AN IMPROVED PROCEDURE FOR THE DETERMINATION OF ASCORBIC ACID BASED UPON THE USE OF A XYLENE SOLUTION OF 2,6-DICHLOROPHENOL INDOPHENOL

by

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INTRODUCTION

The concentration of ascorbic acid in most plant and animal tissues is so low that, as in the case of many biological substances, its practical determination by means other than isolation is necessary.

One of the most outstanding properties of ascorbic acid is its strong reducing power, and the principles of most of the chemical methods for its determination are based on this property.

Of the chemical methods, those involving the reduction of the dye, 2,6-dichlorophenol indophenol are most numerous.

Data presented by Gibbs, Cohen, and Cannon [1] in 1928, on the dichloro indophenols showed that they could be used in the determination of the state of oxidation and reduction of biological systems within physiological hydrogen ion concentration ranges. These workers showed that the dichloro substitution products of phenol indophenol retain their brilliant blue color in mildly acid solution; that in the salt form they are quite stable, and that they are easily prepared and purified.

This work also showed that the measurement of E'_o of these indophenols at different pH levels is possible between pH 5 and pH 11. Precipitation occurs below pH 5 and negative drifts occur above pH 11. They found that of the products studied, 2,6-dichlorophenol indophenol shows the least drift. The equation relating electrode potential to hydrogen ion concentration, at 30 °C., was found to be:

$$E_h = E_o - 0.03006 \log \frac{[S_r]}{[S_o]} + 0.03006 \log \left[K_r K_2 [H^+] + K_r [H^+]^2 + [H^+]^3\right] - 0.03006 \log \left[K_o + [H^+]\right]$$

E_h is the observed potential. E_o is the potential when H = 1 and the ratio $\frac{S_r}{S_o}$ = 1. S_r is the concentration of the total reductant. S_o is the concentration of the total oxident. K_o is the acid dissociation constant of the oxident. K_r is the acid dissociation constant of the reductant. K_2 is the dissociation constant of the phenolic group created by reduction.

Since this work was done these substances have often been used in biological oxidation-reduction studies.

Tillmans, Hirsch and Hirsch [2] showed that reversible dyes of high oxidation potential (indophenols) can be used for the determination of ascorbic acid in acid solution if the titration is carried out rapidly since the rate of reoxidation of these dyes is quite slow.

The method used by Harris and Ray [3] was as follows: A 0.01 molar solution of 2,6-dichlorophenol indophenol was brought to pH 2.5 with acetic acid previous to use. The dye was initially run into a known volume of the solution to be analyzed until it was no longer reduced. Later these workers reversed the procedure and a measured volume of standardized indicator was titrated with the unknown solution. This procedure was reported to give no titration values when tested against other naturally occurring reducing agents including pyrogallol, catechol, tannic acid, quinol, reduced glutathione and fresh and boiled solutions of glucose, fructose and sucrose.

Roussy and King [4] used essentially the same method for ascorbic acid assay as that used by Tillmans, Hirsch and Hirsch [2] and Harris and Ray [3]. They substituted 6% trichloracetic acid for glacial acetic acid in determinations on plant and animal tissues and titrated the acid

solution with the dye. They found, however, that other substances including strong acid, glutathione, cysteine, pyrogallol, heated alkaline sucrose and glucose solutions and glucic acid cause fading of the dye, though ascorbic acid acts much faster. These workers advised that the titrations be completed within one minute in order to minimize the reducing effect of substances other than ascorbic acid.

Emmerie and van Eekelen [5] precipitated interfering reducing substances with mercuric acetate before titration of ascorbic acid solutions with 2,6-dichlorophenol indophenol. This procedure requires large samples and is lengthy. The dye solution must be accurately standardized as in the other methods referred to above.

Mindlin and Butler [6] developed a plasma ascorbic acid method utilizing the photoelectric colorimeter. The amount of ascorbic acid in plasma was measured by observing the decrease in concentration of oxidized indophenol (fading of the color).

A small amount of potassium cyanide was added to the collection tube for the preservation of the blood ascorbic acid previous to preparation of the filtrate.

Blood filtrates were prepared by precipitation with meta phosphoric acid and were buffered to pH 4.10 with sodium acetate before the addition of the dye.

These workers reported the following advantages of the method over the titration procedures:

1. Elimination of subjective reading of the end point.
2. Elimination of standardized indophenol solution.
3. Elimination of error through better pH control by use of a

buffer.

4. Elimination of oxidation of blood ascorbic acid preliminary to its determination through the use of potassium cyanide.

Farmer and Abt [7] developed a method for the determination of plasma ascorbic acid in which they used filtrates containing 2% meta phosphoric acid and titrated them with 2,6-dichlorophenol indophenol. They claimed that filtrates of plasma containing 2% meta phosphoric acid may be kept 24 hours in the ice box without loss of the vitamin. They, in later work [8], invalidated the use of potassium cyanide as advocated by Windlin and Butler [6] stating that filtrates containing it give high values.

Bessey [9] described a modification of the photoelectric method as used by Windlin and Butler [6]. He used 5% meta phosphoric acid extracts of plant and animal tissues which were buffered to pH 3.6-3.7 with a citrate buffer. He claimed determination of both reduced ascorbic acid and dehydro ascorbic acid through the use of H_2S. Bessey's method eliminates the necessity of absolutely clear and colorless filtrates through adjustment of the colorimeter with blank tubes. Bessey recommends that colorimeter readings on the solutions be made within fifteen seconds after adding the dye.

Bukatsch [10] found that oxidized 2,6-dichlorophenol indophenol can be quantitatively extracted from acid solution with xylene, and developed a rapid method for the determination of ascorbic acid based on this fact. The method does not require special care in the preparation of filtrates and requires only one photoelectric reading. The dye is permitted to react with the ascorbic acid for only a short time and is then taken up in xylene where it is not acted upon by interfering reducing substances.

The xylene solution of unreduced dye is then read in the photometer.

Stotz [11] also published a method employing the use of xylene for extraction of the dye. Filtrates of blood plasma containing 3% meta phosphoric acid are brought to pH 4 with sodium hydroxide and buffered by a phosphate-citrate buffer to prevent fading of the dye and yet allow oxidation of ascorbic acid. The solution is permitted to remain in contact with the dye for 15 to 30 seconds, extracted into xylene by shaking, centrifuged and the xylene layer read colorimetrically.

The work reported in this thesis is concerned with the development of a simple general method for the determination of ascorbic acid, utilizing the solubility of oxidized 2,6-dichlorophenol indophenol in xylene. This problem was suggested by the work of Stotz [11] referred to above. The method developed by the writer represents a definite improvement over that of Stotz. While Stotz [11] added the indophenol to the solution to be tested, followed by extraction of unreduced dye with xylene and colorimetric reading, the method reported here utilizes a solution of dye in xylene which is shaken directly with the solution to be analyzed and then the unreduced dye remaining in the xylene layer is estimated in the photoelectric colorimeter.

Problems arising in connection with the development of the method were:

1. Determination of the stability of the 2,6-dichlorophenol indophenol in xylene.

2. Determination of the capacity of the xylene solution of the dye to oxidize ascorbic acid when shaken with aqueous solutions of the acid, and optimum conditions for the reaction.

3. Determination of the effect of reducing substances other than ascorbic acid upon xylene solutions of 2,6-dichlorophenol indophenol.

4. Determination of the applicability of the method to various biological solutions.

EXPERIMENTAL WORK

METHODS OF DETERMINING ASCORBIC ACID IN AQUEOUS SOLUTIONS BY USE OF A XYLENE SOLUTION OF 2,6-DICHLOROPHENOL INDOPHENOL.

APPARATUS

Klett-Summerson Photoelectric Colorimeter. Principle [13].

The Klett-Summerson photoelectric colorimeter contains two Weston electronic photoelectric cells.

The Weston electronic photoelectric cell consists of a thin metal disc on which there is a film of light sensitive material. This forms the positive terminal. A metal collector ring in contact with the light sensitive surface forms the negative terminal.

Both cells are activated by a focused beam of light from a 100 Watt lamp, and this light is ordinarily passed through certain color filters in order to obtain the wave lengths most satisfactory for the solution to be examined. Since a single light source activates both cells, variations in current strength and light intensity do not affect the readings.

The test tube containing the solution to be examined is placed in the path of light striking one cell, termed the working cell. Only light which has passed through the solution in the test tube strikes the surface of the working cell and the voltage developed by this cell and the current in the circuit becomes a measure of the light absorbing power of the solution.

The second cell, the reference cell, is continually activated by the same source of light as the working cell. Its purpose is to furnish a source of potential and current against which changes in the potential

and current output of the working cell are measured.

Color measurements are made by means of a logarithmic scale attached to a potentiometer.

The potential and current output of the working cell are directly proportional to the optical density of the solution.

A general expression of Beer's law is, "The absorption of monochromatic light by a solution is proportional to the thickness of the layer traversed and to the molecular concentration of the light absorbing molecules in the layer". As commonly applied to colored solutions involved in ordinary colorimetric chemical analysis the law is approximately stated, "The concentration of colored substance in solution is directly proportional to the color concentration (optical density). The use of color filters facilitates colorimetric analysis by limiting the wave lengths used and causing the light absorbed to follow Beer's law more closely over a wider range.

REAGENTS

1. 0.03 N. HCl.

2. Xylene, C. P.

3. Saturated solution of rosin in kerosene.

An excess of powdered rosin is allowed to stand in kerosene for several days. The solution is then filtered.

4. 2,6-dichlorophenol indophenol in xylene.

0.1 gram 2,6-dichlorophenol indophenol is extracted with two 25 cc. portions of boiling water, filtered, and diluted to 200 cc. When cool the solution is acidified until red with 0.03 N HCl, 200 cc. of xylene are added, and the mixture is shaken. The indophenol xylene sol-

ution is washed by shaking with several 200 cc. portions of 0.03 N HCl. Careful washing prevents fading of the dye by the acid present in the solutions to be analyzed. The xylene layer is separated and placed in a clean dry flask. The solution is dried by shaking with anhydrous sodium sulphate.

This solution generally gives a reading of about 950 in the colorimeter. It is diluted with xylene until it reads 150. If the dilution is carried too far the proper amount of the more concentrated solution is added. Some of the concentrated solution should be reserved for this purpose.

After each analysis the xylene dye and aqueous solutions are poured into a bottle. When considerable volume has accumulated the mixture is treated as follows: The xylene is separated from the aqueous layer, washed several times with 0.03 N. HCl, shaken with anhydrous sodium sulphate and brought to standard reading by the addition of a concentrated solution of indophenol in xylene.

Mixtures containing stannous chloride could not be adequately purified by this procedure. The dye solutions recovered in such cases were always faded by shaking with 0.03 N HCl alone.

METHOD

1. Procedure for solutions not containing protein.

1 cc. of ascorbic acid solution containing not more than 0.06 mgms. is pipetted into a 75 cc. 6" x 1" test tube. 10 cc. of 0.03 N HCl are added, followed by the addition of 10 cc. of the indophenol xylene solution reading 150, or any other standardized value. The test tube is fitted with a rubber stopper and the mixture is shaken for 15 seconds

(50-60 ninety degree arm sweep excursions), poured into a 50 cc. centrifuge tube and centrifuged for 5 minutes at 700 R.P.M. 5 cc. or more of the indophenol xylene layer are then poured into a colorimeter tube and read after the photoelectric colorimeter has been zeroed to xylene.

The above procedure is applicable to the determination of ascorbic acid in deeply colored fruit juices, (blackberry, huckleberry), citrus fruit juices, blood filtrates, urine, and other aqueous solutions not containing protein.

2. Procedure for protein containing solutions.

1 cc. of the solution to be tested containing not more than 0.06 mgms. ascorbic acid is pipetted into a 75 cc. 6" x 1" test tube. 10 cc. of 0.05 N HCl are added followed by the consecutive addition of 1 cc. of the kerosene-rosin mixture and 10 cc. of the xylene dye solution reading 168. The test tube is fitted with a rubber stopper and the tube is shaken for 15 seconds (50-60 ninety degree arm sweep excursions), making certain that the first few excursions are especially violent and sweeping. This prevents the formation of a permanent emulsion. The solution is poured into a 50 cc. centrifuge tube, centrifuged for 5 minutes at 700 R.P.M. The xylene layer is read in the photoelectric colorimeter.

The above procedure has been found applicable to the determination of ascorbic acid in blood serum and plasma. It is undoubtedly also applicable to other biological solutions which contain protein.

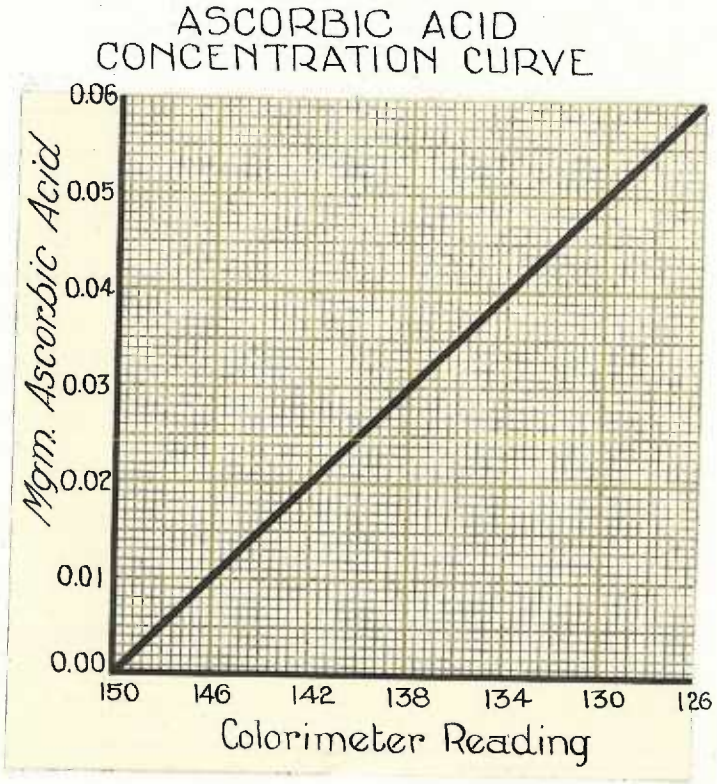
The following curve is valid when the kerosene-rosin mixture is used, if the concentration of dye in xylene is increased sufficiently to exactly compensate for the dilution effect of the kerosene-rosin solution. The

saturated kerosene-rosin solution employed in this work necessitated a dye solution reading 168 in the colorimeter. 1 cc. of kerosene solution added to 10 cc. of this dye solution gives a reading of 150.

TABLE II

Table for calculating the concentration curve of ascorbic acid when 1 cc. containing not more than 0.06 mgms. of ascorbic acid, is diluted to 11 cc. and shaken with 10 cc. of indophenol xylene solution.

mgms.% ascorbic acid soln. cc.	HCl 0.03 N cc.	xylene-dye cc.	colorimeter reading
0 (blank)	11	10	150
1 (0.01 mgm.)	10	10	140
2 (0.02 mgms.)	9	10	142
3 (0.03 mgms.)	8	10	138
4 (0.04 mgms.)	7	10	134
5 (0.05 mgms.)	6	10	130
6 (0.06 mgms.)	5	10	126


ASCORBIC ACID
CONCENTRATION CURVE
Mgm. Ascorbic Acid
0.06
0.05
0.04
0.03
0.02
0.01
0.00
150
146
142
138
134
130
126
Colorimeter Reading

PRELIMINARY INVESTIGATIONS

UPON WHICH THE METHODS WERE BASED

DETERMINATION OF THE STABILITY OF 2,6-DICHLOROPHENOL INDOPHENOL IN XYLENE.

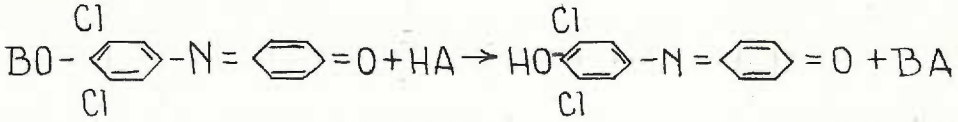
Bukatsch [10] found that 2,6-dichlorophenol indophenol can be extracted into xylene from acid solutions and although the methods of Bukatsch [10] and Stotz [11] employ the extraction of the dye from solutions of quite high acidity, pH 4, the hydrogen ion ranges of solutions which will permit the extraction of the dye are not indicated.

The determination of the relation of pH to extraction of the dye from aqueous solutions constitutes the first part of the problem.

A description of the color changes of 2,6-dichlorophenol indophenol may be advantageously given at this point in the discussion.

1. Acid-base indicator change.

The basic color of the oxidized dye is blue, while the acid color is red.

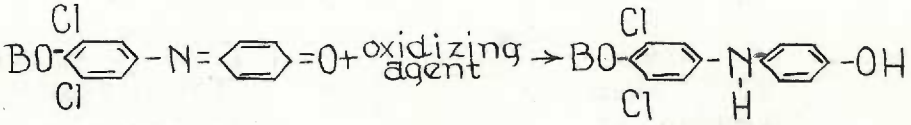


salt (blue) acid (red)

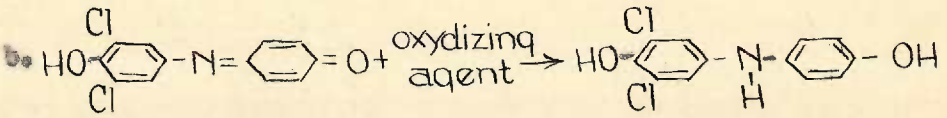
2. Oxidation-reduction change.

The color of the oxidized form of the dye (red or blue) disappears as the dye is reduced.

a.

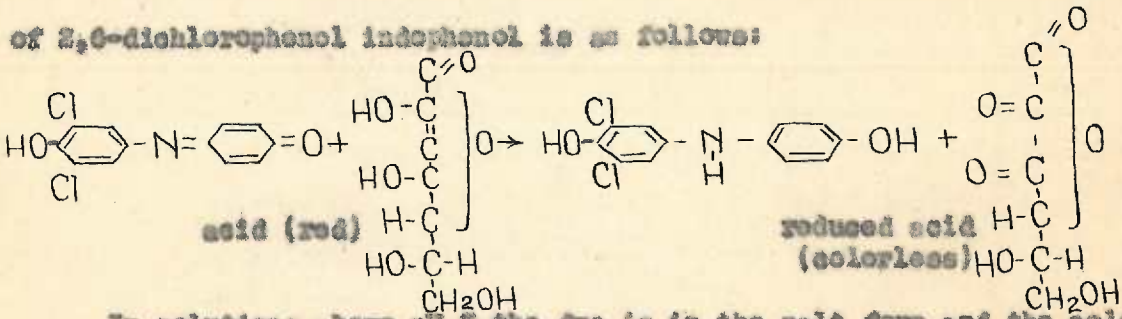


salt (blue) reduced salt (colorless)



acid (red) reduced acid (colorless)

The equation for the oxidation of ascorbic acid by the acid form of 2,6-dichlorophenol indophenol is as follows:



In solutions above pH 5 the dye is in the salt form and the color of the solution is blue. As the pH drops below 5 the color rapidly changes to red and the H ion replaces the basic ion.

It was found that if a solution of dye at or above pH 5 is shaken with xylene no absorption into the xylene takes place, but if acid is gradually added until the oxidized dye is in the acid form it can be quantitatively extracted into the xylene. The elevation of the pH to 5 or above by the addition of base immediately causes the indophenol to pass into the aqueous layer. Therefore, any ascorbic acid solution to be assayed by a xylene extraction method must have a pH below 5.

After establishing the conditions for the extraction of 2,6-dichlorophenol indophenol by xylene, a water solution of it was brought to pH 3.5; xylene was added and the dye extracted. The xylene was separated from the acid solution, placed in a clean dry flask and a reading was taken in the photoelectric colorimeter. Progressively lower readings were observed on this solution for three consecutive days after which the readings were constant. Solutions of the dye in xylene were tested over

a period of two months and gave constant readings.

It was found that the fading of the solution during the first three days is due to water droplets in the solution which are only slowly separated out. Shaking the freshly prepared xylene indophenol solution with anhydrous sodium sulphate results in an initial stable reading.

The following table shows readings of xylene indophenol samples taken after various lengths of time:

TABLE I

Colorimeter readings showing the stability of 2,6-dichlorophenol indophenol in xylene.

Sample	24 hrs.	48 hrs.	72 hrs.	168 hrs.	336 hrs.	1008 hrs.
1	136	133	130	130	130	130
2	157	154	152	150	150	150
3	150	146	146	146	146	146
4 (Na_2SO_4)	147	147	147	147	147	147

DETERMINATION OF THE CAPACITY OF A XYLENE SOLUTION OF 2,6-DICHLOROPHENOL INDOPHENOL TO OXIDIZE ASCORBIC ACID WHEN SHAKEN WITH AN AQUEOUS SOLUTION OF THE ACID AND DETERMINATION OF THE OPTIMUM CONDITIONS FOR THE REACTION.

As previously mentioned in the introduction, Bukatsch [10] and Stotz [11] both determined ascorbic acid by adding an excess of 2,6-dichlorophenol indophenol to the solution to be analyzed, permitting the reaction to proceed for 15-30 seconds and then extracting the unreduced dye with xylene. The dye in the xylene was estimated by use of either a photometer or photoelectric colorimeter. This procedure suggested to the writer the possibility of using a standardized solution of 2,6-dichlorophenol indo-

phenol in xylene and shaking a definite volume of this solution directly with the solution to be analyzed, followed by determination of the unreduced dye in the xylene by the photoelectric colorimeter. Since the investigations showed the dye in xylene to be quite stable further work was done in order to establish the applicability of the method.

After publication of the paper by Fujita and Iwataka [12] reporting the stabilizing effect of meta phosphoric acid upon ascorbic acid solutions, most ascorbic acid methods have included meta phosphoric acid as the acidifying agent. It is also widely used as a deproteinizing agent in preparing extracts of biological materials for ascorbic acid determination. Meta phosphoric acid was used as acidifying agent in the first part of the work reported in this thesis.

Previous work referred to showed that there are reducing substances other than ascorbic acid present in many biological solutions but that these substances generally reduce more slowly than does ascorbic acid. Because of this situation, less error, due to these non-ascorbic acid substances, is encountered if the dye is in contact with the solution analyzed for only a short time. In the work reported the reaction time chosen was fifteen seconds.

One tenth gram of crystalline ascorbic acid was dissolved in a 5% solution of HPO_3. 1 cc. of this solution containing 1 mgm. of ascorbic acid was shaken for fifteen seconds with 10 cc. of indophenol solution in xylene arbitrarily concentrated to read 150 after the colorimeter was zeroed to xylene. After brief centrifugation, the xylene layer was poured into a colorimeter tube and a reading made. Considerable fading of color was observed. Repitition of this experiment using 2 cc. (2 mgms.) of

ascorbic acid, resulted in further fading of the color but not to a proportional degree. It was found that the use of relatively high concentrations of ascorbic acid would cause fading to a certain point when shaken for 15 seconds with the dye solution but that higher concentrations had no effect in decreasing color beyond this point.

This suggested that ascorbic acid in very small amounts, but in quite large volumes of aqueous solution, might be quantitatively determined by the above procedure.

An 8" x 1" 75 cc. test tube fitted with a rubber stopper, was chosen for shaking the mixtures. 0.1 gm. ascorbic acid was dissolved in a 5% solution of HPO_3 and diluted to 100 cc. 1 cc. of this solution (1 mgm.) was diluted to 100 cc. with a 5% solution of HPO_3 making a concentration of 0.01 mgm. per cc. Since the indophenol is extracted into xylene only from an acid solution, a 5% solution of HPO_3 was used to dilute the samples for analysis to the desired volume.

The concentration of ascorbic acid, volumes of HPO_3 solution, volumes of xylene dye solution and colorimeter readings are included in the following table:

TABLE II

Relation of ascorbic acid concentration to scale readings on the photoelectric colorimeter when the ascorbic acid solution is shaken with the xylene-dye reading 150.

mgms.% ascorbic acid solution cc.	HPO_3 5% cc.	xylene-dye cc.	colorimeter reading
0 (blank)	11	10	150
1 (0.01 mgm.)	10	10	146

table II continued:

mgms.% ascorbic acid solution cc.	HPO_3 5% cc.	xylene-dye cc.	colorimeter reading
2 (0.02 mgms.)	9	10	142
3.(0.03 mgms.)	8	10	138
4 (0.04 mgms.)	7	10	134
5 (0.05 mgms.)	6	10	130
6 (0.06 mgms.)	5	10	126
7 (0.07 mgms.)	4	10	124
10 (0.10 mgm.)	1	10	124

Since a slight drift toward lower readings of the tubes was observed when they were allowed to stand for a short time a solution of ascorbic acid containing 0.01 mgm. per cc. in 5% HPO_3 was prepared and the effect of time of shaking and time of centrifugation were observed. Table III shows the results obtained.

TABLE III

Effect of time of shaking and time of centrifugation upon colorimeter readings.

mgms.% ascorbic acid cc.	HPO_3 5% cc.	dye cc.	time of shaking seconds	time of centrifugation minutes	colorimeter reading
1	10	10	15	5	146
1	10	10	15	3	146
1	10	10	30	5	146
1	10	10	30	3	146
1	10	10	15	2	147
1	10	10	15	1	149

A 5% HPO_3 solution of ascorbic acid containing 0.045 mgms. per cc. was prepared. When 1 cc. of this solution was shaken for 15 seconds with xylene containing varying concentrations of the dye and centrifuged for 5 minutes at 700 R.P.M. the following results were obtained:

TABLE IV

ascorbic acid 0.045 mgms.% cc.	HPO_3 5% cc.	xylene reading	colorimeter reading	difference
1	10	150	132	18
1	10	200	176	24
1	10	100	88	12
1	10	50	44	6
1	10	188	165	23

As a result of the preceding data the following conditions for the determination of ascorbic acid in pure solution were chosen:

1. Ascorbic acid concentration not exceeding 0.06 mgms. per sample.
2. Aqueous volume of 11 cc.
3. Volume of indophenol xylene solution, 10 cc.
4. Time of shaking, 15 seconds.
5. Time of centrifugation, 5 minutes at 700 R.P.M.
6. Concentration of dye in xylene arbitrary when reading is near the middle of the scale.

When the above conditions are met the concentration of ascorbic acid is directly proportional to the reduction in scale reading on the photoelectric colorimeter, which is directly proportional to decrease in color of the dye solution caused by oxidation of ascorbic acid.

DETERMINATION OF THE EFFECT OF REDUCING SUBSTANCES OTHER THAN ASCORBIC ACID UPON XYLENE SOLUTIONS OF 2,6-DICHLOROPHENOL INDOPHENOL

It was found that aqueous solutions of stannous chloride, high concentrations of tannic acid, pyrogallol, and strong acids would cause fading of a xylene solution of 2,6-dichlorophenol indophenol when shaken with it for fifteen seconds.

Aqueous solutions containing 4 mgms.% of reduced glutathione, cysteine hydrochloride, tannic acid, pyrogallol, and high dilutions of strong acids do not fade the dye-xylene solution when shaken with it for fifteen seconds.

THE DETERMINATION OF THE APPLICABILITY OF THE METHOD TO VARIOUS BIOLOGICAL SOLUTIONS.

1. Determination of ascorbic acid in fruit juices.

Preliminary work with fruit juices, including orange, lemon, grapefruit, blackberry and huckleberry juice, showed that they cause fading of color when shaken with a solution of indophenol in xylene.

Since these juices contain pigments it was necessary to determine the solubility of the pigments in xylene.

1 cc. of the juice to be tested was placed in an 8" x 1" test tube fitted with a rubber stopper. 10 cc. of 5% meta phosphoric acid and 10 cc. of xylene were added. The mixture was shaken for 15 seconds, centrifuged for 5 minutes at 700 R.P.M. and the xylene layer was placed in the colorimeter and read.

All of the juices gave zero readings and therefore the pigments of these juices are insoluble in xylene, and do not interfere directly in the estimation of ascorbic acid by this method.

Each of the above juices and cucumber juice were tested for ascorbic acid. 1 cc. of whole juice or a dilution of it was placed in an 8" x 1" test tube fitted with a rubber stopper; 10 cc. of 5% meta phosphoric acid and 10 cc. of standardized indophenol in xylene, reading 150 were added. The mixture was shaken for 15 seconds, centrifuged for 5 minutes at 700 R.P.M. and the xylene layer placed in the colorimeter and read. Table V gives the results obtained.

TABLE V

Concentration of ascorbic acid in various juices.

juice	sample cc.	dilution	HPO_3 5% cc.	dye cc.	colorimeter reading	ascorbic acid mgms.%
orange	1	(1-15)	10	10	138	49
lemon	1	(1-15)	10	10	136	56
grapefruit	1	(1-15)	10	10	140	37
blackberry (canned)	1	none	10	10	138	3
huckleberry (canned)	1	none	10	10	126	6
cucumber	1	none	10	10	144	1.5

Orange and lemon juices were assayed for ascorbic acid by the xylene-dye method and by the usual indophenol titration method. Table VI shows comparative results.

TABLE VI

Comparison of the method with the indophenol titration procedure.

sample	xylene-dye method mgms.%	indophenol titration method mgms.%
orange juice	52	51
lemon juice	57	57
lemon juice	49	49.8

2. Determination of blood ascorbic acid.

The method as developed and used for the assay of pure ascorbic acid solutions and fruit juices was found applicable to the determination of ascorbic acid in 5% meta phosphoric acid filtrates of whole blood, blood plasma and blood serum. However, since the concentration of ascorbic acid in these filtrates is very low, 0.4 mgms.% to 2.5 mgms.%, it is necessary to use an amount of filtrate equivalent to at least 1 cc. of the original fluid tested.

Direct measurement of ascorbic acid in whole blood, blood plasma, and serum was attempted by the present method. It was found in each case that precipitation of the blood proteins by the 5% meta phosphoric acid added, resulted in the formation of an emulsion during shaking which prevented separation of the xylene-dye solution by centrifugation. Attempts to prevent the formation of these stable emulsions by the addition of various substances were unsuccessful.

It was found that when 0.03 N HCl is substituted for 5% meta phosphoric acid no precipitation of proteins occurs but a stable emulsion is formed during shaking which prevents the separation of the xylene-dye solution by centrifugation.

It was also found that a solution of rosin in kerosene may be used to prevent this difficulty, when such a solution is added to the well cooled sample previous to addition of the xylene-dye solution, and if after the dye is added the mixture is violently shaken.

The stability of ascorbic acid in 0.03 N HCl was investigated. No destruction was observed over a period of one hour. Shaking the indophenol xylene solution 15 seconds with 0.03 N HCl causes no fading

of the dye. These findings led to the substitution of 0.03 N HCl for 5% HPO_3 in the determination of ascorbic acid in all solutions which are not required to stand more than one hour before assay.

Further attempts were made to determine ascorbic acid directly in whole blood, plasma, and serum. It was found that whole blood contains substances which change the color of the indophenol xylene solution, often resulting in a reading higher than that of the standard and that when a lower reading is obtained the results are erratic.

Plasma from oxalated blood which had been agitated as little as possible and immediately chilled, and serum taken as soon as clotting permitted, followed by chilling, were tested by the method as used for fruit juices, after the addition of the kerosene-rosin mixture to the sample.

Since kerosene is soluble in xylene it causes a change in concentration of the standard solution through dilution, and correction for this dilution must be made by means of blank determinations.

If an indophenol-xylene solution is standardized to give a blank reading of 150 when 10cc. of it are diluted with 1 cc. of the kerosene rosin mixture, a concentration curve may be constructed which will serve in all determinations. Table VII shows concentrations of ascorbic acid in plasma and serum obtained by the method.

TABLE VII

Concentration of ascorbic acid in samples of plasma and serum.

sample cc.	HCl 0.03 N cc.	dye cc.	colorimeter reading	ascorbic acid mgms.%
serum 1 (old)	10	10	150	0.00

table VII continued:

sample	HCl 0.03 N cc.	dye cc.	colorimeter reading	ascorbic acid mgms.%
1 (2 days in icebox)	10	10	148	0.50
1 (fresh)	10	10	144	1.60
plasma 1 (fresh)	10	10	144	1.50
1 (fresh)	10	10	145	1.25
1 (shaken)	10	10	150	0.00

1 cc. of resin in kerosene was added to each sample before the addition of the dye solution.

Blood plasma was assayed for ascorbic acid by the xylene indophenol method and by the method of Farmer and Abt [7]. Comparative results are shown in Table VIII.

TABLE VIII

Comparison of the xylene indophenol method of ascorbic acid determination with Farmer and Abt's [7] method.

Sample	xylene indophenol method mgms.%	Farmer and Abt's method mgms.%
1	1.5	1.59
2	1.0	0.912
3	1.25	1.32
4	0.75	0.72
5	0.87	0.80

3. Urine ascorbic acid determination.

The method as used for fruit juices was also used for the determination of ascorbic acid in samples of fresh urine. Table IX shows some values

obtained.

TABLE IX

Concentration of ascorbic acid in samples of urine.

sample cc.	condition	HCl 0.03 N cc.	dye cc.	colorimeter reading	ascorbic acid mgms.%
1	fresh	10	10	138	3.00
1	fresh	10	10	143	1.75
1	fresh	10	10	139	2.75
1	24 hr.	10	10	150	0.00
1	fresh	10	10	141	2.25
1	24 hr.	10	10	143	1.75

same

Many preservatives were used in an attempt to prevent destruction of ascorbic acid in urine. Specimens containing 5% HPO_3, kept at room temperature, and those containing 2% HPO_3, kept in the ice box, showed no destruction of ascorbic acid in 24 hours. Since the cost of HPO_3 is high its use for a large number of samples is rather excessive.

It is reported that 24 hour specimens of urine containing Sendroy's Reagent show only 5% destruction of ascorbic acid when kept at room temperature, and only 2% destruction when kept in the ice box.

Sendroy's Reagent as used for the preservation of ascorbic acid in urine is as follows: In each quart jar used for collection there are placed 75 cc. 5 N sulfuric acid, 1 cc. 8-hydroxyquinoline (1.45 gms. of 8-hydroxyquinoline in 100 cc. alcohol), 5 cc. toluene.

The reagent has not been used in this laboratory. Its efficacy will be tested in later work.

As the result of experimental data two variations of a general method for the determination of ascorbic acid have been developed as described.

SUMMARY

1. The acid form of 2,6-dichlorophenol indophenol is stable in xylene.
2. A xylene solution of 2,6-dichlorophenol indophenol will oxidize ascorbic acid in aqueous solution when shaken with it.
3. A photoelectric colorimetric method for the determination of ascorbic acid in aqueous solutions of low concentration is described.
4. Advantages offered by the method are:
   a. Elimination of daily standardization of indophenol solution.
   b. Elimination of subjective errors in reading the colored solutions.
   c. Elimination of blank corrections in the analysis of colored solutions.
   d. Elimination of filtrates in the analysis of blood plasma and serum.
   e. All reagents are very stable and inexpensive.
   f. A number of analyses may be run simultaneously since the xylene indophenol solution does not undergo change.

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