The Use of Homocysteine in the Estimation of Dehydroascorbic Acid

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Two main methods are in current use for the estimation of dehydroascorbic acid. In the one, developed by Roe and his colleagues (Roe & Kuether, 1943; Roe, Mills, Oesterling & Damron, 1948), the dehydroascorbic acid is condensed with 2:4-dinitrophenylhydrazine and the product treated with sulphuric acid to give a red colour, the intensity of which is measured photoelectrically. In a second technique (Tillmans, Hirsch & Siebert, 1932; Eekelen, Emmerie, Josephy & Wolff, 1933; Bessey, 1938), dehydroascorbic acid is measured as ascorbic acid after reduction with hydrogen sulphide and removal of excess of reductant. Disadvantages are associated with both methods; they are non-specific for dehydroascorbic acid, time- and labour-consuming, and in the method involving reduction with hydrogen sulphide it may be difficult to ensure the removal of excess of reductant before estimating the ascorbic acid formed with 2:6-dichlorophenol-indophenol.

In this paper an account is given of a new method, based on the two findings that homocysteine rapidly reduces dehydroascorbic acid at pH 7·0, and that under certain simple conditions 2:6-dichlorophenol-indophenol can be used to estimate ascorbic acid in the presence of homocysteine without interference from the latter compound.

EXPERIMENTAL

Preliminary observations

Reduction of dehydroascorbic acid by homocysteine. At pH values greater than 6·8 homocysteine rapidly reduces dehydroascorbic acid to ascorbic acid. In Fig. 1 the reduction at pH 7·2 of dehydroascorbic acid by three sulphydryl compounds (homocysteine, glutathione and cysteine) is recorded: reduction by homocysteine is complete after a few minutes at room temperature (about 18°), whereas glutathione and cysteine act much more slowly. It was found difficult to measure accurately the rate of the reaction in the presence of cysteine owing to the rapid rate at which cysteine reduces the indophenol dye used in the estimation of the ascorbic acid formed. In these, as in all the experiments described in this paper, the ascorbic acid was measured by the photoelectric method of Bessey (1938).

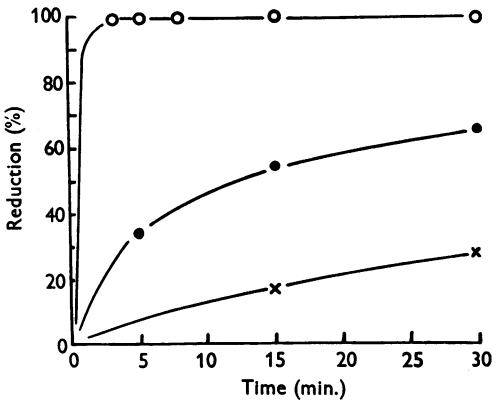

Fig. 1. Reduction of dehydroascorbic acid (0·045 mg./ml.) by various sulphydryl compounds in phosphate buffer of pH 7·2, a molecular ratio (reductant to dehydroascorbic acid) of 40:1 being present in each case. ○, Homocysteine; ●, glutathione; ×, cysteine.

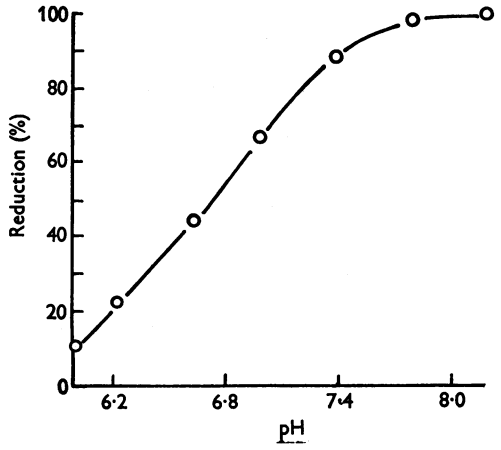

Fig. 2. Percentage reduction, at different pH values, of 0·05 mg. of dehydroascorbic acid by 2·0 mg. of homocysteine. The total volume of the reaction mixture was 6 ml. and the time 8 min.

Table 1. *Effect of different homocysteine:dehydroascorbic acid ratios on the degree of reduction of dehydroascorbic acid*

Time (min.)	Percentage reduction of dehydroascorbic acid: Expt. 1. Molecular ratio homocysteine:dehydroascorbic acid = 10	Expt. 2. Molecular ratio homocysteine:dehydroascorbic acid = 28
3	68	89
6	82	96
10	91	98
15	94	98

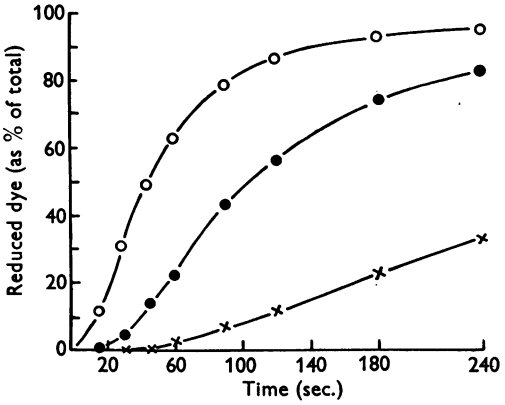

Fig. 3. Reduction of 4 ml. of 2:6-dichlorophenolindophenol dye (1 ml. ≡ 0·02 mg. ascorbic acid) by 0·40 mg. of homocysteine at different pH values. ○, Na_2HPO_4–citric acid buffer, pH 4·5; ●, Na_2HPO_4–citric acid buffer, pH 3·5; ×, Na_2HPO_4–citric acid buffer, pH 2·5.

Below pH 6·8 the rate of the reduction of dehydroascorbic acid by homocysteine is measurably slower; Fig. 2 gives the percentage reduction occurring after 8 min. in buffered solutions of different pH values, the reduction being stopped after 8 min. by acidifying the reaction mixture with 30% (w/v) metaphosphoric acid. For the experiments described above a relatively high homocysteine:dehydroascorbic acid ratio was maintained; this is necessary for complete reduction of the dehydroascorbic acid. Table 1 records the effect of two such ratios upon the reduction of dehydroascorbic acid.

Reduction of 2:6-dichlorophenolindophenol by homocysteine. Sulphydryl compounds reduce the indophenol dye used in the estimation of ascorbic acid at a rate that is dependent upon the pH of the reaction mixture (Mapson & Harris, 1947; Bland, Constable, Harris & Hughes, 1951). It was important to examine the rate of the reduction of the dye by homocysteine in order to ascertain whether the homocysteine would be likely to interfere in the estimation of the ascorbic acid formed. Fig. 3 shows the reaction between 2:6-dichlorophenolindophenol and homocysteine at three pH values, and it will be observed that at pH 2·5 there is negligible reduction of the dye within the first minute; at this pH ascorbic acid reacts with the dye to completion within 2·5 sec. (Mapson & Harris, 1947; Bland *et al.* 1951). This means that homocysteine will not interfere in the estimation of ascorbic acid with 2:6-dichlorophenolindophenol, provided that the reaction is carried out at pH 2·5 and that the galvanometer is read within 30 sec. of the addition of the dye. Estimating the ascorbic acid at a pH below 2·5 would still further reduce the possibility of interference by homocysteine, but would, on the other hand, introduce an error due to the spontaneous fading of the dye which occurs sufficiently rapidly below pH 2·0 to be a source of error.

These findings form the basis of a method for the estimation of dehydroascorbic acid, a preliminary account of which has already appeared (Hughes, 1954*a*).

METHOD

Reagents. (*a*) Homocysteine (Light and Co. Ltd.) (2·0 mg./ml.). (*b*) 2:6-Dichlorophenolindophenol (1 ml. ≡ 0·02 mg. of ascorbic acid). This was usually prepared by diluting a stock solution of which 1 ml. is equivalent to 0·40 mg. of ascorbic acid. The exact strength of the stock solution is determined initially and checked from time to time by titration against a standard, freshly prepared solution of ascorbic acid in 4% metaphosphoric acid. (*c*) Buffer of pH 2·3, 500 ml. of which contains 188·0 g. of citric acid and 30·8 g. of anhydrous Na_2HPO_4. (*d*) 3% (w/v) metaphosphoric acid, used as extractant. (*e*) 45% (w/v) K_2HPO_4 (pure; British Drug Houses, Ltd.). (*f*) Dehydroascorbic acid (used in checking the method). This was prepared by oxidizing ascorbic acid with bromine and removing the excess of bromine by bubbling air through the solution (Association of Vitamin Chemists, 1947).

Procedure. The tissue to be examined is extracted with 3% metaphosphoric acid (or, for plasma, with a solution containing 2% of oxalic acid and 4% of metaphosphoric acid), and a clear solution is obtained by centrifuging and filtration. The method involves the estimation of ascorbic acid in portions of this extract before and after treatment with homocysteine; the difference between the two values, after correcting for volume changes, gives the dehydroascorbic acid content.

Estimation of the ascorbic acid content of the extract. This is estimated by measuring photoelectrically the reduction of a known amount of the indophenol dye by a portion of the solution by a method that is essentially that described by Bessey (1938) being used. Ostwald blow-out pipettes are used to measure 4 ml. of the buffer (pH 2·3) and 2 ml. of the extract into one of the 10 ml., 3 cm. cells of a photoelectric colorimeter. A direct-reading photoelectric colorimeter, with a spot galvanometer, sensitivity of 170 mm./μA, and a 61N Kodak filter (max. transmission at 520 mμ.), was employed. With water in the second cell, the galvanometer is set to 100, and 4 ml. of the indophenol dye (of which 1 ml. ≡ 0·02 mg. of ascorbic acid) is blown into the cell containing the buffered extract. The mixture is stirred with a small glass rod, the reaction cell moved into position and the galvanometer read within 30 sec. of the addition of the dye. The amount of ascorbic acid is calculated by reference to a standard curve constructed by treating known amounts of ascorbic acid with the dye under the conditions of the estimation. Turbidity, if present, may be corrected for by decolorization as described by Bessey (1938).

Estimation of ascorbic acid + dehydroascorbic acid. The next step is the estimation of the total ascorbic acid present after the conversion of any dehydroascorbic acid present into ascorbic acid with homocysteine. A portion (3 ml.) of the extract is treated in a test tube with 1 ml. of the homocysteine solution. The amount of K_2HPO_4 necessary to change the pH to 6·8 is slowly added from a 5 ml. microburette, the test tube being shaken during the addition. The amount of K_2HPO_4 required may be determined beforehand by titrating 3 ml. of the extractant against the K_2HPO_4 in the presence of bromothymol blue; 3 ml. of a solution containing 3% metaphosphoric acid should require about 1·0 ml. of the K_2HPO_4. The reaction mixture is allowed to stand at room temperature for about 15 min., after which period any dehydroascorbic acid present will have been changed to ascorbic acid (see Fig. 4). The total ascorbic acid is estimated by using 2 ml. of the homocysteine-treated solution in the photoelectric procedure outlined above, care being taken to read the galvanometer within 30 sec. of the addition of the dye, so as to avoid interference by the homocysteine. The volume changes are corrected for and the dehydroascorbic acid content is calculated by subtracting the initial value for the ascorbic acid from the total obtained after treatment with the homocysteine.

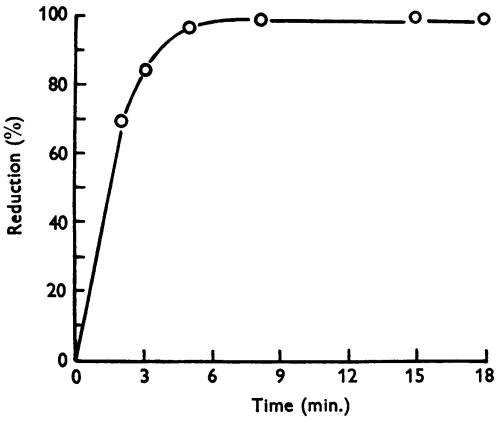

Fig. 4. The rate of reduction of dehydroascorbic acid (0·0048 mg./ml.) by homocysteine. 1 ml. of the homocysteine solution (1·2 mg./ml.) was added to 3 ml. of the dehydroascorbic acid solution, which was then treated with K_2HPO_4 as described in the text. The reaction was stopped after the required time by the addition of 2 ml. of 30% metaphosphoric acid.

If the concentration of ascorbic acid plus dehydroascorbic acid in the extract is low (less than 0·75 mg./100 ml.), then the method may be modified slightly. In such cases 4 ml. of buffer, 3 ml. of the extract and 3 ml. of indophenol dye (strength 1 ml. ≡ 0·01 mg. ascorbic acid) should be used in the estimation, and a homocysteine solution containing 1·3 mg./ml. is sufficiently strong for the reduction of the dehydroascorbic acid.

This technique has been applied to a large number of chemically prepared samples of dehydroascorbic acid and to mixtures of ascorbic acid and dehydroascorbic acid; typical results of such experiments are given in Table 2.

The purity of the potassium phosphate used in the neutralization procedure is of importance; certain samples appear to contain an impurity which, by destroying or removing some of the dehydroascorbic acid, lowers the percentage recovery. Fresh samples of the salt should always be checked by its use in the recovery of standard amounts of chemically prepared dehydroascorbic acid before it is used in the estimation of unknown amounts of the compound.

Specificity of method: the use of boric acid. As is to be expected, the method is not completely specific, certain substances in addition to dehydroascorbic acid being reduced by homocysteine to give reducing products which react with the indophenol dye. Such compounds include alloxan and the products obtained when certain ene-diol substances such as reductic acid, glucoreductone (prepared as described by Kertesz, 1934) and *iso*ascorbic acid

Table 2. *Typical recoveries obtained on applying the homocysteine technique to standard solutions of dehydroascorbic acid and to mixtures of dehydroascorbic acid and ascorbic acid*

Taken		Recovered	
Ascorbic acid (mg./ml.)	Dehydroascorbic acid (mg./ml.)	Ascorbic acid (mg./ml.)	Dehydroascorbic acid (mg./ml.)
0	0·1000	—	0·0960 (96%)
0	0·0800	—	0·0791 (99%)
0	0·0763	—	0·0735 (96%)
0	0·0450	—	0·0441 (98%)
0	0·0382	—	0·0374 (98%)
0	0·0092	—	0·00915 (99%)
0	0·0046	—	0·00452 (98%)
0	0·0047	—	0·00451 (96%)
0·0205	0·0205	0·0202 (99%)	0·0200 (98%)
0·0137	0·0274	0·0137 (100%)	0·0272 (99%)
0·0274	0·0137	0·0278 (101%)	0·0127 (93%)
0·0193	0·0193	0·0187 (97%)	0·0193 (100%)
0·0243	0·0146	0·0247 (101%)	0·0142 (98%)
0·0282	0·0106	0·0290 (102%)	0·0105 (99%)

are oxidized with bromine. These compounds are not believed to be of any biological importance but, even so, some attention was paid to possible improvements by means of which the specificity of the homocysteine reaction could be increased.

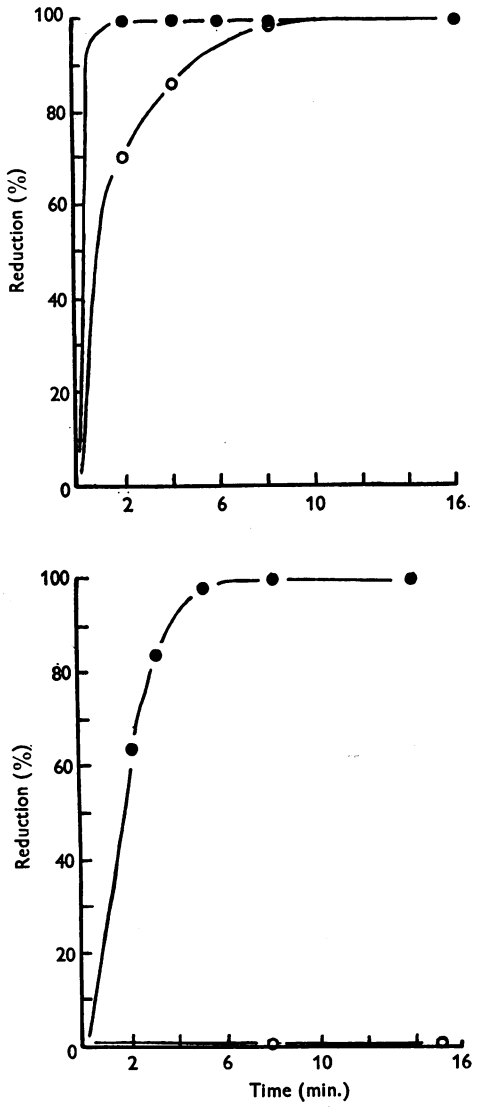

Fig. 5. Effect of boric acid on the reduction by homocysteine of: (*a*) dehydroreductic acid (upper graph) and (*b*) dehydroascorbic acid (lower graph). ●, Normal reduction; ○, reduction in the presence of boric acid. 3 ml. of the dehydroascorbic acid (0·050 mg./ml.) or of the dehydroreductic acid (0·034 mg./ml.) was treated with 1 ml. of homocysteine solution (2·0 mg./ml.) and 1 ml. K_2HPO_4 (30%, w/v) in the presence of 1 ml. aqueous solution of boric acid (about 3%, w/v). The 'normal' values were obtained from a parallel set of experiments in which water was substituted for the boric acid.

It has been known for some time (e.g. Penney & Zilva, 1943; Huelin, 1949) that at alkaline pH values boric acid rapidly destroys dehydroascorbic acid; such a destruction occurs rapidly at pH 7·0, at which the homocysteine reduction is carried out, and if boric acid is added to an acid extract of dehydroascorbic acid there is no formation of ascorbic acid after subjecting such a treated extract to the homocysteine technique. Boric acid, however, has no appreciable destructive effect upon at least some of the interfering compounds listed above. In Fig. 5 the effect of boric acid (in a final concentration of about 0·5%) upon the reduction of dehydroascorbic acid by homocysteine is compared with its effect upon the reduction of dehydroreductic acid (a possible interfering compound) by homocysteine; after 15 min. there is zero reduction of dehydroascorbic acid in the presence of boric acid, whereas the final degree of reduction of the dehydroreductic acid is not appreciably affected, although it may be noted that boric acid causes an initial retardation of the rate. Similar considerations apply with 'dehydroglucoreductone' and alloxan. Hence it can safely be assumed that any compound which is reduced by homocysteine in the presence of boric acid cannot be dehydroascorbic acid. It has been found best when applying this boric acid modification to add 1 ml. of a 3% solution of boric acid to 3 ml. of the acid extract before effecting the change in pH with K_2HPO_4.

As these interfering compounds are believed not to be of widespread occurrence in Nature, it is recommended that in routine estimations of dehydroascorbic acid the unmodified procedure (i.e. without boric acid) be used.

APPLICATIONS

The homocysteine technique has been applied to a number of natural substances, notably to the examination of the dehydroascorbic acid content of plasma and of certain plant tissues. Reference will be made here to the result of one such application, namely the examination of the cotyledonous tissue of germinating peas, a tissue reported by several workers (Robertson, 1947; Sreenivasan & Wandrekar, 1950) to contain comparatively large amounts of dehydroascorbic acid. Such an occurrence of the compound would be unexpected, in view of the findings that pea cotyledons contain a comparatively high concentration of sulphydryl compounds and are also one of the best sources of the enzyme dehydroascorbic acid reductase, which catalyses the reduction of dehydroascorbic acid by glutathione to ascorbic acid (Crook & Morgan, 1944; Hughes, 1954*b*). Accordingly, the dehydroascorbic acid content of pea cotyledons in the initial stages of germination was examined by applying both the homocysteine technique and the dinitrophenylhydrazine method (Roe & Kuether, 1943) to portions of extracts of the cotyledonous tissue, so that a value was obtained for the dehydroascorbic acid by two methods.

Peas were soaked for 12 hr. and allowed to germinate on pads of moist cotton wool covered with gauze. The testas

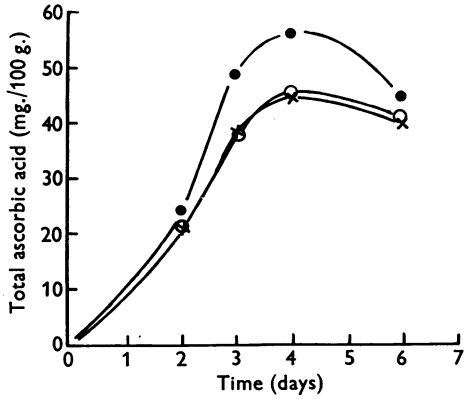

Fig. 6. Formation of dehydroascorbic acid in the cotyledons of germinating peas as measured by the homocysteine and dinitrophenylhydrazine methods. ×, Ascorbic acid by indophenol method; ○, total vitamin C (i.e. dehydroascorbic acid plus ascorbic acid) as measured by the homocysteine method; ●, total vitamin C as measured by the dinitrophenylhydrazine method.

were removed from the peas before germination to enable a greater and more regular formation of ascorbic acid to occur (Hughes, 1954*b*). After timed periods the embryos were removed from 20–30 of the peas and the cotyledons dried between filter paper, weighed and extracted with a solution of 3% metaphosphoric acid. Ascorbic acid was estimated in a portion of the filtered extract and a further portion (3 ml.) was subjected to the homocysteine procedure to obtain a value for the dehydroascorbic acid content. A third portion was diluted with 5% trichloroacetic acid and shaken with charcoal; this was used for the estimation of total vitamin C (i.e. dehydroascorbic acid plus ascorbic acid) by the dinitrophenylhydrazine procedure (Roe & Kuether, 1943).

From the results (Fig. 6) it is seen that there is no increase in ascorbic acid after treatment with homocysteine; this suggests that there is no dehydroascorbic acid present, but there does appear to be present an interfering substance which behaves like dehydroascorbic acid towards the reagents used in the dinitrophenylhydrazine procedure. It is conceivable that the interfering compound is dioxogulonic acid, the first breakdown product of dehydroascorbic acid, but the fact that other workers (Sreenivasan & Wandrekar, 1950) have obtained results which suggest that it is reducible by hydrogen sulphide would appear to militate against this interpretation. The experiment indicates the somewhat greater degree of specificity associated with the homocysteine technique.

DISCUSSION

The time consumption and lack of specificity associated with the methods in current use for the estimation of dehydroascorbic acid have, to some extent, limited the nature and range of investigations into the distribution and metabolism of this substance. The main advantages associated with the technique described in this paper are that there is no need to remove the excess of reductant before measuring the formed ascorbic acid with indophenol dye, and that a single estimation need take no longer than 15 min. at room temperature, as compared with a time of some hours required by other methods. Furthermore, the fact that the reduction occurs rapidly at physiological pH values means that dehydroascorbic acid in certain tissues, plasma and urine can be converted into ascorbic acid by adding homocysteine without any preliminary preparation of an acid extract as would be required in the classical methods; such a direct addition of homocysteine gives information as to the state of any dehydroascorbic acid that might be present, for free dehydroascorbic acid would be changed to ascorbic acid, whereas any 'bound' dehydroascorbic acid would not be reduced until freed by acid extractants. Preliminary work with different plasmas suggests that dehydroascorbic acid exists in plasma in a 'bound' form, possibly in combination with one or more of the proteins of the plasma.

The method, although not in itself completely specific for dehydroascorbic acid, can be made more specific by making use of the knowledge that at pH 7·0 (the pH of the homocysteine reduction) boric acid rapidly and completely destroys dehydroascorbic acid but is without any such effect upon at least some of the interfering compounds.

SUMMARY

1. At pH values above 6·8 homocysteine rapidly reduces dehydroascorbic acid to ascorbic acid.

2. The ascorbic acid so formed can be estimated by reduction of indophenol dye, without any appreciable interference from the excess of homocysteine present, provided that the reaction is carried out within 30 sec. at pH 2·5.

3. Based on these observations, a routine method is described for estimating dehydroascorbic acid which is more rapid and specific than other available methods. The technique involves titration with indophenol dye both before and after the reaction with homocysteine. When the presence of interfering substances is suspected, the specificity can be increased further by titration with and without the addition of boric acid.

4. As an example of the application of the method, tests on pea cotyledons are described.

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