# INVESTIGATIONS ON THE STABILITY AND DETERMINATION OF DEHYDROASCORBIC ACID

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*Summary*

The time intervals for complete reduction of dehydroascorbic acid by hydrogen sulphide at pH 4, 5, 6, and 7 at temperatures of 0°, 25°, and 40°C. have been determined.

The times are given for half destruction of dehydroascorbic acid at pH 0, 1, 2.2, 3, 4, 5, 6, and 7 and at temperatures of 0°, 25°, 40°, 70°, and 100°C. The rate of destruction is least at pH 2.2. Borate considerably accelerates the destruction of dehydroascorbic acid, particularly at higher pH.

A procedure is described which increases the specificity of the determination of dehydroascorbic acid by indophenol titration. This involves extraction at pH 2, reduction with hydrogen sulphide at pH 5.3, and destruction of dehydroascorbic acid with borate to obtain a blank. This blank is considerable in extracts from a number of fruit and vegetable products.

Little dehydroascorbic acid was found in most of the fresh fruits and vegetables and in the processed juices examined, but appreciable concentrations were found in the fresh juices.

## I. Introduction

This paper is concerned with the determination of the stability of dehydroascorbic acid over a wide range of temperature and pH. Such data can be used for predicting the retention of dehydroascorbic acid by various processed foods, and can also be applied in the determination of dehydroascorbic acid by reduction with hydrogen sulphide (to ascorbic acid) and subsequent indophenol titration. It is desirable to extract at the pH of maximum stability and to obtain a blank titre after destruction of the dehydroascorbic acid under suitable conditions.

Penney and Zilva (1943) found that the rate of conversion of dehydroascorbic acid to 2, 3-diketogulonic acid in citrate-phosphate buffers at 38°C. increased with pH over the range 4.0-7.4. In a more acid range, the rate was found to increase with increasing concentration of hydrochloric acid from 0.1N to 1.0N at 25°C. In these experiments the loss of dehydroascorbic acid was followed both directly and by determination of the diketogulonic acid formed. The two methods gave very similar results except at pH 7.4, where there was evidence of further change. Borate buffer at pH 7.4 was found to stabilize the diketogulonic acid and give results which were in substantial agreement for the two methods. Roe and others (1948) found maximum stability of dehydroascorbic acid at pH 2-3.

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## II. Reduction of Dehydroascorbic Acid

Before proceeding with the main investigation, it was necessary to determine the conditions for complete reduction of dehydroascorbic acid by hydrogen sulphide. The solutions of dehydroascorbic acid were obtained by oxidizing ascorbic acid (20 mg./100 ml.) in citrate-phosphate buffer with an exact equivalent of iodine. After bubbling hydrogen sulphide through the solution for fifteen minutes, it was allowed to stand in a stoppered flask. At intervals aliquots were pipetted into metaphosphoric acid solution (to reduce the pH to about 1). The hydrogen sulphide was then removed by passing carbon dioxide through the solution for fifteen minutes, and the reduced ascorbic acid was determined by titration. The minimum periods after saturation with hydrogen sulphide (by bubbling for fifteen minutes) necessary under these conditions for complete reduction are given in Table 1.

Table 1

MINIMUM TIME OF STANDING FOR COMPLETE REDUCTION OF DEHYDROASCORBIC ACID WITH HYDROGEN SULPHIDE

| | Minimum Time (min.) at | | |
|---|---|---|---|
| pH | 0°C. | 25°C. | 40°C. |
| 4 | 90 | 15 | 0 |
| 5 | 45 | 5 | 0 |
| 6 | 30 | 0 | 0 |
| 7 | 15 | 0 | 0 |

The rates of reduction were not significantly affected by adding boric acid to the buffers to give a final concentration of 0.1M. The importance of boric acid will be shown in subsequent sections.

## III. Stability of Dehydroascorbic Acid

### (*a*) *Effect of Temperature and pH*

The destruction of dehydroascorbic acid was estimated at pH levels from 0.0 to 7.0 and at temperatures from 0° to 100°C. The solutions of dehydroascorbic acid were obtained by mixing 40 ml. of the appropriate buffer, 2 ml. of 0.5 per cent. ascorbic acid, and the exact equivalent of 0.01N iodine. In the experiments at 0°, 25°, and 40°C. the solutions were held at each temperature and 5 ml. aliquots were withdrawn at intervals. The data at 70° and 100°C. were obtained by keeping the bulk solutions at a low temperature, and holding each aliquot for the required period at the higher temperature. Each aliquot was reduced with hydrogen sulphide as described in the previous section and then titrated with 2, 6-dichlorophenolindophenol (aliquots which were more acid than pH 4 were brought to pH 4 by addition of sodium acetate or disodium phosphate before reduction).

The precise investigation of the kinetics is complicated by the fact that the end products when treated with hydrogen sulphide give rise to some indo-

phenol-reducing material, but it is probable that the course of destruction of dehydroascorbic acid approximates a first order reaction. The results of Penney and Zilva (1943), confirmed by the author, show that diketogulonic acid gives about one-tenth the titre of the equivalent amount of dehydroascorbic acid. At pH 7, further changes result in the production of reducing material; after 30 minutes at 40°C. a solution of this pH developed, without any hydrogen sulphide treatment, a reducing titre equivalent to 7 per cent. of that of the original solution after reduction.

The results can be most usefully expressed as the time required for destruction of half the original dehydroascorbic acid. The titre after reduction should be approximately 55 per cent. of the original (50 per cent. due to dehydroascorbic acid and 5 per cent. due to diketogulonic acid). The times of half destruction, reported in Table 2, were obtained in 1.0N hydrochloric acid (pH 0), 0.25M oxalic acid (pH 1), and McIlvaine's citrate-phosphate buffers (pH 2.2-7). Results obtained in 1.4N sulphuric acid (pH 0), 0.1N hydrochloric acid (pH 1), 0.1M sodium acetate adjusted with sulphuric acid (pH 1-2), phthalate buffers (pH 2.2-6), and phosphate buffers (pH 6-7) did not differ by more than 10 per cent.

TABLE 2

TIMES OF HALF DESTRUCTION OF DEHYDROASCORBIC ACID

| | Time of Half Destruction at | | | | |
|---|---|---|---|---|---|
| pH | 0°C. | 25°C. | 40°C. | 70°C. | 100°C. |
| 0 | 24 hr. | 2 hr. | 30 min. | 6 min. | 1½ min. |
| 1 | 5½ days | 12 hr. | 3 hr. | 30 min. | 7 min. |
| 2.2 | 15 days | 35 hr. | 9 hr. | 80 min. | 17 min. |
| 3 | 14 days | 34 hr. | 8 hr. | 50 min. | 8 min. |
| 4 | 12 days | 24 hr. | 6 hr. | 30 min. | 3 min. |
| 5 | 9 days | 15 hr. | 3 hr. | 8 min. | 1 min. |
| 6 | 5 days | 4½ hr. | 35 min. | 2 min. | < 1 min. |
| 7 | 24 hr. | 45 min. | 6 min. | < 1 min. | < 1 min. |

At all temperatures, dehydroascorbic acid has maximum stability at approximately pH 2. The stability decreases at both higher and lower pH levels. With increasing pH, the temperature coefficient of the reaction increases. From 0° to 40°C., the $Q_{10}$ is approximately 2.5 at pH 0-4, 3.0 at pH 5, and 4.0 at pH 6-7.

The stability of dehydroascorbic acid is shown to vary greatly with both temperature and pH. The stability at 0°C. suggests the possibility of appreciable retention in many cold stored foods, particularly frozen foods. With regard to canned foods, the heat treatments used for sterilization would usually destroy most of the dehydroascorbic acid. Exceptions are most likely to be found in acid foods, particularly canned fruit juices. In such products, which usually have a pH of 2.5-4.0, dehydroascorbic acid is comparatively stable and only a short heat treatment is required for sterilization.

The destruction of dehydroascorbic acid does not involve atmospheric oxidation, and the rate of destruction was not significantly affected by bubbling either air or nitrogen through the solution. The addition of ten parts per million of copper (as copper sulphate), which catalyses the atmospheric oxidation of ascorbic acid, had no significant effect.

### (*b*) *Effect of Borate*

Penney and Zilva (1943) have shown that diketogulonic acid is rendered more stable at pH 7.4 by the presence of borate, and the formation of reducing substances on treatment with hydrogen sulphide is thus eliminated. The possibility of using borate in the determination of dehydroascorbic acid was considered, as it would enable dehydroascorbic acid to be removed without giving rise to a residual titre.

Borate was found to accelerate the destruction of dehydroascorbic acid, an effect which was not apparent from the data of Penney and Zilva (1943), as destruction at pH 7.4 is extremely rapid whether in the presence or absence of borate.

The effect of borate was investigated at pH 0-7 by adding boric acid to the appropriate buffers to give a final concentration of 0.1M. As the borate prevented interference by diketogulonic acid, the time taken to reach half the original titre can be regarded as that necessary for half destruction of dehydroascorbic acid. The results are given in Table 3.

TABLE 3

EFFECT OF BORATE ON DESTRUCTION OF DEHYDROASCORBIC ACID

| pH | Temp. (°C.) | Time of Half Destruction | |
|---|---|---|---|
| | | Control | Borate |
| 0 | 40 | 30 min. | 30 min. |
| - | 40 | 3 hr. | 3 hr. |
| 2.2 | 40 | 9 hr. | 4 hr. |
| 3 | 40 | 8 hr. | 2 hr. |
| 4 | 40 | 6 hr. | 40 min. |
| | 0 | 9 days | 3½ hr. |
| 5 | 25 | 15 hr. | 30 min. |
| | 40 | 3 hr. | 11 min. |
| | 0 | 5 days | 60 min. |
| 6 | 25 | 4½ hr. | 7 min. |
| | 40 | 35 min. | 1½ min. |
| 7 | 40 | 6 min. | 1 min. |

The accelerating effect of borate appears at approximately pH 2 and increases with increasing pH. The effect of boric acid both in accelerating the decomposition of dehydroascorbic acid and in stabilizing the diketogulonic acid formed is probably associated with its ability to react with hydroxyl groups.

## IV. Determination of Dehydroascorbic Acid

### (*a*) *Methods of Obtaining Blank*

Determination of dehydroascorbic acid in foods involves extraction, reduction with hydrogen sulphide, and titration with 2,6-dichlorophenolindophenol. The extracting solution should contain a stabilizer for ascorbic acid, e.g. oxalic acid, and should give an extract with a pH of approximately 2. This pH combines maximum stability of dehydroascorbic acid with satisfactory stability and extraction of ascorbic acid. It is desirable to increase the pH before reduction with hydrogen sulphide.

As substances other than dehydroascorbic acid may give indophenol-reducing material on treatment with hydrogen sulphide, it is essential to obtain a satisfactory blank. The blank is a measure of interfering substances and is obtained by destroying the dehydroascorbic acid before treatment of the solution with hydrogen sulphide and titration. The blank titre is subtracted from the total titre.

In preliminary investigations, a solution containing one per cent. of oxalic acid and 0.75 per cent. of sodium acetate was used for extraction. This gave an extract of approximately pH 2. The uncorrected figure (a) (see Table 4) was obtained by adding 2 ml. of 50 per cent. sodium acetate to a 10 ml. aliquot (to bring the pH to 5.3), reducing with hydrogen sulphide, and titrating. The pH of 5.3 combined adequate stability with sufficiently rapid reduction. Slight variations in the amount of sodium acetate added did not affect the pH appreciably.

Blank titres were obtained on 10 ml. aliquots after treating as follows:

($b_1$) Add 2 ml. of 50 per cent. sodium acetate (to bring the pH to 5.3), allow to stand for 16 hours at 40°C. in an atmosphere of carbon dioxide, and subsequently reduce with hydrogen sulphide.

($b_2$) Add 2 ml. of a solution containing 50 per cent. of sodium acetate and 3 per cent. of boric acid (which brings the pH to 5.3), allow to stand for one hour at 40°C. in a current of carbon dioxide, and reduce with hydrogen sulphide.

($b_3$) Add 2 g. of $Na_2HPO_4.12H_2O$ (to bring the pH to 7), allow to stand for one hour at 40°C. in a current of nitrogen, and reduce with hydrogen sulphide.

Carbon dioxide does not affect the pH in the first two procedures, but reduces the pH in the third procedure. Hence nitrogen was used instead.

Of the three methods for destroying dehydroascorbic acid, method ($b_1$) required an excessive time for destruction, but it was useful as a control for comparing the effect of borate. Method ($b_2$), which involved the use of borate, gave comparatively rapid destruction and a low residual titre. However, as there was a possibility of borate causing interference through combination with other substances, procedure ($b_3$), which gave as rapid destruction without the use of borate, was included. It was necessary to raise the pH to 7 to obtain rapid destruction in the absence of borate.

Solutions of dehydroascorbic acid and a number of possible interfering substances were analysed to obtain the uncorrected figure and each of the three blank figures. Each of the blanks was subtracted from the uncorrected figure to obtain a corrected figure for dehydroascorbic acid. All the results were calculated as mg. of ascorbic acid per 10 ml. aliquot.

The solutions contained in a 10 ml. aliquot 0.1, 0.2, 0.5, and 1.0 mg. of dehydroascorbic acid and the following amounts of possible interfering substances: 1.0 mg. of ascorbic acid; 0.2 mg. of tin (as $SnCl_2.2H_2O$); 0.2 mg. of iron (as $FeSO_4.(NH_4)_2S0_4.6H_2O$); 1.0 mg. of pyruvic acid; 1.0 mg. of 1,4-naphthoquinone; and 1.0 mg. of diketogulonic acid. The ferrous and stannous solutions had become partially oxidized before analysis. Both tin and iron may be found in canned foods. Derivatives of 1,4-naphthoquinone, such as vitamin K and juglone, are known to occur naturally. The diketogulonic acid was obtained by allowing dehydroascorbic acid to stand in 0.1N hydrochloric acid.

The results, which include original reducing material (without reduction by hydrogen sulphide), uncorrected figure after reduction, blanks, and corrected figures for dehydroascorbic acid, are given in Table 4.

TABLE 4

COMPARISON OF BLANKS AND CORRECTED FIGURES FOR DEHYDROASCORBIC ACID

| Substance | Mg. in 10 ml. Aliquot | Original Reducing Material | Reducing Material after Reduction by Hydrogen Sulphide | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Uncorrected Figure | Blanks | | | Corrected Figure for Dehydroascorbic Acid | | |
| | | | (a) | ($b_1$) | ($b_2$) | ($b_3$) | (a-$b_1$) | (a-$b_2$) | (a-$b_3$) |
| Dehydroascorbic acid | 0.1* | 0 | 0.109 | 0.023 | 0.011 | 0.018 | 0.086 | 0.098 | 0.091 |
| „ | 0.2* | 0 | 0.210 | 0.034 | 0.012 | 0.041 | 0.176 | 0.198 | 0.169 |
| „ | 0.5* | 0 | 0.514 | 0.051 | 0.014 | 0.062 | 0.463 | 0.500 | 0.452 |
| „ | 1.0* | 0 | 1.005 | 0.106 | 0.028 | 0.097 | 0.899 | 0.977 | 0.908 |
| Ascorbic acid | 1.0 | 1.000 | 1.000 | 0.990 | 1.000 | 0.941 | 0.010 | 0 | 0.059 |
| Tin | 0.2 | 0 | 0.012 | 0.012 | 0.012 | 0.012 | 0 | 0 | 0 |
| Iron | 0.2 | 0.120 | 0.314 | 0.314 | 0.314 | 0.233 | 0 | 0 | 0.081 |
| Pyruvic acid | 1.0 | 0 | 0.015 | 0.015 | 0.015 | 0.030 | 0 | 0 | −0.015 |
| 1,4-Naphtho-quinone | 1.0 | 0.038 | 0.886 | 0.610 | 0.827 | 0.784 | 0.276 | 0.059 | 0.102 |
| Diketogulonic acid | 1.0 | 0 | 0.123 | 0.058 | 0.066 | 0.150 | 0.065 | 0.057 | −0.027 |

* Expressed as ascorbic acid.

The best results were generally obtained with blank ($b_2$), in which borate was used for accelerating the destruction of dehydroascorbic acid. The figure obtained by subtracting this blank gave maximum recovery of dehydroascorbic acid (98-100 per cent.) and no interference by ascorbic acid, tin, iron, or pyruvic acid. Interference by 1,4-naphthoquinone was only 7 per cent. of the uncorrected figure and was considerably less than that obtained by subtracting

either of the other blanks. Interference by diketogulonic acid was reduced to about half the uncorrected figure.

It is of interest that the residual titre after destruction of dehydroascorbic acid in the presence of borate was much lower than that given by pre-formed diketogulonic acid in the presence of borate. This suggests that a boro-diketogulonic acid complex was formed directly by the action of borate on dehydroascorbic acid.

The slight interference by ascorbic acid in ($a$-$b_1$) and the greater interference in ($a$-$b_3$) were probably due to oxidation of ascorbic acid. Any ascorbic acid oxidized during the period of standing naturally appeared as dehydroascorbic acid. It is difficult to avoid slight oxidation on standing for sixteen hours at pH 5.3. If the period of standing was reduced to one hour by the use of borate, oxidation was negligible. At pH 7 (blank $b_3$) oxidation of ascorbic acid was more rapid, and it was difficult to obtain nitrogen completely free from oxygen. The figure given in Table 4 was obtained by displacing the air above the solution with nitrogen purified by passing through alkaline pyrogallol. If the nitrogen was bubbled continuously, more rapid oxidation occurred.

Sulphur dioxide seriously interfered with all of the possible procedures for determining dehydroascorbic acid. Solutions of sulphur dioxide gave a variable titre after treatment with hydrogen sulphide, and holding at pH 5-7 increased this variability.

### (*b*) *Suggested Procedure*

The following solutions are required:

(i) 10 g. of oxalic acid, $(COOH)_2.2H_2O$, and 7.5 g. of sodium acetate, $CH_3COONa.3H_2O$, per litre.

(ii) 100 g. of sodium acetate per 200 ml.

(iii) 6 g. of boric acid, $H_3BO_3$, and 100 g. of sodium acetate per 200 ml.

(iv) 5N sulphuric acid.

(v) 0.2 g. of 2,6-dichlorophenolindophenol per litre (equivalent to approximately 0.1 mg. of ascorbic acid per ml.). Make up in phosphate buffer of pH 7.2 and standardize with ferrous ammonium sulphate.

Extract 20 g. of material with solution (i) and make up to 200 ml. with solution (i). Procedures (a) and ($b_2$) are to be carried out on 10 ml. aliquots in 50 ml. conical flasks.

(a) Add 2 ml. of solution (ii), and immediately bubble $H_2S$ for 15 min. Stopper, and allow to stand for 30 min. Then add 2 ml. of 5N $H_2SO_4$ (which reduces the pH to 1.3), bubble $CO_2$ for 15 min., and titrate.

($b_2$) Immerse 50 ml. conical flask containing 2 ml. of solution (iii) in bath at 40°C. for 5 min. Pipette 10 ml. of extract, and bubble $CO_2$ for one hour. Then bubble $H_2S$ for 15 min. and continue as for (a).

Calculate concentration of dehydroascorbic acid from ($a$-$b_2$).

This procedure is suggested as one method of determining dehydroascorbic acid by indophenol titration. It is certainly more specific than simple reduction

and titration without use of a blank. Without extensive analyses, including biological assays, it is not possible to compare this method adequately with alternative procedures, such as those of Lugg (1942) and Roe and others (1948).

### (c) *Estimation of Dehydroascorbic Acid in Various Products*

The suggested procedure was applied to a number of fresh and processed foods which were likely to contain dehydroascorbic acid. They included fresh apples, fresh and canned apple juice, fresh tomatoes, fresh and canned tomato juice, fresh and canned orange juice, fresh beetroot, and honey. The apples (Granny Smith variety) were semi-ripe. The canned juices had been stored for about twelve months.

Fresh beetroot and honey were included, as the presence of interfering substances has been suggested in these products. Beetroot extracts were difficult to titrate either visually or potentiometrically, and approximate titres were obtained by the chloroform method of McHenry and Graham (1935). The results, which include ascorbic acid by direct titration, are given in Table 5. The uncorrected figure for dehydroascorbic acid is obtained by subtracting ascorbic acid (obtained by direct titration) from (a).

TABLE 5
DETERMINATION OF DEHYDROASCORBIC ACID IN FRESH AND PROCESSED FOODS

| Product | Ascorbic Acid (mg. per 100 g.) | Dehydroascorbic Acid (as mg. of Ascorbic Acid per 100 g.) | | | |
|---|---|---|---|---|---|
| | | (a) | ($b_2$) | Uncorrected Figure | Corrected Figure (a-$b_2$) |
| Fresh apple (very small) | 15.1 | 21.2 | 15.4 | 6.1 | 5.8 |
| Fresh apple (medium) | 8.6 | 13.2 | 9.9 | 4.6 | 3.3 |
| Fresh apple juice (very small) | 0 | 17.2 | 7.3 | 17.2 | 9.9 |
| Fresh apple juice (medium) | 0 | 8.6 | 5.5 | 8.6 | 3.1 |
| Canned apple juice | 0 | 3.2 | 2.4 | 3.2 | 0.8 |
| Fresh tomato | 20.2 | 22.0 | 21.1 | 1.8 | 0.9 |
| Fresh tomato juice | 12.1 | 17.7 | 14.5 | 5.6 | 3.2 |
| Canned tomato juice | 6.4 | 12.6 | 11.7 | 6.2 | 0.9 |
| Fresh orange juice | 37.5 | 41.7 | 40.7 | 4.2 | 1.0 |
| Canned orange juice | 51.7 | 54.1 | 54.1 | 2.4 | 0 |
| Fresh beetroot | 25.4 | 31.2 | 31.2 | 5.8 | 0 |
| Honey | 1.5 | 5.1 | 8.0 | 3.6 | – |

In all products the corrected figure for dehydroascorbic acid was less than the uncorrected figure. Interference was considerably reduced by the use of a blank. Honey gave somewhat anomalous results in which ($b_2$) was greater than (a).

On examining the corrected figures, it is seen that the immature fresh apples contained an appreciable concentration of dehydroascorbic acid. The larger apples, which were still comparatively immature, had less dehydroascorbic acid, and the concentration in fresh tomatoes and beetroot was negligible. As expected, the fresh juices all contained significant levels of dehydroascorbic acid resulting from oxidation during extraction. The concentration in orange juice was, however, slight, as oxidation of ascorbic acid proceeds slowly in this product. Practically complete oxidation occurred during the extraction of apple juices.

In the canned juices, the concentration of dehydroascorbic acid was slight in tomato, and negligible in apple and orange. The heat sterilization used in canning, combined with the long storage period, was sufficient to destroy practically all the dehydroascorbic acid.

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