A Note on the Estimation of Dehydro-L-Ascorbic Acid in Plant Tissues by the Roe & Kuether Procedure

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Dehydro-L-ascorbic acid is usually found in plant tissues in low concentrations and is always associated with ascorbic acid in much higher concentrations. One of the most reliable and direct methods for its estimation is that originally proposed by Roe & Kuether (1943) which is based on the formation of a 2:4-dinitrophenylosazone.

Many plant tissues, however, contain so little dehydro-L-ascorbic acid that after extraction and dilution with the phenylhydrazine reagents the extracts may contain less than 0.5 μ g. of dehydro-L-ascorbic acid/ml. and even lower values may be encountered if the tissue has been exposed to anaerobic conditions. If more concentrated extracts are prepared interference is often observed on the addition of the 85 % sulphuric acid reagent, owing to formation of coloured products. A more serious disadvantage, and one which makes it impossible to use the method at all, is that with some plant extracts hydrazones of other compounds are formed which absorb strongly in the same region (530 $m\mu$) as the red 2:4-dinitrophenylosazone formed from dehydro-L-ascorbic acid. The method has been modified to permit of the more accurate estimation of dehydro-L-ascorbic acid when present in small amounts and to effect the separation of the osazone of dehydro-L-ascorbic acid from other hydrazones formed from other compounds in plant tissues.

REAGENTS

2:4-Dinitrophenylhydrazine, metaphosphoric acid and thiourea were analytical-grade reagents. The acid-activated alumina (Woelm) was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. For routine estimation of dehydro-L-ascorbic acid (DHA) in plant tissues the dinitrophenylosazone formed from DHA was separated from other hydrazones or osazones on columns (5 cm. $\times$ 1 cm.) of alumina.

RESULTS

Concentration of the osazone. The red dinitrophenylosazone formed from DHA is almost insoluble in the sulphuric acid-phenylhydrazine reagent and may be quantitatively collected from extracts by centrifuging or filtration or both. By this means the total DHA from 100 g. or even larger

amounts of tissue may be conveniently prepared. In this way the osazone may be concentrated, and at the same time separated from unchanged phenylhydrazine and many other undesirable constituents of the tissue.

After extraction of the plant tissue, carried out by the methods described by Barker & Mapson (1959) to avoid any oxidation of ascorbic acid during extraction, the usual procedure of Roe & Kuether is followed whereby the extract is incubated with 2:4-dinitrophenylhydrazine in the presence of thiourea for 3 hr. at 37°. When microgram quantities of DHA are present with milligram quantities of ascorbic acid it is advisable and even essential to remove all O_2 from the solution before incubation with the phenylhydrazine reagent.

Procedure. The tissue is extracted with metaphosphoric acid (5 %, w/v) at -3° under anaerobic conditions (Barker & Mapson), and to this extract 4 ml. of 2:4-dinitrophenylhydrazine (2 %, w/v) in 9N- H_2SO_4 is added together with 200 mg. of thiourea to every 20 ml. of extract. The solution is freed from O_2 by bubbling with N_2 and is then incubated for 3 hr. at 37°.

After incubation the solution is centrifuged at 20 000g for 30 min. to aggregate the insoluble hydrazones and osazones. The supernatant is passed through sintered-glass filters, and any precipitate collected is combined with that centrifuged down. The combined precipitates are washed with 0.1N- H_2SO_4 to remove most of the unchanged 2:4-dinitrophenylhydrazine reagent. The washed hydrazones are dissolved in a small volume of ethyl acetate, and the solution is transferred to a separating funnel. If, as is the case with many plant extracts, hydrazones of the α -oxo acids are also present, these may with advantage be removed at this stage by extracting the ethyl acetate solution twice with 0.2M- $NaHCO_3$. By this treatment these latter hydrazones are rendered soluble in the aqueous phase, whilst the red osazone formed from DHA remains in the non-aqueous phase. The ethyl acetate solution is washed again with 0.1N- H_2SO_4 and, after carefully separating the aqueous phase, the ethyl acetate solution is concentrated *in vacuo* to dryness; the hydrazones are dissolved in 1 ml. of ethyl acetate to which is added 5 ml. of H_2SO_4 (50 %, w/v). The concentration of the red osazone is determined from the difference between the absorption at 530 $m\mu$ of this solution and a similar solution in which the DHA had been previously reduced by H_2S in the usual Roe & Kuether method. Dehydroascorbic acid standards (prepared by the oxidation of ascorbic acid with Br_2) are prepared and are carried through the same procedure.

Tests have been carried out with the above procedure with the osazone prepared from DHA present in amounts varying from 5 to 75 $\mu\text{g.}$ in 20 ml. of solution. Fig. 1 shows that, after the standard procedure described above, a linear relation exists between the amounts of DHA taken and the amount of osazone collected over the range 10–75 $\mu\text{g.}$ of DHA/20 ml. of solution. With less than 10 $\mu\text{g.}$ of DHA there was a departure from this linear relationship. This we attribute mainly to a small loss of the osazone due to a low but finite solubility in the reagent solution; this loss appears to be constant under our standard conditions. Fortunately with this procedure more concentrated extracts may be prepared so as to increase the concentration of the osazone, and we suggest that a suitable amount of tissue should be extracted to yield, if possible, an extract containing not less than 0.5 $\mu\text{g.}$ of DHA/ml.

Separation on acid alumina. With some plant tissues (e.g. peas) the absence of compounds forming hydrazones or osazones or both which interfere in the estimation makes it possible to proceed simply as described above. With other plant tissues (strawberry leaves) this procedure is inapplicable since during the treatment with 2:4-dinitrophenylhydrazine other hydrazones or osazones or both are formed which absorb strongly in the region of 530 $m\mu$ and cause serious interference.

It is possible to separate these compounds from that of the red osazone formed from DHA by chromatography on acid-activated alumina. Columns of acid alumina are prepared with ethyl acetate containing 2% (v/v) of acetic acid, the hydrazones from the tissue are added in solution in ethyl acetate, and the chromatogram is developed

with the ethyl acetate–acetic acid solvent. Any unchanged 2:4-dinitrophenylhydrazine is first eluted but this need not be separated, unless desired, from the following band of the red osazone of DHA which is readily eluted from the column by the ethyl acetate–acetic acid reagent. Other hydrazones are firmly held on the column, and include those formed from α -oxo acids, which makes it unnecessary to use the extraction with NaHCO_3 described above if separation on alumina is employed.

The eluates are concentrated *in vacuo* and the concentration of the osazone formed from DHA is determined as described above. Tests carried out with solutions of the osazone prepared from DHA show that the osazone is quantitatively recovered from the column, if this is well washed with the ethyl acetate–acetic acid reagent. With this method good recoveries of DHA added to extracts of potatoes and strawberry leaves have been obtained (Table 1). It is also possible to separate quantitatively the osazone formed from DHA by paper chromatography, by using an aqueous solvent containing phenol (10%, w/v), acetic acid (10%, v/v) and glycerol (10%, v/v), and by eluting the osazone from the appropriate area of the chromatogram with ethyl acetate. This method possesses no advantage over that outlined above and is more tedious to operate. Analysis by paper chromatography can, however, serve as a valuable qualitative check on the results obtained by the alumina method.

Formation of open- and closed-chain osazones from dehydroascorbic acid

Herbert, Hirst, Percival, Reynolds & Smith (1933) first pointed out the numerous possibilities for structural isomerism and stereoisomerism of the phenylhydrazine derivatives of DHA. Hirst (1933) noted the presence of two different phenyl-osazones obtained from the oxidized form of DHA

Fig. 1. Relation between the concentration of DHA and the dinitrophenylosazone formed.

Table 1. Accuracy of the method as determined by the estimation of known amounts of dehydro-L-ascorbic acid added to plant extracts

Plant extract	DHA added ($\mu\text{g./20 ml.}$ of extract)	DHA estimated ($\mu\text{g./20 ml.}$ of extract)	Error (%)
Strawberry leaf	30	32	+6
	50	52	+4
	20	19	-5
	20	21	+5
	30	30	0
	20	18	-10
	30	29	-3
Potato	20	19	-5
	20	21	+5
	40	39	-2.5

and phenylhydrazine and Ohle & Bockman (1934) found that, in the presence of mineral acids, osazones corresponding to the open- and closed-chain forms of dehydroascorbic acid (I and II) could be obtained.

In our experiments we have also found evidence that two similar osazones are formed as a result of the reaction between 2:4-dinitrophenylhydrazine and DHA under the conditions of the Roe & Kuether procedure. These two forms have been separated by paper chromatography by using as solvent the phenol-acetic acid-glycerol mixture. The open-chain compound, an orange-yellow form with R_f 0.43, is easily separated from the red lactone form with R_f 0.66. These two compounds may also be separated on the alumina column in the manner described above, under which conditions the orange-yellow component is adsorbed strongly on the alumina and may, after removal of the red component with the ethyl acetate-acetic acid solvent, be eluted with acetone containing 0.2M-oxalic acid. After elution and on standing (24 hr. at 20°) in the presence of oxalic acid the orange-yellow compound is completely converted into the red component as shown by the change, revealed by paper chromatography, from an orange-red component with R_f 0.43 to a red component with R_f 0.66. A similar occurrence was reported by Probst & Schultze (1950) after chromatography of 2:4-dinitrophenylhydrazine derivatives on magnesium phosphate columns.

The red lactone form isolated after chromatography on alumina and recrystallized from an ethanol-acetone mixture (1:1) appears to be identical with the derivative prepared by the above workers, giving in 9N-H₂SO₄ an absorption spectrum with maxima at 525 and 370 mμ and in which the ratio of the extinctions measured at 530:450, 370:450, and 370:530 were 2.1, 2.25 and

1.08 respectively, values which are almost identical with those reported by Probst & Schultze.

The lactone form may be reconverted into the open-chain form by shaking an ethyl acetate solution of the red derivative with 0.1N-NaOH, when the orange-yellow compound formed becomes soluble in the aqueous phase and may be recovered, after acidification with acetic acid, by extraction into ethyl acetate. These changes have likewise been demonstrated by paper chromatography.

Fortunately, from the point of view of estimating DHA, the formation of the red osazone predominates, under our conditions, over that of the orange-yellow osazone. The relative amounts of the two compounds formed have been estimated after separation on alumina and conversion of the open-chain form into the lactone form. Under the standard conditions used here for the formation of the osazone, 97-98 % of the total osazone formation is in the lactone form. Since, moreover, the amount of the lactone form is proportional to the original concentration of DHA present, the failure to estimate the small amount of the orange-yellow component is of little consequence as far as the estimation of DHA is concerned.

SUMMARY

1. A modification of the Roe & Kuether (1943) procedure for the estimation of dehydroascorbic acid in plant tissues is described. The dinitrophenyl-osazone formed from dehydroascorbic acid may be separated from other interfering hydrazones or osazones by chromatography on acid alumina.

2. Evidence of the formation of an open-chain osazone and of a lactone form has been obtained when dehydroascorbic acid reacts with 2:4-dinitrophenylhydrazine under the conditions of the Roe & Kuether test.

REFERENCES

- Barker, J. & Mapson, L. W. (1959). *New Phytol.* **58**, 58.
 Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W. & Smith, F. (1933). *J. chem. Soc.* p. 1270.
 Hirst, E. L. (1933). *J. Soc. chem. Ind., Lond.*, **52**, 221.
 Ohle, H. & Bockman, G. (1934). *Ber. dtsch. chem. Ges.* **67**, 1750.
 Probst, G. W. & Schultze, M. O. (1950). *J. biol. Chem.* **187**, 453.
 Roe, H. H. & Kuether, C. A. (1943). *J. biol. Chem.* **147**, 399.