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RAPID METHOD FOR DETERMINING ASCORBIC ACID CONCENTRATION

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ABSTRACT

In the course of investigations on the effect of citrus fruits upon the human organism it was found necessary to develop a method of determining the vitamin C content of the blood of a large number of individuals in a short time. In this method the plasma is not deproteinized but is diluted with 5-percent acetic acid and used directly in a specially constructed photoelectric comparator with the dye 2,6-dichlorophenolindophenol.[2] The ascorbic acid content of the plasma sample is read directly from empirical calibration curves determined by adding known amounts of ascorbic acid to blood plasma. The comparator is operated so as to compensate automatically for the usual variation in the turbidity and color of plasma samples. While the method is quite rapid, once it is in operation, some time must be spent in preparing calibration curves. The maximum total error in the determination on plasma samples containing from 0 to 3.5 mg of reduced ascorbic acid per 100 ml of plasma was estimated to be ± 0.1 mg/100 ml.

CONTENTS

I. INTRODUCTION

In the course of investigations upon the effect of citrus fruits on the human organism it often becomes necessary to determine the ascorbic acid content in the blood of a large number of subjects in a relatively short time. Only by investigating a large group of test cases and another large group of controls can the effect of random statistical fluctuations and individual idiosyncrasies be made small. The time is limited by the necessity of performing the determinations on the entire group of subjects within the same fasting period and by the instability of ascorbic acid [3] in the presence of atmospheric oxygen [1].[4]

A chemical rather than a biological method must necessarily be used when speed of analysis is desired. The most specific and generally applicable reagent for ascorbic acid in use at present is the dye 2,6-dichlorophenolindophenol [2].[5] Tillmans [3] showed that the reducing substance in food that could be determined by titration with 2,6-

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[2] The sodium salt; also called sodium 2,6-dichlorobenzenoneindophenol.
[3] In this paper the words "ascorbic acid" always indicate the unoxidized compound.
[4] Figures in brackets indicate literature references at the end of this paper.
[5] See footnote 2.

dichlorophenolindophenol was approximately equal to the reported vitamin C content of the food. The identity of vitamin C with ascorbic acid was established by Waugh and King [4]. Tillmans and his associates [5], Harris and his associates [6] and Bessey and King [7] showed a close correlation to exist between the reducing power of plant and animal tissues on 2,6-dichlorophenolindophenol and the biological assay of the vitamin C content of these tissues. King [8] has given additional evidence to support the view that a properly conducted titration of natural products with the dye 2,6-dichlorophenolindophenol gives a reasonably specific test for vitamin C. This dye has been used regularly to titrate ascorbic acid in blood plasma [9, 10].

A simplification of existing methods for determining ascorbic acid in blood plasma by working directly upon the plasma without previously subjecting it to a deproteinization [9, 10] is introduced in the present paper. The time-consuming procedure of deproteinizing the plasma is open to the objection that some of the ascorbic acid is occluded by the precipitated proteins and is furthermore subject to atmospheric oxidation during the manipulations [11]. The primary purpose of the deproteinization is to obtain a clear solution for titrating or for absorption measurements in the photoelectric comparator. However, it has been shown [12] that turbid and colored solutions can be used, in general, with a photoelectric comparator for determinations of ascorbic acid with 2,6-dichlorophenolindophenol. It is shown in the present paper that when proper calibration curves have been made it is possible to determine directly the ascorbic acid content of blood plasma. Such analyses of the plasma are complicated, however, in about 5 percent of the samples by exceptional turbidity, which renders the determination less accurate. These samples may be analyzed by preparing special calibration curves, as discussed later, or by one of the longer chemical precipitation and extraction methods [9] if an accurate determination is necessary. Since the approximate determinations showed the ascorbic acid content to be uncorrelated with exceptional turbidity, these cases can merely be omitted in a study involving large numbers of analyses. It was observed that the number of plasma samples having excess turbidity was greater during the winter months, at which time the experiment was started, than later during the summer. From 5 to 10 percent of the samples were excessively turbid during February and March but less than 1 percent in May and June.

The amount of turbidity in blood samples is lowered if they are taken as long a time as possible after meals and the diet of the subjects is not too high in fats. No difficulties with hemolysis were experienced when the proper technique was used for obtaining the blood samples. If the samples are taken with a syringe, it is advisable to remove the needle from the syringe before the sample is transferred to a centrifuge tube in order to avoid rupturing the red blood cells by forcing them too rapidly through the needle. If the blood sample is stirred or shaken, these operations should be very gentle. The tube in which the sample is collected should be dry, except for 2 drops of a 20-percent solution of potassium oxalate.

II. APPARATUS

The photoelectric comparator was constructed in this laboratory along lines generally in use and is similar to one described by Evelyn [13]. It differs only in the light source, filters used, and control of the galvanometer deflection. The light source (*A*, fig. 1) was a 120-volt, 60-watt, clear, daylight, straight-filament, Mazda lamp (*A*–19, *CC*–b). The power was supplied through a constant-voltage regulator [6] (*R*) which kept the fluctuations of intensity of the light source to less than 1 percent when used on the regular a–c supply line. The image of the filament was focused through a condenser lens (*B*), through the filter (*F*), and the absorption cell (*C*) on the photoelectric cell (*D*). The absorption cells were carefully selected

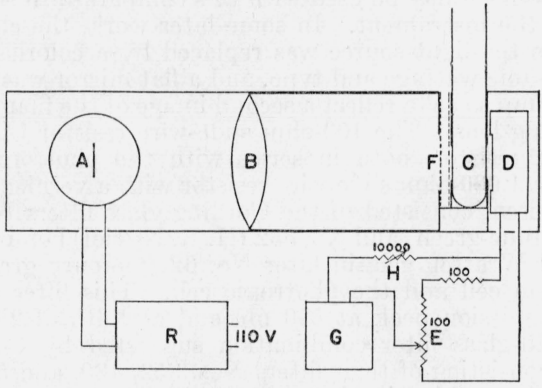


FIGURE 1.—*Schematic diagram of photoelectric comparator used for ascorbic acid analyses.*

calipered test tubes of 1-inch outside diameter by 6 inches long. When filled with a standard colored solution and rotated to a calibrated position marked on each test tube, the transmissions differed by less than 1 percent. This calibration took care of minor variations in wall thickness and roundness of the tubes. The photoelectric element was a Weston barrier-layer photronic cell, model 594, type 1. The galvanometer (*G*) was a Rubicon "spotlight" galvanometer having a coil resistance of 1,100 ohms, sensitivity of 0.005μa/mm, and critical damping resistance of 10,000 ohms. In figure 1, (*E*) is a 100-ohm slide-wire resistance by means of which the sensitivity of the galvanometer can be controlled without appreciably affecting the external resistance in series with the photronic cell. This method of controlling the deflection of the galvanometer has an advantage over the method which regulates the temperature of the filament of the light source [13] because, unlike the latter, it does not change the spectral distribution of the energy transmitted by the filters. In figure 1, (*H*) is a resistance used to effect critical damping of the galvanometer.

The light from the source passed through a yellow Wratten filter (No. 12) and then through a medium-blue glass filter before entering the absorption cell. The filter system, consisting of the two filters and the blue bulb, passed a spectral band with a transmission peak at

[6] Raytheon, 120-watt voltage regulator.

540 mμ and a width of 85 mμ at half peak. The band cut off sharply on the short-wave side, but tailed off on the long-wave side. The dye 2,6-dichlorophenolindophenol has in the acidified solution used in this work, a maximum absorption at 520 mμ and a half-peak width of 140 mμ, so that the light passed by the filters falls well within the region that the dye absorbs sensitively. The fraction of incident energy absorbed by a 1-in. layer of the blood plasma itself, in the dilution used, is about 10 percent at 600 mμ and increases to 25 percent at 515 mμ and 50 percent at 470 mμ. The filters were, accordingly, chosen to pass a spectral band toward the long-wave side of the region absorbed strongly by the unreduced dye (at pH 3) in order to have a minimum of absorption by the plasma solution alone.

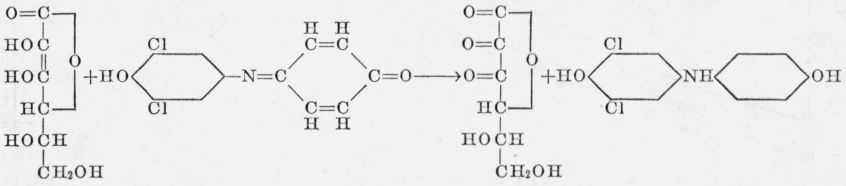
Filter systems transmitting more nearly homogeneous energy than that just described may be used with this comparator if some changes are made in the instrument. In some later work, the clear daylight lamp used as the light source was replaced by a colorless clear-bulb lamp of the same wattage and type, and a flat mirror was placed close behind the lamp so as to reflect a second image of the filament through the condensing lens. The 100-ohm slide-wire resistor (*E*, fig. 1) and the 100-ohm resistor, both in series with the photronic cell, were replaced by a 1,000-ohm slide-wire resistor with a vernier adjustment. The filter system consisted of the Corning glass filters No. 429 (medium-shade, blue-green) and No. 352 (H. R. Noviol) before the absorption cell and Wratten gelatin filter No. 62 (mercury green) between the absorption cell and the photronic cell. This filter combination gives a transmission peak at 530 mμ and a width of 28 mμ at half peak. An all-glass filter combination suggested by Corning Glass Works and consisting of their filters Nos. 352, 430, and 502 may also be used. This combination has a transmission peak at 518 mμ and a half-peak width of about 32 mμ. The substitution of the 1,000-ohm resistance for the 200-ohm resistance in series with the photronic cell will mean some sacrifice in the linearity of the response curve of the cell, even at the low radiant-flux densities used here. This situation can be improved, if necessary, by using a more sensitive galvanometer and leaving the resistance in series with the photronic cell at 200 ohms. One of the Rubicon high-sensitivity "spotlight" galvanometers could be used for this purpose. Their "spotlight" galvanometer, having a critical damping resistance of 40,000 ohms and a sensitivity of 0.0006 μa/mm, happened to be available and was found to be satisfactory. However, with the circuit shown in figure 1 their "spotlight" galvanometer having a critical damping resistance of 10,000 ohms and sensitivity of 0.0015 μa/mm would be better, both in sensitivity and period. The sensitivity of the photocell was improved by the use of one of the latest type barrier-layer photoelectric cells in the comparator.

The stock solution of 2,6-dichlorophenolindophenol was prepared by dissolving 100 mg of the dye crystals in warm distilled water, filtering, and diluting to 200 ml. The dye solution was kept in a refrigerator when not in use, and a fresh solution was prepared every few days. This was necessitated by the fact that after standing for 1 or 2 weeks (especially if warm) the dye is not quite completely bleached from reduction by ascorbic acid.

III. PROCEDURE

In determining the concentration of ascorbic acid, 10-ml samples of blood were collected in centrifuge tubes containing 2 drops of a 20-percent solution of potassium oxalate. After very gentle stirring, the blood was centrifuged for 10 minutes. Three (or, if necessary, two) milliliters of plasma was then transferred to a colorimeter cell and 15 (or 16) ml of 5-percent acetic acid was added, making a total volume of 18 ml. This gave a solution of pH 3.1. After stirring the solution, the cell was placed in the colorimeter and the slide wire (*E*), which was capable of being set to 1 part in 1,000, was carefully adjusted to give a deflection of 100 on the galvanometer. This procedure compensates for differences in the transmission of the original plasma samples, so that they may all be referred to the same calibration curve. The solution was then quickly poured into a test tube containing 1 ml of standard dye solution and at once poured back into the absorption cell. These mixing operations were performed in about 3 seconds. The galvanometer deflection can be read at any desired time intervals after 10 seconds from mixing, 20 seconds being adopted as the standard time necessary to allow the reaction to take place between the dye and the ascorbic acid in the plasma solution.

The chemical reaction is represented by the following equation:



Ascorbic acid. | 2,6-Dichlorophenolindophenol (blue in alkali, red in acid). | Dehydro-ascorbic acid. | Reduced indicator (colorless).

The determinations were made on groups of 20 samples. All the reagents were added in a uniform manner from transfer pipettes with enlarged tips and equipped with rubber bulbs. The necessary chemicals were added to a battery of 20 absorption cells while the first batch of blood samples was being centrifuged. A second batch of samples was centrifuged by the time the preceding batch was analyzed and the absorption tubes were again prepared. The operation was thus continuous and minimized the length of time during which the blood samples were kept. The analysis of a batch of 20 samples was completed within 40 minutes after they were taken from the ice box. The plasma samples originally were placed in the ice box within 10 minutes after they were collected, and the period of standing in the ice box was never more than 3 hours. The plasma was always removed from the centrifuge tube and acidified within 10 minutes after its separation from the whole blood. The stock dye solution was diluted to one-fourth (dye 4) its original strength when used for determinations on plasma with concentrations of ascorbic acid from 1 to 2 mg/100 ml, and to one-eighth (dye 8) of its original strength for plasma containing less than 1 mg/100 ml. The amount of ascorbic acid in the sample was read directly from the calibration curves in mg/100 ml of plasma.

IV. CALIBRATION CURVES

1. BLOOD ANALYSES

Figure 2 contains calibration curves prepared by various methods based on the use of 1 ml of dye 4 and 3-ml samples of the unknown solution. The data for curve 1 were obtained by adding successive increments of ascorbic acid to a 5-percent acetic acid solution containing no plasma and taking the galvanometer reading 20 seconds after mixing the solution with the dye. Curve 2 gives the results obtained by adding successive increments of ascorbic acid to the

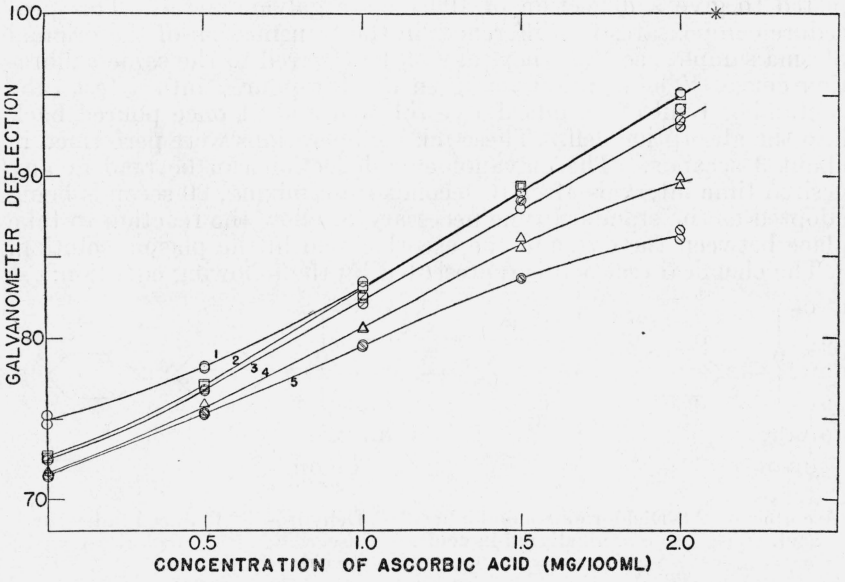


FIGURE 2.—*Calibration curves for photoelectric comparator, dye dilution 4.*

1. Solvent, 20-second reaction period. 2. Plasma, 30-second reaction period. 3. Plasma, 20-second reaction period. 4. Plasma, 15- to 30-second extrapolation. 5. Plasma, 10- to 20-second extrapolation.

solutions of acetic acid containing 3 ml of plasma free of ascorbic acid (see below) and allowing a 30-second interval between mixing with the dye and reading the galvanometer. Curve 3 is the same as 2, except that a 20-second interval was used instead of 30 seconds. Curve 4 differs from curve 2 in that the galvanometer readings were taken at 15 and 30 seconds and these values extrapolated linearly to zero time. Curve 5 was obtained similarly, except that 10- and 20-second readings were used for the extrapolation instead of the 15- and 30-second ones. The point designated by a star on the 100 ordinate represents the concentration of ascorbic acid which is stoichiometrically equivalent to 1 ml of the dye used. If the increments of ascorbic acid were totally oxidized within the time allowed for taking the readings, and if the plasma had no effect on the dye or on the ascorbic acid, then the calibration curves should all pass through the starred point. The fact that they do not shows that factors such as those just mentioned do complicate the reaction and points to the advantage of using an empirical calibration curve. For working with blood samples containing from 2 to 3.5 mg of ascorbic acid per 100 ml, a curve similar

to curve 3 but containing 2-ml plasma samples was prepared. For working with blood samples containing less than 1 mg of ascorbic acid per 100 ml, greater precision is obtained by using a more dilute dye solution. Figure 3 contains the calibration curve for 3-ml plasma samples, using the 20-second reaction period and dye dilution 8.

The preparation of a satisfactory blank plasma, for use in obtaining the calibration curves, presented some difficulties. The blank should contain no ascorbic acid but should have as nearly as possible the normal amount of the slowly reacting interfering substances. The

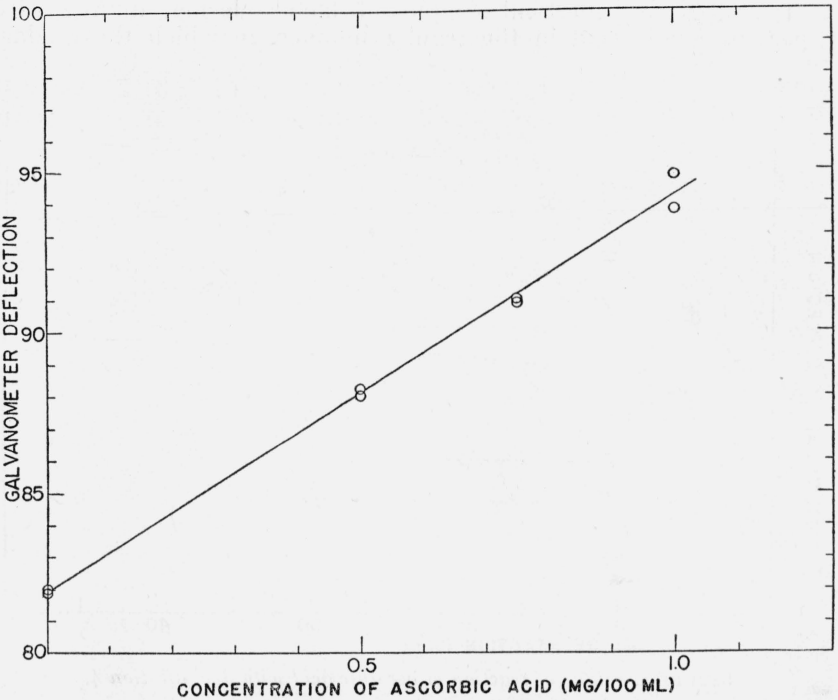


FIGURE 3.—*Calibration curve for blood plasma, dye dilution 8.*

blank plasma was prepared by two separate methods. The standard method was to titrate the fresh plasma, acidified with acetic acid, with dye as long as the photoelectric colorimeter showed the added dye to fade completely in 20 seconds. An alternative method was to let the plasma stand in the ice box for about 6 days and depend on the atmosphere to oxidize the ascorbic acid. These two independent methods for preparing plasma free of ascorbic acid gave samples so nearly the same that calibration curves prepared from them checked to within 0.1 mg of ascorbic acid per 100 ml of plasma.

Figure 2 shows that the calibration curves prepared from data which had been extrapolated to zero time (curves 4 and 5) deviated considerably from linearity in the region of higher concentration, while curves prepared from data obtained by using a straight 20- or 30-second reading (2 and 3) were nearly linear and came much closer to passing through the starred point. Important also is the

fact that the curves from the 10- to 20- and 15- to 30-second extrapolations have smaller slopes and hence give less sensitivity at high concentrations. The fact that the curves extrapolated to zero time are appreciably below those in which a definite reaction period was allowed shows that the reaction of ascorbic acid with the dye in blood plasma is not instantaneous. Figure 4 gives curves of galvanometer deflection versus time, for various solutions when 1 ml of dye 4 is added to them. These curves (and a duplicate check set not given here) furnish the data from which some of the points on the curves in figure 2 were obtained. The curves in figure 4 labeled 0, 1, and 2 were for 3-ml samples of blood plasma, diluted with 5-percent acetic acid in the regular manner, in which the original

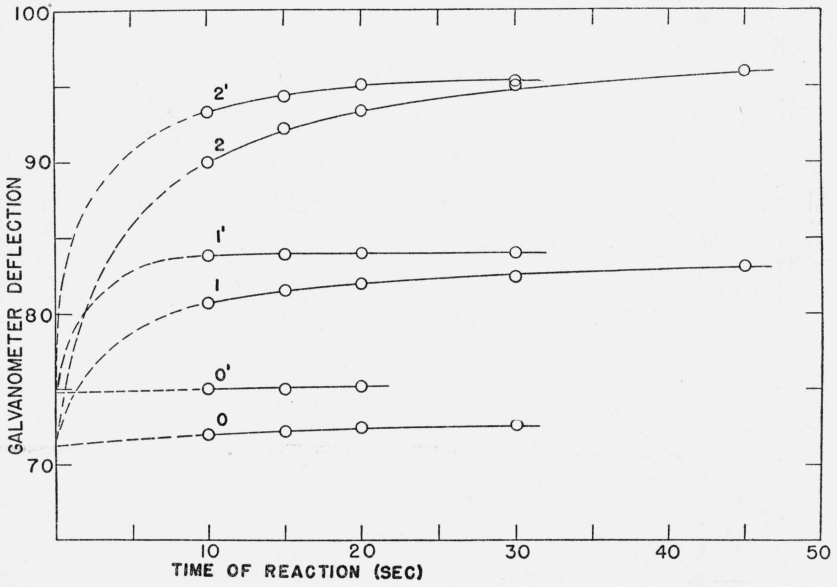


FIGURE 4.—*Rate of reaction of ascorbic acid with dye, dilution 4.*

Curves 0, 1, and 2 are for ascorbic acid concentrations of 0, 1, and 2 mg/100 ml, respectively, in acetic acid, blood plasma solution; curves 0′, 1′, and 2′ are the same as 0, 1, and 2, respectively, except no blood plasma was present.

ascorbic acid concentration was, respectively, 0, 1, and 2 mg/100 ml. The curves labeled 0′, 1′, and 2′ are given for comparison and were the same as 0, 1, and 2, except that they were made with the 5-percent acetic acid solution without the blood plasma present. It can be seen that at least 15 seconds is required for the reaction in the plasma solutions when the ascorbic acid concentration is of the order of 1 mg/100 ml, and at least 20 seconds is necessary when the concentration is 2 mg/100 ml. A comparison of the curves made with plasma solutions with those made with 5-percent acetic acid only show that the plasma reduces the rate of the reaction. As the ascorbic acid concentration approaches the stoichiometric equivalent of the dye, the reaction time lengthens appreciably. However, since the blood plasma also contains slowly reacting interfering substances [14], the reaction time must be kept to a minimum. A compromise must, accordingly, be reached to allow sufficient time for the ascorbic acid

to react but to minimize the reaction of the interfering substances. Calibration curves prepared on the basis of a 20-second reaction time were used in this work, since a linear curve was obtained for the type of plot used in figure 2 and 20 seconds appears to be the minimum time necessary for the oxidation of ascorbic acid in blood plasma. An extrapolation to zero time from readings taken on a 30- to 60-second basis gave essentially the same results as the 20-second readings. This extrapolation has an advantage in that it takes into account a second-order error due to slight differences in the amount of interfering substances between various blood plasma samples. Its chief disadvantage in this work is that it takes an appreciably longer time to analyze each sample. This time factor is important

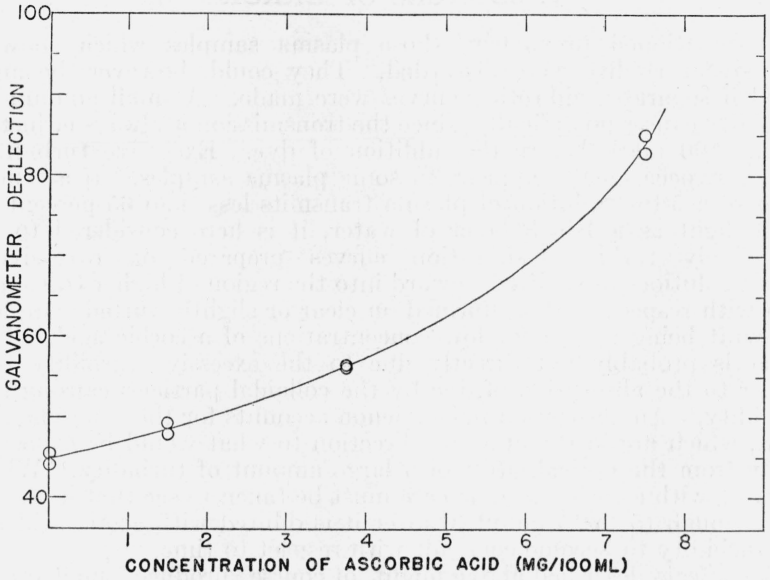


FIGURE 5.—*Calibration curve for urine, stock dye solution.*

when it is necessary to analyze large numbers of samples before oxidation by the air has a chance to affect appreciably their ascorbic acid content. With the method described here, two operators can analyze 40 blood samples in an hour.

2. URINE ANALYSES

The photoelectric colorimeter may also be used to determine the ascorbic acid in urine [12, 15], which contains many more interfering substances than does blood plasma. These substances are partially removed by precipitation with barium acetate [16, 17]. In the procedure for urine, ½ ml of glacial acetic acid and 2 g of dry barium acetate are added to 10 ml of the fresh sample. After shaking, the sample is centrifuged and 1 to 3 ml (depending on the ascorbic acid contained) is analyzed in the colorimeter in the same manner as the blood plasmas. However, instead of the 20-second reaction time used for blood analyses, it is necessary to extrapolate to zero time in order to minimize the effect of the slowly reacting interfering substances which remain even after the precipitation with barium

acetate. In these experiments a 10- to 20-second extrapolation was found most satisfactory. Dye dilution 4 is used for urine samples containing less than 2 mg of ascorbic acid per 100 ml of urine, and the undiluted dye is used for samples containing 2 mg or more per 100 ml. Here again empirical calibration curves were found to be preferable to the stoichiometric method. These curves were prepared by adding known increments of ascorbic acid to urine that contained no reduced ascorbic acid. Figure 5 contains the calibration curve prepared by using the undiluted dye and 3-ml samples of urine. The urine analyses may be made in batches of 20 and with about the same speed as the analyses of ascorbic acid in blood.

V. SOURCES OF ERROR

As mentioned previously, those plasma samples which showed excessive turbidity were discarded. They could, however, be analyzed if separate calibration curves were made. A small amount of turbidity causes no difficulty, since the transmission is always adjusted to the 100 mark before the addition of dye. Excessive turbidity, however, occasionally appears in some plasma samples. If a 1-inch layer of a 5-to-1 dilution of plasma transmits less than 65 percent as much light as a 1-inch layer of water, it is here considered to be excessively turbid. Calibration curves prepared on excessively turbid solutions are shifted upward into the region of higher transmission with respect to those formed on clear or slightly turbid samples, the shift being largest for low concentrations of ascorbic acid. The effect is probably not directly due to the excessive turbidity but rather to the absorption of dye by the colloidal particles causing the turbidity. An absorption phenomenon accounts for the experimental facts, which are in the opposite direction to what would be expected solely from the optical effect of a large amount of turbidity. When working with a turbid sample care must be taken to see that it stands long enough (5 to 15 minutes) after it is diluted with acetic acid for the turbidity to become constant with respect to time.

The effects described above might, of course, produce small errors even in slightly turbid plasmas. A good test for such errors, arising from variation in the natural color and in the turbidity, is obtained by comparing the final galvanometer readings for a series of plasmas, free of reduced ascorbic acid, to each of which 1 ml of dye 8 has been added. The results on plasmas of 11 individuals taken at random are given in table 1. The first column gives the original percentage transmission, compared with distilled water, of a 5-to-1 dilution of plasma with 5-percent acetic acid.

TABLE 1.—*Effect of turbidity on calibration curves*

$T_{orig.}/T_{H_2O}$	Final galvanometer reading	$T_{orig.}/T_{H_2O}$	Final galvanometer reading
Percent		*Percent*	
74	82.0	80	82.7
76	82.1	80	82.4
78	81.3	81	81.6
78	81.9	83	81.7
79	82.0	85	81.1
79	81.9		
			Mean....81.9±0.3

The galvanometer is then adjusted to 100 and 1 ml of of dye 8 added, the resulting galvanometer reading being recorded in the second column of the table. The latter galvanometer readings, except for the errors discussed above, should all be identical. The average deviation of 0.3 from the mean (81.9) corresponds (see fig. 3) to an uncertainty of approximately 0.03 mg/100 ml of ascorbic acid in the undiluted plasma.

Atmospheric oxidation of the ascorbic acid will introduce systematic errors if not controlled. However, in these experiments the samples were never kept more than 3 hours in the refrigerator nor, upon dilution and immediate acidification, for as much as 40 minutes at room temperatures. It is known [1, 9] that no appreciable oxidation by air takes place in 3 hours in the refrigerator. Furthermore, no difference in the ascorbic acid content could be detected in diluted and acidified plasma samples because of standing for 40 minutes at room temperatures. The only other possibility for oxidation by air is in the centrifuge and while the plasma samples are being acidified, especially since ascorbic acid is known to be less stable in plasma than in whole blood. Although appreciable oxidation of the ascorbic acid in blood plasma is known to take place when the plasma stands for more than 4 hours at room temperature [1, 18], the amount of oxidation in the 15 minutes taken to centrifuge and acidify a batch of 20 samples will be small, especially since it takes a part of the 15 minutes for the samples to warm up to room temperature. Roe [19] has discussed various sources of error in his and other methods.

Errors due to a slow fading of the dye in 5-percent acetic acid solution, to nonlinearity of the photoelectric-cell response, and possible incompleteness of the reaction of ascorbic acid with dye in 20 seconds are practically eliminated by the use of empirical calibration curves instead of stoichiometric equivalents. Air bubbles introduced by mixing the solutions cause no difficulty in the transmission measurements if the readings are taken 10 seconds or more after mixing.

The maximum total error in these determinations of ascorbic acid in plasma containing 0 to 3.5 mg/100 ml is estimated as ± 0.1 mg/100 ml.

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