and possible functions. In earlier work we were guided by the hypothesis that the apparent vitamin C resembled that found in other foods and was probably an enediol of the reductone type. This hypo-

thesis was supported by a close resemblance to reductone in its slow rate of reaction with the dye, a criterion which Harris & Mapson (1947) used to differentiate between various non-specific dye reductants. However, its failure to react with ascorbic acid oxidase, under conditions such that reductone would react, led us to doubt this hypothesis, and finally to abandon it on discovering (Wokes, 1946a) that, in contrast with reductone, the apparent vitamin C of walnuts showed a marked increase in dye titration value as the pH was reduced below 2. This was confirmed by our failure to detect reductone spectroscopically in numerous extracts of different walnut tissues, especially buds and catkins. The observation that a yellow colour developed when the apparent vitamin C was titrated with dye, or oxidized with the walnut enzymes, led us to investigate this oxidation product; and when we found that it had a similar spectrum to that of juglone, the possibility arose that some at least of the apparent vitamin C in walnuts is a reduced form of juglone. Although there was a high correlation between the increase in intensity of yellow colour and the concentration of apparent vitamin C in different tissues, occasional discrepant results suggested that in some instances at least there might be a second non-specific dye reductant present. Spectroscopic studies of numerous walnut extracts failed to identify the apparent vitamin C, the absorption curves showing evidence of fine structure which needed for its complete elucidation more accurate and more sensitive spectroscopic apparatus than was at our disposal.

The occurrence of apparent vitamin C in so many of the walnut tissues, especially those in which ascorbic acid is also found, suggests that it cannot be merely a waste product, but almost certainly plays an active part in some metabolic process, possibly one concerned with ascorbic acid. Had it proved to be reductone, as earlier postulated, it might then have been a precursor of ascorbic acid. Since reductone has been formed *in vitro* by the action of ultraviolet light on glyoxal, which is itself a possible product of photosynthesis, an attractive hypothesis could be developed for the photosynthesis of ascorbic acid from glyoxal via reductone. Probably in most plants synthesis of ascorbic acid occurs in green photosynthetic tissues, to be followed by translocation to the fruits. This could explain our failure to find apparent vitamin C in fruits or leaves of other plants. In the walnut, synthesis of ascorbic acid in the leaves might be incomplete, some of the reductone being translocated as such to the endocarp there to be converted into ascorbic acid. Such a picture would fit many observations, especially those concerning concentration gradients, which are more favourable to the translocation of apparent than of true vitamin C from leaf to fruit.

The above theory was discredited by our failure to obtain a significant increase in vitamin C concentration during the storage of immature fruits in which the concentration of apparent vitamin C was falling. It was abandoned when we obtained clear evidence that the apparent vitamin C is not reductone, but is more likely to be a juglone derivative, possibly a reduction product. Such a compound could hardly be a precursor of ascorbic acid, though it might be linked with the latter in an oxidation system. Further speculation must, however, be deferred until the chemical nature of apparent vitamin C has been more clearly established. Work on this problem is still in progress.

Since the above work was completed evidence has been obtained (Daglish & Wokes, 1948) that the apparent vitamin C in resting buds and catkins is mainly, if not entirely, a derivative of hydrojuglone (1:4:5-trihydroxynaphthalene).

SUMMARY

1. Apparent vitamin C has been detected in numerous samples from the walnut (*Juglans regia*).

2. In fruits the highest concentrations occurred in the epicarp and mesocarp, especially in very young fruits, in which it could form half of the total vitamin C, as measured by dye titration.

3. In leaves the highest concentrations occurred in the mesophyll, from which there was usually a definite concentration gradient through the vascular tissue, rachis and petiole towards the stem.

4. In the stem the highest concentration was in the phloem, in which the concentration gradient was generally from older to younger tissue during the early summer, but in the reverse direction in the winter.

5. Remarkably high concentrations of apparent vitamin C were found in buds and catkins, in which it usually formed most of the total vitamin C.

6. The rate of reaction of apparent vitamin C with the indophenol dye was found to be much slower than that of ascorbic acid, dihydroxymaleic acid, diketogulonic acid, hydroxytetronic acid or isoascorbic acid, and to lie between those of reductic acid and reductone, all determined at the normal titration pH of about 0.7.

7. The apparent vitamin C in walnut tissues differs from reductone in (a) its stability towards ascorbic acid oxidase; (b) the increase of its dye titration value as the titration pH is reduced below 2; (c) the development, by the action of the indophenol dye or of the walnut enzymes, of a yellow compound which spectroscopically seemed to resemble juglone.

We are indebted to Prof. T. Reichstein for specimens of reductone and of reductic acid, to Dr F. Bergel for specimens of reductone, reductic acid, diketogulonic acid, hydroxytetronic acid and isoascorbic acid, to the late Dr F. C. Jacoby for many of the spectroscopic estimations and to Miss J. G. Organ, Miss E. M. James and Miss E. Finnegan for assistance.

Note Since this paper was sent to the press we have been shown by Prof. Mirimanoff a thesis for Doctor of Pharmacy dealing with vitamin C in the walnut and confirming some of our findings. This thesis (Stadelmann, 1949) refers to work carried out in Germany during the war by J. Breinlich (Breinlich, 1942), in which the suggestion is put forward that the ascorbic acid in walnuts is oxidised to dehydroascorbic acid by juglone, which is itself reduced to hydrojuglone. No proof is provided of the existence of hydrojuglone in walnut tissues.

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