VI. A CRITICAL INVESTIGATION OF THE TILLMANS METHOD FOR THE DETERMINATION OF ASCORBIC ACID

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During the past few years the Tillmans method for the determination of ascorbic acid has been utilized in a large number of investigations. The method is capable of great precision, but both the original (1) and modified forms (2, 3) may involve large constant errors. While King (4) has shown how interference from other reducing materials in plant tissues can be minimized, a volumetric oxidation method cannot be expected to be highly specific. The problem is further complicated because (a) ascorbic acid may be oxidized to dehydroascorbic acid which is physiologically active but does not react with the titration reagent, (b) while the oxidation of ascorbic acid is reversible, dehydroascorbic acid is unstable and undergoes a further irreversible decomposition (5). Ascorbic acid exists in the tissues of freshly harvested vegetables only in the reduced state (6), but oxidation and further decomposition may occur during the process of extraction.

EXPERIMENTAL

Procedure for Extracting Ascorbic Acid from Vegetable Tissues—If oxidation has been allowed to proceed during extraction, the total amount of ascorbic acid can be recovered for titration by immediately reducing the dehydroascorbic acid with hydrogen

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sulfide (1, 5). But the necessity of removing excess hydrogen sulfide is a serious objection to the use of this method for routine analysis (7).

The analytical process would be much simpler if, instead of reversing the reaction, the oxidation were prevented throughout the determination. Ascorbic acid is not autoxidizable at pH values below 6.8 (8). Its instability has been ascribed to two catalysts, an oxidase present in practically all vegetables (9) and copper present as an impurity in the reagents (10, 11). The prob-

### Table I
**Relation between pH of Extraction Medium and Amount of Unoxidized Ascorbic Acid Obtained from Vegetables**

<table>
<thead>
<tr>
<th>Extraction medium</th>
<th>String bean pH of extract</th>
<th>Carrot pH of extract</th>
<th>Carrot Ascorbic acid mg. per gm.</th>
<th>String bean Ascorbic acid mg. per gm.</th>
<th>Carrot Ascorbic acid mg. per gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>6.2 0.00</td>
<td>6.8 0.00</td>
<td>6.3 0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18% CH₃COOH + 7.5% K₂C₂O₄</td>
<td>4.3 0.05</td>
<td>4.0 0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td>3.7 0.10</td>
<td>3.4 0.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18%</td>
<td>2.1 0.15</td>
<td>2.4 0.20</td>
<td>2.8 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8%</td>
<td>1.2 0.19</td>
<td>1.3 0.26</td>
<td>1.2 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8%</td>
<td>0.8 0.20</td>
<td>0.8 0.26</td>
<td>0.8 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>0.5 0.20</td>
<td>0.5 0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15%</td>
<td>0.2 0.20</td>
<td>0.2 0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

lem, then, is one of inactivating or inhibiting the two catalysts of the oxidation reaction.

The catalytic action of copper may be inhibited satisfactorily by adding 2 per cent metaphosphoric acid to the extracting medium (10, 11). The activity of the enzyme is a maximum at a pH value of approximately 5.5 and decreases rapidly as the hydrogen ion concentration is increased. It occurred to us that if the organic acids used in the extraction were replaced by a strongly ionized acid, the pH of the resulting extract would be so low that the enzyme action might be completely inhibited. We investigated the possibility of using sulfuric acid for this purpose, and
compared it with various organic acids which had previously been used. Recently (12) hydrochloric acid has been recommended and should also prove to be quite satisfactory.

Duplicate samples of a number of vegetables were ground with various extraction media by exactly the same technique. The extraction was found to be complete in all cases. In Table I it may be noted that as the acidity of the extract increases the amount of titratable ascorbic acid also increases to a maximum value. Further examples of the influence of pH upon the amount of ascorbic acid obtained from different vegetables are shown in Fig. 1.

The maximum amount of ascorbic acid is obtained when the enzyme system catalyzing the oxidation of ascorbic acid is completely inhibited by strong acid. Consequently, the same maximum value is obtained whether dehydroascorbic acid is reduced with hydrogen sulfide or stannous chloride (13) or whether the
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Oxidation is prevented by inhibiting the reaction with alcohol (6) or with strong acid or heat as shown in Table II.

Examination of Hydrogen Sulfide Treatment for Ascorbic Acid Extracts—The proposed modification of the extraction procedure is not intended to eliminate the hydrogen sulfide treatment in exploratory work. Indeed, it will now be shown that the use of stronger acid increases the reliability of the results obtained after reduction with hydrogen sulfide.

King (4) has postulated that hydrogen sulfide might reduce substances other than dehydroascorbic acid, which will then re-

### Table II

Comparison of Effect of Heat and pH upon Activity of Oxidizing Enzymes in Parsnip Extract

<table>
<thead>
<tr>
<th>Extraction medium</th>
<th>pH of extract</th>
<th>Heating time</th>
<th>Ascorbic acid per gm. parsnip</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>min.</td>
<td>Before reduction</td>
</tr>
<tr>
<td>15% H₂SO₄ + 2% HPO₃</td>
<td>0.2</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>4% lactic acid</td>
<td>2.7</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>Water</td>
<td>6.0</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.0</td>
<td>5</td>
<td>0.13</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.0</td>
<td>7</td>
<td>0.13</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.0</td>
<td>12</td>
<td>0.11</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.0</td>
<td>20</td>
<td>0.12</td>
</tr>
</tbody>
</table>

...act with the titration reagent. We have discovered an instance in which such interfering substances cause a serious error in the usual ascorbic acid determination. Freshly sliced carrots were cooked in boiling water for 15 to 17 minutes and drained. 25 cc. portions of the cooking water were mixed with equal volumes of ice-cold 8 per cent acetic, 8 per cent trichloroacetic, and 10 per cent sulfuric acids. All mixtures contained 2 per cent metaphosphoric acid to inhibit catalytic oxidation by copper. Duplicate 25 cc. samples were titrated without further treatment. Other samples were saturated with hydrogen sulfide for 10 minutes. One-half of these were corked and set aside for 20 hours before removing excess hydrogen sulfide. From the remaining samples excess hydrogen sulfide was removed as quickly as possible with carbon dioxide.
The hydrogen sulfide treatment caused (a) no increase in the titration value of any of the sulfuric acid samples, (b) a small increase in the trichloroacetic acid sample treated 20 hours and in the acetic acid sample treated for 10 minutes, (c) a very great increase of over 80 per cent in the acetic acid sample saturated with \( \text{H}_2\text{S} \) for 20 hours. To show whether the seeming increases in ascorbic acid were real or only apparent, the differential enzymic method of Tauber, Kleiner, and Mishkind (14) was utilized.

**Table III**

**Effect of Hydrogen Sulfide Treatment upon Total Reducing Material in Cooking Water from Carrots Determined in Presence of Various Acids and Expressed As Ascorbic Acid**

<table>
<thead>
<tr>
<th>Time saturated with ( \text{H}_2\text{S} )</th>
<th>Material titrated</th>
<th>Apparent ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td>5% sulfuric acid</td>
</tr>
<tr>
<td>0</td>
<td>Total reducing material</td>
<td>mg. per l.</td>
</tr>
<tr>
<td>10</td>
<td>Total reducing material</td>
<td>16</td>
</tr>
<tr>
<td>Reducing material unoxidized by enzyme</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid (by difference)</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>40</td>
<td>Total reducing material</td>
<td>18</td>
</tr>
<tr>
<td>Reducing material unoxidized by copper</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid (by difference)</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>1200</td>
<td>Total reducing material</td>
<td>16</td>
</tr>
<tr>
<td>Reducing material unoxidized by enzyme</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Ascorbic acid (by difference)</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

The results given in Table III show definitely that the increased titration value of the cooking water from carrots after hydrogen sulfide treatment cannot be ascribed to reduced ascorbic acid. A similar differential method of analysis with copper as the oxidizing catalyst gave confirmatory results after a short period of saturation with hydrogen sulfide. This method could not be used when the hydrogen sulfide treatment was prolonged for 20 hours. A sample containing reducing substances equivalent to 32 mg. of ascorbic acid per liter of cooking water increased in
3 minutes after the addition of 50 parts per million of copper to about 60 mg. per liter, followed by a slow decrease to about 40 mg. per liter. Obviously, such anomalous behavior could not be caused by ascorbic acid. Treatment with mercuric acetate according to the improved procedure of van Eekelen and Emmerie (15) removed the interfering material from the acetic acid extract.

An aqueous extract of Lima beans also was found to give an erroneously high titration value after prolonged treatment with hydrogen sulfide.

In their original work on the hydrogen sulfide treatment, Tillmans, Hirsch, and Jackisch (1) state that the reduction was usually complete in a few minutes but that in certain cases a further slow reaction occurred, so that merely for the sake of uniformity all samples were saturated with hydrogen sulfide for 24 hours. Later workers (15, 16) adopted this procedure without question, but our results, in agreement with those of Bukin (12), indicate that the hydrogen sulfide treatment should only be applied to strongly acidified extracts and for a period of not more than 30 minutes. Cabbage and snap bean extracts treated with hydrogen sulfide for 10, 30, 120, and 1440 minutes showed no significant differences in the amount of ascorbic acid reduced.

State of Ascorbic Acid in Plant Tissues—It has recently (16, 17) been argued that ascorbic acid occurred in a bound or esterified condition in natural foodstuffs, and that it could be liberated by heat or acid. One of us (6) attempted to show by heating duplicate samples of cabbage before and after extraction in an aqueous medium that the apparent increase in ascorbic acid is due to the inactivation of the enzyme. It was conclusively demonstrated that this hypothesis accounted for the greater part of the increase. However, when an aqueous cabbage or pea extract containing no unoxidized ascorbic acid is heated, a small but definite and reproducible amount of reducing material is formed. Further experiments now indicate that this material is not ascorbic acid.

100 mg. of synthetic or natural crystallized ascorbic acid of tested purity were dissolved in 40 cc. of water, acidified with 10 cc. of glacial acetic acid, completely oxidized with 5 gm. of activated charcoal, neutralized with calcium carbonate, and
filtered. The final filtrate contained about 2.5 mg. of dehydro-
ascorbic acid per cc. and did not react with the dye. 2 cc. por-
tions of this solution were mixed with 10 cc. of Sørensen's phos-
phate buffer mixtures adjusted to pH 6 to 7. Heating these
mixtures for 5 minutes in a boiling water bath produced a quan-
tity of reducing material equivalent to 0.29 mg. of ascorbic acid.
This is equal to 0.06 mg. of apparent ascorbic acid per mg. of
dehydroascorbic acid and is comparable to the value of 0.04 mg.
per gm. obtained from both cabbage and pea extracts. These
reducing substances were not observed on heating acidified ex-
tracts containing dehydroascorbic acid; hence they are probably
identical to the physiologically inactive decomposition products
of dehydroascorbic acid described by Borsook and coworkers (5).

We wish to express our appreciation to H. Tauber for supplying
the purified oxidase used in some of these experiments, and to
Z. I. Kertesz for many helpful suggestions during the progress of
this work. We are also indebted to F. Fenton, R. B. Dearborn,
and S. L. Gould for portions of the experimental data.

**SUMMARY**

1. The extraction procedure in the Tillmans method for the
determination of ascorbic acid has been modified to include
the use of an acid which is ionized sufficiently to prevent enzymic
oxidation of ascorbic acid.

2. For routine analysis the practicability of preventing the
oxidation of ascorbic acid throughout the determination is demon-
strated. The consequent elimination of the hydrogen sulfide
treatment greatly simplifies the analytical procedure.

3. Substances other than dehydroascorbic acid were reduced
by prolonged hydrogen sulfide treatment in weak acid solutions.
The use of a strongly ionized acid prevented the interfering ma-
terial from reacting with the titration reagent.

4. The apparent increase in ascorbic acid on heating an aqueous
cabbage extract is caused by the decomposition of dehydro-
ascorbic acid.

**BIBLIOGRAPHY**

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