the chains with a greater number of these are not digested down so far as those with fewer.

The action of pancreatin on this peptide has revealed that it terminates in a peptide containing DNP-glycine, isoleucine, valine and glutamic acid, which Sanger has described as a *N*-terminal peptide sequence from the glycy chains of insulin. It also contains an additional leucine residue. The fraction is therefore probably derived from the *N*-terminal end of the original glycy chains and digestion by the enzyme apparently occurs from the other end of the chain.

This fraction 1/DNP 2/2 is unaffected by other enzymes such as pepsin and papain; this distinguishes it from the glycy chains in another complex fraction 1/DNP 1, which are digested with pepsin. This suggests that not all the glycy chains in the core are alike. Unless rearrangements are brought about by the enzymes the latter glycy chains must therefore come from the phenylalanyl chains of insulin. The chains having valyl or leucyl end groups may be derived from either type of original chain.

SUMMARY

1. The dinitrophenyl derivative of the 'core' resulting from chymotryptic digestion of insulin has

been oxidized with performic acid, and three fractions, which differ in solubility, have been isolated.

2. The core itself has been oxidized, and the main product after treatment with fluorodinitrobenzene or benzoyl chloride has in each case been separated into four fractions.

3. These benzoyl or dinitrophenyl fractions have been analysed for sulphur.

4. The dinitrophenyl fractions, with the exception of one, are complex, having a variety of *N*-terminal amino-acid residues.

5. One dinitrophenyl fraction, about 13 % of the total product, has a glycy end group, a molecular weight of about 2000, and a high cysteic acid content. A terminal peptide, yielding on acid hydrolysis *N*-dinitrophenylglycine, isoleucine, valine, glutamic acid and leucine, has been split from it by pancreatin.

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The Spectrophotometric Determination of Ascorbic Acid in Tissue Extracts, Particularly those of the Walnut (*Juglans regia*)

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Although ascorbic acid shows a well defined absorption band in the ultraviolet region of the spectrum, this property has received little attention for the determination of the vitamin in tissue extracts. The reasons for this would seem to be that the band is in a region where many other substances interfere, that the position of the band varies with pH (Karrer, Salomon, Morf & Schöpp, 1933) and that beyond certain limits the intensity of absorption is not directly proportional to the concentration (Herbert, Hirst, Percival, Reynolds & Smith, 1933).

Previously described spectrophotometric methods of assay have depended upon the measurement of absorption, before and after the destruction of ascorbic acid, by oxidation in the presence of copper (Johnson, 1936) or by irradiation (Chevallier & Choran, 1937). These methods are not applicable to extracts which contain a second reducing agent which absorbs in the same region as the ascorbic acid. Loureiro (1936) suggested that the shift with pH of the absorption curve might be utilized for determining ascorbic acid, but gave few details.

The factors affecting the position and intensity of absorption have been studied, and a spectrophotometric method of assay developed, details of which are described below.

EXPERIMENTAL

General. Absorption measurements were made with a Beckman spectrophotometer, using 1 cm. quartz cells and a hydrogen discharge lamp. Solutions were prepared with water freshly distilled from glass. The buffer solutions used were those described by Daglish, Baxter & Wokes (1948), and acetate buffers to cover the range pH 3.8–5.7. pH measurements were made with a Cambridge pH meter.

Fig. 1. The effect of instrumental irradiation on the stability of ascorbic acid solutions at different pH values. —, with KCN; ---, without KCN. Figures denote pH values.

Stability of ascorbic acid during assay. A stock solution (1 mg./ml.) of ascorbic acid in 0.01 N-HCl was diluted to 6–8 $\mu\text{g./ml.}$ in various buffers, with and without the addition of KCN solution (1 mg./ml.), to give a final concentration of 10 $\mu\text{g./ml.}$ The optical density at the absorption maximum was read at 60 sec. intervals. The results (Fig. 1) showed that irradiation of ascorbic acid solutions during measurement caused considerable losses. Traces of heavy metals may have been responsible in part for these losses which were prevented by KCN at pH values less than 6, but above this it was less effective.

Effect of pH on the absorption curve of ascorbic acid

The absorption curve was determined from 220–300 $\text{m}\mu.$ for dilutions of ascorbic acid and KCN in buffers covering the range pH 0–11. With unstable solutions, the density at the maximum was deter-

mined at timed intervals, and the value at zero time obtained by extrapolation.

The results, when plotted (Fig. 2), gave a family of absorption curves passing through an isosbestic point at 250 $\text{m}\mu.$, where $E_{1\text{ cm.}}^{1\%}$ is 485.3. This is due to the transition from the non-ionized form of the molecule absorbing at 245 $\text{m}\mu.$, to the totally ionized

Fig. 2. The effect of pH upon the absorption curve of ascorbic acid. (Figures adjoining curves denote pH.)

anion absorbing at 265 $\text{m}\mu.$ The pK_1 value of ascorbic acid calculated from the data is 4.16, which agrees with 4.17 given by Rosenberg (1942), where

$$\text{pK}_1 = \text{pH} + \log \frac{[\text{Unionized form}]}{[\text{Ionized form}]},$$

and from which the dissociation constant is 6.92×10^{-5} .

PROPOSED METHOD OF ASSAY

Fig. 2 shows that if the absorption of ascorbic acid is measured first at a pH below 3.5 and then above 4.5, two distinct curves will be obtained with peaks at 245 and 265 $\text{m}\mu.$ respectively as in Fig. 3a. The differences in readings of the two curves at 245 and 265 $\text{m}\mu.$, x and y , will depend only on the concentration and the selected pH values. Hence, if these last are kept constant, x and y will be proportional to concentration and the ratio y/x will be a constant for ascorbic acid.

The assay of ascorbic acid in any extract consists then of diluting into two buffers and measuring the values of x and y . If these are known for the pure compound, its proportion in the extract can be readily calculated. When the ratio y/x for the extract

is the same as that for ascorbic acid, the method is specific for this compound, and any discrepancy will indicate the presence of interfering substances.

The choice of buffers will depend largely upon the stability of the other constituents of the extract, and upon any shift in absorption they might exhibit. Two buffers giving an y/x ratio of the extract approximating that for ascorbic acid should be used.

Fig. 3. *a*, absorption curve of ascorbic acid at (—) pH 3 and (---) pH 4.6. *b*, absorption curve of mesocarp extract at (—) pH 3 and (---) pH 4.6; and of endocarp extract at (— · —) pH 3 and (....) pH 4.6.

Application of assay to walnut extracts

Walnut extracts are unstable above pH 5 (Daglish, 1950*a*) so buffers of pH 3.0 and 4.6 were chosen for the assay. Dilutions in these of two extracts gave the curves of Fig. 3*b*, whilst ascorbic

acid gave those of Fig. 3*a*. The fact that the y/x ratio determined for a number of extracts did not differ significantly from the value 3.62 found for ascorbic acid (Table 1) confirmed that these buffers were satisfactory. A change of pH in this range gave negligible shift to the curves of isolated hydrojuglone glucoside, tannins and their accompanying colouring matters.

Ethanolic hydrochloric acid had proved the most satisfactory solvent for stabilizing hydrojuglone glucoside (Daglish, 1950*a*). In this, or in ethanol, ascorbic acid solutions are unstable at room temperature, but more stable when refrigerated (Table 2). To avoid losses before assay, the following procedure was adopted.

Freshly prepared extract (2 ml.), diluted with 0.01*N*-hydrochloric acid to a final volume of 10 ml., was chilled to precipitate and coagulate chlorophyll and other water-insoluble constituents. The clear solution, clarified by spinning if necessary, was diluted into the buffers with sufficient potassium cyanide solution (1 mg./ml.) to give a final concentration of 10 μg./ml. of this compound, and the absorption measured at 245 and 265 mμ., then at 5 mμ. intervals to cover the range 230–280 mμ. The absorption curves of dilutions of a freshly prepared ascorbic acid solution (1 mg./ml. in 0.01*N*-hydrochloric acid) were determined for each group of assays to avoid variations due to slight changes in the buffer solutions.

This spectrophotometric method of assay and the formaldehyde dye titration, previously described (Daglish, 1950*b*) were applied to extracts prepared from different tissues of the walnut throughout 1949 and 1950. This was to determine whether a similarity

Table 1. *Comparison of spectrophotometric assay results with those of formaldehyde dye titration*

No. of assays	Ascorbic acid	Walnut extracts			
		Mesophyll	Epicarp	Mesocarp	Endocarp
Spectrophotometric assay × 100	18	18	15	15	15
Dye titration assay	—	93.5	101.7	87.5	103.5
Deviation of mean	—	6.5	3.1	4.7	1.2
y/x	3.62	3.48	3.70	3.57	3.33
Deviation of mean	0.16	0.55	0.29	0.24	0.21

Table 2. *Stability of 0.07 % (w/v) solution of ascorbic acid in ethanol and ethanolic hydrochloric acid*

(The results are expressed as a percentage of the amount originally present.)

HCl (N)	Temp. (°)	Percentage after				
		3 hr.	22 hr.	46 hr.	70 hr.	120 hr.
0	18	88	59	21.7	—	—
0	4	100	100	100	98.2	83.6
0.3	18	70.6	0	—	—	—
1.5	18	98.8	71	25	—	—
1.5	4	100	97.6	87.4	69.7	40.0
3.0	18	101.8	90.9	46.3	—	—
Catkin extract	4	—	96.3	96.3	85.3	—
0.3 <i>N</i>						

in results was obtained for the distribution of ascorbic acid in the plant, which could be compared with the results of earlier workers who had used only the dye titration. Although a detailed account of this work will be given in a future paper, the results of sixty-three such duplicate assays are summarized in Table 1. Fig. 4 shows the seasonal variation in concentration of ascorbic acid in two tissues when determined by both methods.

Dehydroascorbic acid. Because this does not absorb at 245 or at 265 $m\mu$, it was determined by the difference in the reduced ascorbic acid values found after and before treatment with hydrogen sulphide at pH 3.

Fig. 4. Seasonal variation of ascorbic acid concentration in endocarp and mesocarp. —, endocarp by spectrophotometric assay; ---, endocarp by indophenol dye titration;, mesocarp by spectrophotometric assay; - · - · -, mesocarp by indophenol dye titration.

Accuracy of the method. The accuracy depends upon the least density difference at 245 and 265 $m\mu$, caused by the change in pH, which can be measured, and upon the x and y values of ascorbic acid in the selected buffers. At pH 3.0 and 4.6, the changes in $E_{1\text{ cm}}^{1\%}$ at 245 and 265 $m\mu$ are -131.4 and $+474$ respectively. If the reproducible least density difference is taken as 0.005 (which is equivalent to 0.038 and 0.011 mg./100 ml. ascorbic acid at the two wavelengths), and if the concentration for assay is about 0.8 mg./100 ml., the error is 1.3 and 4.7 % at 265 and 245 $m\mu$ respectively.

DISCUSSION

Owing to the complex nature of most natural products, it is desirable to minimize the number of steps in any assay method to prevent the formation of non-specific substances and the destruction of ascorbic acid. To overcome these difficulties, a general procedure has arisen of determining the total effect (indophenol titration, ultraviolet light absorption, etc.) before and after the destruction of

ascorbic acid. Should a second substance be present in the extract as, for example, hydrojuglone glucoside in the walnut, which is affected by the oxidation step, the results are often doubtful.

Loureiro (1936) suggested that the shift with pH of the ultraviolet absorption might be applied to the determination of ascorbic acid. Although the shift was characteristic of a group of enediols, the positions of the maxima of those investigated were sufficiently far removed from that of ascorbic acid to avoid interference. Reductone, for example, would not interfere unless present to the extent of 30 % the amount of ascorbic acid. He proposed measurement at pH 1 and 6–7, but gave no values for the extinction of ascorbic acid, save that it was lower at the shorter wavelengths.

After investigating the effect of pH on the absorption curve of ascorbic acid it has been found possible to develop a method of assay applicable to walnut extracts. Measurements were made at pH 3.0 and 4.6. A change of pH in this range gave negligible shift to the absorption curves of other constituents of the extracts as shown by the agreement of the y/x values of these when compared with that of ascorbic acid. This agreement indicates that the method is specific for this compound, and a deviation, the presence of interfering substances. A further and more rapid check on the specificity of the method is the intersection of the curves at 250 $m\mu$, the isosbestic point of ascorbic acid (Fig. 2).

In this assay no purification step was necessary save that of removing water-insoluble substances from acid ethanol extracts. It was found to be much quicker than the indophenol dye titration in that only 6–8 min. were required for determining the two sets of absorption readings. Comparison of the results by the two methods (Table 1) showed that good agreement was reached when ascorbic acid alone was present as in endocarp extracts, but greater variations were experienced when a second dye reductant (hydrojuglone glucoside) was present as in the mesocarp and epicarp.

Whilst the present work was in progress, a similar method of assay was proposed by Vacher & Fauquembergue (1949). The treatment of the results by these workers differs from that above, in that the 'shift curve' is plotted and identified with that of ascorbic acid, when the pH is moved from below pH 3.5 to slightly above 5.5. They suggest that accurate control of the pH is not necessary below pH 3.5 and above 5.5 provided 7.5 is not passed. Fig. 2 shows that the extinction values of the maxima in these regions vary greatly with pH, and for accurate work the pH must be closely controlled.

Although applied in the present work only to walnut extracts, the method of assay should be applicable, as found by the French workers, to the determination of ascorbic acid in most tissue extracts.

SUMMARY

1. The absorption characteristics of ascorbic acid as affected by pH have been determined.
2. A method of assay utilizing the shift of the absorption curve has been suggested.
3. The results of this method when applied to

walnut extracts have been compared with those obtained by the indophenol dye titration.

I wish to thank Dr F. Wokes, the Director of the Laboratories, for affording me the opportunity to publish this work (which, together with that described in the following paper, will form part of the thesis for a Ph.D. degree of the University of London), and Miss N. Baxter for technical help in the preparation of the many extracts used.

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The Occurrence of Ascorbic Acid in the Walnut (*Juglans regia*)

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In an earlier paper (Daglish, 1950*a*) it was shown that hydrojuglone glucoside was more resistant to atmospheric oxidation in walnut extracts than in solutions prepared therefrom by chromatography. The properties of the antioxidant suggested that it was ascorbic acid, high concentrations of which are known to occur in the walnut (Gerghelezhiu, 1937).

Breinlich (1942) postulated that ascorbic acid and juglone formed part of an oxidation-reduction system. If there is a similar interaction between ascorbic acid and hydrojuglone glucoside, then, as the concentration of the latter shows definite seasonal variations in the different tissues of the plant (Daglish, 1950*b*), it would be expected that ascorbic acid would exhibit corresponding variations. In order to determine if this is so, the occurrence of ascorbic acid has been determined throughout the 1949 and 1950 growing periods.

EXPERIMENTAL

Preparation of extracts. In order to avoid variations due to location, age of trees, soil, etc., samples were taken from one mature tree in 1949. The ascorbic acid concentration in the different plant parts exhibited definite seasonal fluctuations. A second tree in a different location was used in 1950 to demonstrate that these variations were characteristic of the walnut as a species and not just a single member.

Within 30 min. of collection, the plant material was extracted into ethanolic HCl (Daglish, 1950*a*), and then assayed for ascorbic acid, dehydroascorbic acid and hydrojuglone glucoside by the spectrophotometric methods previously described (Daglish, 1950*b*, 1951). All results were calculated on a fresh weight basis.

RESULTS AND DISCUSSION

Male catkins. To minimize errors caused by the variation of individual catkins, at least twenty of these were used for each assay. Duplicate assays at any one time differed by not more than 8.5 %, which was less than the variation of successive weekly samples.

When first distinguishable from the vegetative buds, the male catkin buds weighed 30–50 mg. each, and contained approximately 30 mg./100 g. of ascorbic acid. This level was maintained throughout the winter. In the spring there was an initial gradual increase in weight to 0.1–0.15 g. and with this, the concentration of ascorbic acid increased to 160 and 386 mg./100 g. in 1950 and 1949, respectively. This was followed by a rapid decrease in concentration which was not due to a loss of ascorbic acid, but due solely to the 20-fold increase in weight which had taken place. In fact, the amount of ascorbic acid present showed an increase as indicated