MEDICAL EVALUATION OF NUTRITIONAL STATUS[1]

XI. AN ANALYSIS OF SOURCES OF ERRORS IN THE PHOTELOMETRIC MACROMETHOD OF DETERMINING ASCORBIC ACID IN PLASMA

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DETERMINATION of ascorbic acid in plasma by use of the photoelectric colorimeter, according to the method of Mindlin and Butler (1), has provided a convenient, reliable, and objective procedure. This procedure, reported in 1938, has already been widely adopted. A macromethod and a micromethod were described by Mindlin and Butler, and this has made the procedure available in situations in which only small samples of blood could be obtained. There is little information available to indicate the accuracy of colorimetric determinations of ascorbic acid concentrations derived by either the macro or micromethod. In the study, "Medical Evaluation of Nutritional Status," which is a cooperative investigation by the New York City Department of Health, the United States Public Health Service, the Cornell University Medical College, and the Milbank Memorial Fund, one of the principal objectives has been the appraisal of procedures for determining nutritional status (2). A detailed analysis of the colorimetric determination of ascorbic acid in plasma, therefore, was undertaken.

In the Nutrition Study, the macromethod was used routinely and this report presents a statistical analysis of sources of error in the determination of ascorbic acid content. The quantitative contribution of each source of variation to the errors of the values required by the method is described and an estimate is made of the error

[1] This paper is the eleventh of a series from a cooperative investigation by the New York City Department of Health; the Division of Public Health Methods, National Institute of Health, United States Public Health Service; the Cornell University Medical College, Department of Public Health and Preventive Medicine and Department of Pediatrics; and the Milbank Memorial Fund.

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of the determined ascorbic acid content when the procedure used is most favorable in that certain potential sources of error are controlled.

A similar analysis of errors in the micromethod is to be published shortly. There is also in progress a report on comparisons of determinations by the photelometric methods and the titrimetric methods.

Method of Photelometric Determinations

The estimation of ascorbic acid is based upon the color change resulting from reduction of 2.6 dichlorophenol-indophenol. By means of the photoelectric colorimeter, objective measurements are made of the intensity of a light beam passed through a solution of the dye (dye blank) and of the light intensity when a solution containing ascorbic acid has been added to the dye solution. The diminution of color in the dye from the addition of ascorbic acid permits an increased transmission of light, and the difference in galvanometer readings of light intensities for the original dye solution and the dye plus a solution containing ascorbic acid affords a measure of the change in the amount of light transmitted. The quantitative relationship between the change in light transmission and the amount of ascorbic acid added to the dye has been established and is expressed in the formula $C = K\ (\log. G_s - \log. G_b)$, where G_s is a value read from the galvanometer scale for the dye with the specimen solution added, G_b is the galvanometer reading of the prepared dye solution before the specimen solution is added, K is the constant for a given volume of the solutions which expresses the relationship between reduction of color in the dye and amount of ascorbic acid, and C is the determined amount of ascorbic acid in the specimen solution. The galvanometer scale is in units from 1 to 100, and in the macromethod, readings are from the upper half.

The value for K may be determined by transposing the equation to read $K = \frac{C}{\log. G_s - \log. G_b}$ and using weighed amounts of solutions

of crystalline ascorbic acid for the specimen solution, so that the value of C is known.

In the Nutrition Study an Evelyn colorimeter was used, and tables of values for 2 – log. G which come with the instrument were substituted for common logarithms. When this substitution is made the difference computation is reversed[2] and (log. G_s – log. G_b) becomes (2 – log. G_b) – (2 – log G_s). These values for (2 – log. G) are designated as L values, and when substituted in the equations, the formulae become $K = \frac{C}{L_b - L_s}$ and $C = K\ (L_b - L_s)$. Throughout this report, L with the subscript b is used for the value for 2 – logarithm of the galvanometer reading on the dye blank and L_s for the value of 2 – logarithm of the galvanometer reading after addition of a specimen solution to the dye acetate solution. The L table used gave values for one-quarter units of the galvanometer scale and readings were made to the nearest one-quarter unit, although only units are marked on the scale.

It is apparent that each observation required for determining the ascorbic acid content of a specimen of plasma is a potential source of error in the final estimate of the content. Consideration of the above formulae will show that a basic source of error is in the readings made of light intensity on the galvanometer scale. The reliability of the measurement of the transmitted light has been studied for the three types of solutions involved: (1) the prepared dye blank; (2) the dye blank plus a prepared solution of crystalline ascorbic acid; and (3) the dye blank plus the plasma specimen. Observations on the first two solutions provide the constant (K) used in the plasma determination; and those on the first and third, together with K, enter into the plasma determination.

It is obvious that any error in the ratio, K, has an important influence on the accuracy of the determination of ascorbic acid concentration in plasma. Mindlin and Butler suggested that an average

[2] This is shown as follows: (2 – log. G_b) – (2 – log. G_s) = 2 – log. G_b – 2 + log G_s = log. G_s – log. G_b.

K value for a particular colorimeter should be obtained from several determinations made on sample solutions of ascorbic acid of different concentrations, and that the K value so derived for a particular colorimeter could be taken as a constant value. In the course of this Study, K values were obtained daily for a period of nine months, and an analysis of the variation of K under different conditions forms a large part of this report. Although the preparation of solutions of ascorbic acid each day to determine K adds to the laboratory work, our data indicate that this is necessary to keep the error of K at a minimum.

Evidence on another possible source of inaccuracy to be presented relates to the ratio $\frac{C}{L_b - L_s}$ at different concentration levels. How constant is this value throughout the range of ascorbic acid concentration found in human plasma?

Data Used for Analysis

The data utilized in the analysis of errors in the macromethod are, in large part, the daily laboratory records from routine use of the procedure in determining plasma ascorbic acid for over 2,000 high school pupils examined during the eight months from October, 1939 to June, 1940. On the basis of a preliminary analysis, it was decided to determine K each day. The laboratory record provides a galvanometer reading in duplicate or triplicate, usually the latter, on the dye blank for each day and on each of two or three prepared ascorbic acid solutions, usually at concentrations of 1.0, 1.5, and 2.0 mg. per 100 cc. Each reading for a dye blank was from a separate colorimeter tube and each reading for a given concentration was from an independently prepared tube containing the dye blank plus an aliquot of the ascorbic acid solution. On each laboratory record there was recorded the number of the dye stock from which the dye solution was prepared, and the date on which the solution was prepared. The dye acetate solution usually was prepared in quantity to last for several days, but was seldom used more than five days.

Data for the preliminary analysis provided galvanometer readings on prepared solutions of crystalline ascorbic acid at seven concentrations, ranging from 0.1 mg. to 2.0 mg. per 100 cc. These data are referred to in the following discussion as the initial or test series, and have been used chiefly to compare K values derived from lower concentrations than those used in the routine series.

Error of Galvanometer Readings

Dye Blank Readings. From the large series of independent duplicate galvanometer readings on the same dye blank, data are available to show the variation in readings and to estimate the error of a single measurement of light transmission as read from the galvanometer scale. Two readings each day were paired[3] and the difference in L values was obtained. A frequency distribution of differences centering around zero is obtained, since differences were taken in a constant order and the sign of the difference retained. The standard deviation of these differences indicates the extent of the variation in L_b values if several observations are made on the same dye blank and eliminates all variation between different dye solutions. On the assumption that the true L value for each dye blank is approximated by the average of the L values for duplicates, the variance of differences (S.D.^{2}) divided by 2 (since two observations entered into each difference) gives an estimate of the variance and from this of the standard deviation for L_b based on a single galvanometer observation on a dye blank.[4] The standard deviation of an L_b from errors of measurement is given in Table 1 for all routine data combined and separately for observations made on

[3] Only two galvanometer readings were used for days on which three had been reported. With the records filed chronologically, for successive days, differences with signs were taken for: (1) first minus second reading for one day; (2) second minus third for the following day; and (3) first minus third reading for third day; and this order then was repeated.

[4] This standard deviation of an L_b from a series of duplicate readings on different dye blanks is the average standard deviation of L_b for each pair from the mean L_b for the pair and is equivalent to the standard deviation of a series of observations made on the same dye blank.

Dye Stock	Number of Pairs of Readings	Standard Deviation (L) for Difference in Duplicates	Estimated Error of L_b	
			Variance e_b $\left(\frac{\sigma \text{ diff.}^2}{2}\right)$	Standard Deviation σ_{e_b}
All Dye Stocks	135	.00425	.00000902	.00300
Stock I—With 17 Dye Acetate Solutions	55	.00407	.00000828	.00288
5 Different Stocks—16 Dye Acetate Sol.	80	.00437	.00000953	.00309

Table 1. Estimated error of L for a single galvanometer reading on the dye blank in the macrocolorimetric method.

solutions from one dye stock used for several months and for five different stocks each of which was used for a relatively short period.

The standard deviation of a single L_b value[5] based on 270 observations, or 135 pairs of duplicates, was 0.00300. The error of the L_b value is small in relation to the actual value which varied around 0.300, and, conversely, the reliability of the galvanometer measurement of light intensity was high. Some error in reading the galvanometer no doubt occurred, but in this procedure several factors also may affect the measurement, especially any slight imperfection in the tube or interfering particles in the solution or left on the tube after cleaning. The error found for a dye blank reading has been termed the accidental error of L_b, and is designated σ_{e_b}.

Galvanometer Readings on Dye Plus Ascorbic Acid. From galvanometer readings on two colorimeter tubes in which samples from the same dye blank were mixed with an aliquot of the same prepared solution of ascorbic acid of known concentration, a series of differences between paired L_s values was obtained as described above for differences in L_b values. These differences in L_s values

[5] The dye blanks throughout the Study gave G readings between 50 and 55 with the exception of a few readings from 47 to 49, and a few from 56 to 58. The L values, therefore, ranged from 0.328 to 0.231. *Change* in light transmission is the significant measurement, and the density of the dye blank is not precisely controlled. G readings from 50 to 55 are recommended by Mindlin and Butler.

include the type of error described for L_b plus any variability in the reaction of the same dye to the added ascorbic acid. Errors in measuring the ascorbic acid aliquot into the dye solution also would affect the differences. In Table 2 there is shown the standard deviation of the differences between duplicate readings on different concentrations of ascorbic acid with dye, and the estimated standard deviation of L_s from a single reading.

The reliability of a single observation on the reduced dye was very high. The standard deviation of L_s was in every case less than that for the dye blank alone for the same series of solutions, although at 1.0 mg. per cent the error for L_s from five different dye stocks was very nearly as great as the error of L_b. This does not mean that there was less variability in the G readings, for this variability was slightly higher for the reduced dye. The galvanometer value increases as the dye is reduced, or as greater amounts of ascor-

Table 2. Estimated error of L for a single galvanometer reading on the dye solution plus a solution of crystalline ascorbic acid in the macrocolorimetric method.

Dye Stock and Ascorbic Acid Concentration	Number of Pairs of G Readings	Standard Deviation (L) for Differences in Duplicates	Estimated Error of L_s	
			Variance e_s $\left(\frac{\sigma \text{ diff.}^2}{2}\right)$	Standard Deviation σ_{e_s}
All Dye Stocks				
1.00 Mg. Per Cent	126	.00373	.00000695	.00264
1.50 Mg. Per Cent	126	.00347	.00000602	.00245
2.00 Mg. Per Cent	125	.00330	.00000545	.00233
Stock I— 17 Dye Acetate Solutions				
1.00 Mg. Per Cent	52	.00302	.00000457	00214
1.50 Mg. Per Cent	56	.00287	.00000413	.00203
2.00 Mg. Per Cent	53	.00349	.00000609	.00247
5 Stocks— 16 Dye Acetate Solutions				
1.00 Mg. Per Cent	74	.00416	.00000863	.00294
1.50 Mg. Per Cent	70	.00388	.00000753	.00274
2.00 Mg. Per Cent	72	.00313	.00000490	.00221

bic acid are added, and the L value not only becomes smaller but also a unit difference in the G value has a decreasing L value as G increases. The slightly greater variability in G readings was more than compensated for by the smaller L value per G unit. The error of measurement of L_s (σ_{e_s}) for all data at 1.0 mg. per 100 cc. was 0.00264, at 1.5 mg. it was 0.00245, and at 2.0 mg. it was 0.00233. The magnitude of these errors indicates that the reaction of the same dye blank to a given amount of ascorbic acid was very stable.

Variation in K Values

Accidental Error of K. Since the K value[6] is a quotient derived from a known concentration of ascorbic acid and the difference ($L_b - L_s$), the error of a single K from accidental errors in determining L_b and L_s may be determined from the above estimates of the accidental errors of L_b and of L_s. The accidental error of this difference (L_d) is the square root of the sum of the variances of the two values, from the formula σ diff. $= \sqrt{\sigma_1^2 + \sigma_2^2}$. The approximate error of K from this error for L_d may be determined from the formula for the standard deviation of a quotient (3). In this instance, the dividend C, the known concentration, is not a variable but a fixed value for which the error is only that incidental to preparing the solution. If the error for C is assumed to be zero, the standard deviation of a calculated K is obtained as follows:[7]

$$\sigma_K = \frac{C}{L_d}\left(\frac{\sigma_{e_d}}{L_d}\right)$$

[6] The K value, as given in this report, is the constant obtained when C for the 4 cc. of a standard solution used in the determination is taken as the equivalent concentration per 100 cc. of plasma filtrate.

[7] The formula (3) for the standard deviation of a quotient is:

$$\sigma_{X_1/X_2} = \frac{X_1}{X_2}\sqrt{C.V._{.1}^2 + C.V._{.2}^2 - 2r_{1.2}\, C.V._{.1}\, C.V._{.2}}$$

It is reasonable to assume no correlation because the standard deviation to be estimated is for a K value for a specific ascorbic acid solution, and is that associated with errors of

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Ascorbic Acid Solution Mg. Per 100 cc.	Accidental Error of L_d $\sigma_{ed} = \sqrt{(\sigma_{eb})^2 + (\sigma_{es})^2}$	True L Difference L_d When		Coefficient of Variation C.V. $= \sigma_{ed} \div L_d$		Error of K from σ_{ed} $\sigma_K = K \times C.V.$	
		K = 9.7	K = 9.2	K = 9.7	K = 9.2	K = 9.7	K = 9.2
1.00 Mg.	.00400	.103	.109	.0388	.0367	.376	.338
1.50 Mg.	.00388	.155	.163	.0250	.0238	.243	.219
2.00 Mg.	.00380	.206	.217	.0184	.0175	.178	.161

Table 3. Error of estimate for K attributable to accidental error of a single observation for the dye blank and of a single observation for the ascorbic acid solution at three different concentrations. Estimate is derived from data in Tables 1 and 2.

It is apparent from this formula that the size of the standard deviation of K will vary positively with the magnitude of K since it is the product of K and the coefficient of variation of the L difference, or $\sigma_{e_d} \div L_d$. An example of the calculation of the standard deviation of a K value, when only the effect of the accidental error of L_b and of L_s is measured, follows for K derived from a single observation for L_b and for L_s.

(Table 1) variance for L_b $= .00000902$
(Table 2) variance for $L_{1.0}$ mg. $= .00000695$
Variance of L_d Sum $= .00001597$

$$\sigma_{e_d} = \sqrt{.00001597} = .00400$$

$$\text{If } L_d \text{ for 1.0 mg.} = .100, \text{ then } K = \frac{1.00}{.100} = 10.0$$

$$\text{Standard deviation of K} = 10.0 \left(\frac{.00400}{.100}\right), \text{ or } 10.0 \times .0400 = .400$$

In Table 3, the estimated error of K attributable to the accidental variability of L_b and L_s is shown for three concentrations of ascorbic

measurement of L_d (σ_{ed}) for a constant solution. For the standard deviation of K, the formula is:

$$\sigma_K = \frac{C}{L_d}\sqrt{\left(\frac{\sigma_c}{C}\right)^2 + \left(\frac{\sigma_{ed}}{L_d}\right)^2}$$

but if σ_c is taken as 0, $\sigma_K = \frac{C}{L_d}\sqrt{\left(\frac{0}{C}\right)^2 + \left(\frac{\sigma_{ed}}{L_d}\right)^2}$ or $\frac{C}{L_d}\left(\frac{\sigma_{ed}}{L_d}\right)$

acid when K is at two different levels. It is clear that this error in K varies inversely with the concentration from which it is derived. Thus, if K is 9.7, the standard deviation of K is 0.376 at 1.0 mg. per 100 cc., but it is 0.178 at 2.0 mg. Most of this decrease in the error as the concentration increases results from the fact that the difference, L_d, must increase proportionately with the concentration if K is the same at each concentration and the coefficient of variation becomes smaller unless the standard deviation of L_d also increases proportionately. But in Table 3, it is shown that the standard deviation of L_d decreased slightly, as the concentration was increased. From these data, it is apparent that the error of K from these accidental sources is a minimum if K is derived from high concentrations of ascorbic acid within the range of ascorbic acid values to be expected in the material to be analyzed.

The accidental error of K estimated in Table 3 is that which is shown in variability of observations on a dye blank at a given time and of observations on this blank reduced by aliquots of the same prepared ascorbic acid solutions. It is, therefore, the apparent error of K within one day. The true error of K for any day will include the error in C which results from preparing the ascorbic acid solutions. On each day 100 mgs. of ascorbic acid were weighed for the preparation of a stock solution. From this stock solution, independent dilutions were made for the three standard solutions with concentrations equivalent to 2.00, 1.50, and 1.00 mg. per 100 cc. No experimental data were collected to estimate the error in preparing these solutions. However, K values obtained from independently prepared solutions will vary as a result of errors in the solution as well as from the accidental error described. This total accidental variation in K from errors of C and of L_d is found in K values derived on different days. The accidental error of L_d is known, and the error in the solutions can be estimated from the observed day-to-day variability of K if the effect of other factors on the variation in K can be controlled.

The same dye acetate solutions were used for several days, and it has been assumed that, if the standard deviation of L_b for different days on which a given dye solution was used did not exceed that found for the accidental error of L_b, the K values for the different days also should vary within limits of the accidental error of L_d plus the error of the prepared solution. For many dye acetate solutions, the observed standard deviation of L_b for the entire period of use was significantly higher than the accidental error of L_b, and this will be discussed later. There were nine dye solutions used from three to six days for which the standard deviation of L_b was from .00224 to .00332, as compared with the estimated accidental error of a single L_b of .00300. For each of these nine dye acetate solutions, the excess variation in K over that attributable to accidental errors of L_b and L_s is assumed to be due to errors in solutions prepared each day.

The method of estimating the error of C from the observed variation of K for each dye acetate solution and the accidental error of L_d for each dye solution was to substitute these known values in the formula for the standard deviation of K and solve for the unknown value, the error of C. The formula to determine the error of C derived from the formula for the standard deviation of the quotient K, is obtained as follows:

$$\sigma_K = K\sqrt{\left(\frac{\sigma_c}{C}\right)^2 + \left(\frac{\sigma_{e_d}}{L_d}\right)^2}$$

$$\therefore \quad \frac{\sigma_K}{K} = \sqrt{\left(\frac{\sigma_c}{C}\right)^2 + \left(\frac{\sigma_{e_d}}{L_d}\right)^2} \quad \text{and} \quad \left(\frac{\sigma_c}{C}\right)^2 = \left(\frac{\sigma_K}{K}\right)^2 - \left(\frac{\sigma_{e_d}}{L_d}\right)^2$$

where σ_K is the observed standard deviation of K for different days on which the same dye solution was used, σ_{e_d} is the error of the difference (L_d) from the accidental error of L_b for this dye solution and the accidental error of L_s obtained for this dye solu-

tion. The square of the coefficient of variation $\left(\frac{\sigma_c}{C}\right)^2$ was determined for each dye solution by the following calculations:

1. The mean variance of L_s from accidental errors for each specific dye acetate solution was calculated as the *within-the-day* variance from triplicate readings each day and was determined for each of the concentrations.
2. The variance of L_b for all observations on a specific dye solution was used as the variance of a dye blank reading from accidental errors.
3. The variance of the L difference between mean L_b and mean L_s for one day from accidental errors (σ_{e_d}) was calculated for each dye solution as the sum of variances of L_b and L_s divided by 3 since three independent readings were taken each day on the dye blank and on the dye plus ascorbic acid solution.
4. The variance of L_d for each day at a specific concentration was divided by the square of mean L_d at that concentration for all days on which the dye solution was used. This value is $\left(\frac{\sigma_{e_d}}{L_d}\right)^2$ in the above formula.
5. For K values on different days for the same dye solution, the mean K and the standard deviation of K was obtained at each concentration of ascorbic acid. From these, the value $\left(\frac{\sigma_K}{K}\right)^2$ for the above formula was obtained.
6. The difference, $\left(\frac{\sigma_K}{K}\right)^2 - \left(\frac{\sigma_{e_d}}{L_d}\right)^2$ obtained for each dye solution was weighted by the number of days of use and the mean difference taken as the estimate of the square of the coefficient of variation of C.

The mean percentage errors of C obtained from the nine dye acetate solutions used for varying periods over a total of thirty-eight different days were:

For C = 1.00 mg., the percentage error = 2.94[8]
For C = 1.50 mg., the percentage error = 1.90
For C = 2.00 mg., the percentage error = 1.46

For the nine solutions, the variation in the estimated percentages

[8] This estimated percentage error in solutions of 1.0 mg. per cent is very close to an

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was small. These estimates of the error in C are believed to be reasonably good approximations of the percentage variation in C to be expected over a period of time. On any one day, the chances are nearly two to one that the error in C is less than the estimated mean percentage.

The effect of the percentage error of C on the total accidental error of K for one day is not reduced by repeated observations on each prepared solution, since aliquots of the same solution are used in making these observations on the dye blank plus ascorbic acid solution. The accidental error of L_b and L_s is reduced by taking several observations. An estimate of the expected error of K from accidental sources discussed may be expressed as follows:

$$\sigma_{e_K} = \frac{C}{L_d}\sqrt{\left(\frac{\sigma_c}{C}\right)^2 + \left(\frac{\frac{V_{e_b}}{n_b} + \frac{V_{e_s}}{n_s}}{L_d^2}\right)}$$

In this formula, σ_{e_K} is the expected total accidental error of K, V_{e_b} is the square of the estimated error of measurement for L_b, n_b is the number of independent galvanometer readings taken on the dye blank, V_{e_s} is the square of the error of measurement for L_s, n_s is the number of readings on the standard ascorbic acid solution, and L_d is the difference between the mean L_b and mean L_s.

In the present Study, as previously stated, it was usual to make three readings on the dye blank each day and three readings with the standard solution added to obtain a K value at 1.0 mg., 1.5 mg., and 2.0 mg. concentrations. The estimates of variances of L_b and L_s from accidental errors, shown in Tables 1 and 2, and the percentage

estimate made from data from a series of observations on plasma to which a known amount of ascorbic acid was added. To ninety-seven plasma specimens, an amount of ascorbic acid equivalent to 1.0 mg. per cent was added, and the plasma content was determined before and after this addition. The standard deviation of the differences between the initial determinations and those after addition of ascorbic acid was found to be greater than that for duplicate determinations when no addition was made. If the difference between the variance with addition of 1.0 mg. per cent ascorbic acid and the variance without addition is assumed to be the variance contributed by errors incidental to weighing and handling the crystalline ascorbic acid, then the estimated standard deviation for the 1.0 mg. per cent solution added to each specimen was 2.5 per cent.

errors for C may be substituted in the above formula to derive the expected total error of K at each concentration.

The expected total accidental errors of daily K values for each of the three concentrations and at two levels for K have been computed and are shown in Table 4. These estimates of the error of the daily K should approximate the observed standard deviation of K over a period of time unless other factors caused the K to change. However, the average value of K on dye stock I was about 9.2, but the observed standard deviation on 1.0 mg. per cent solutions was 0.379 as compared with the expected standard deviation from accidental errors of 0.334, and on 2.0 mg. per cent, the observed standard deviation was 0.211 as compared with the expected standard deviation of 0.163. Further analysis of the variation in K was made, therefore, to identify, if possible, other factors which affected the K value.

K Variation for Different Dye Solutions. Inspection of the routine laboratory records suggested that K changed when new dye solutions were used. Therefore, an analysis of the variation in daily K values was made to determine the magnitude of this variation under three conditions; namely, when the same dye acetate solution

Table 4. Total error of measurement for K estimated from percentage error for prepared ascorbic acid solutions plus accidental error of L difference when three observations are made for the dye blank and for the blank plus solution.[1]

Ascorbic Acid Solution Mg. Per 100 cc.	$\frac{\sigma_c}{C}$ (1)	$\frac{\sigma_{ed} \div Ld^*}{\sqrt{3}}$ K = 9.7 (2)	$\frac{\sigma_{ed} \div Ld^*}{\sqrt{3}}$ K = 9.2 (3)	Total Accidental Error of K or σ_{eK} K = 9.7 $\sqrt{(1)^2+(2)^2}\times K$	Total Accidental Error of K or σ_{eK} K = 9.2 $\sqrt{(1)^2+(3)^2}\times K$
1.00 Mg.	.0294	.0224	.0212	.359	.334
1.50 Mg.	.0190	.0145	.0137	.232	.216
2.00 Mg.	.0146	.0107	.0101	.176	.163

[1] See text, pages 189-190.

*Since n = 3 for both L_b and L_s, the coefficient of variation (C.V.) for Ld in Table 3 when n is 1 may be divided by $\sqrt{3}$ to obtain C.V. for Ld from three observations.

was used for several days; when different dye solutions prepared from the same dye stock were used; and when different dye stocks were used. The basic data for the analysis included all routine daily laboratory records for the five dye lots from which at least three different dye acetate solutions were prepared. The analysis is made separately at each of the three levels of ascorbic acid solutions in order to eliminate different concentration levels as a source of variation in K. The mean daily K values at each concentration based on three galvanometer readings on the dye blank and three readings after addition of ascorbic acid were used for a variance analysis to show the mean variation of these daily K values for days on which the same dye acetate solution was used, and to compare this variance with the variance for means of the K values derived from each dye acetate solution prepared from the same dye lot. The results of this analysis of K values derived from concentrations of 1.0 mg., 1.5 mg., and 2.0 mg. per 100 cc. are shown in Table 5.

The total variance of all K values obtained on the same dye stock was in most instances greater than the mean variance of K on days for which the same dye acetate solution had been used. This greater total variance reflects the fact that, as shown in Table 5, the variance among mean K values for different acetate solutions was frequently, although not consistently, greater than the variance of K within dye solutions. In ten of the fifteen comparisons, the variance among mean K values for different dye solutions of a single stock is greater than that for daily K values for the same dye solutions; and in the five instances in which the reverse occurred, the variances did not differ significantly. In seven of the ten cases of greater variance among means for specific dye acetate solutions, the statistical probability is less than 5 chances in 100 that this greater variance would occur as a result of the variance of K on the same dye solution. Six of the eight comparisons for which the variance among solutions was not significantly greater than within solutions are for the two dye lots from which only three acetate solutions were prepared (2

Table 5. Significance of variations in K associated with different dye acetate solutions prepared from various dye stocks, by dye stock and concentration of ascorbic acid. Each K is based on three L_b values and three L_s values.

Source of Variation (Dye Acetate Solution = D.A.)	Mean K	Variation of K			Variance Ratio F	P
		D.F.	Mean Square	S.D.		
	1.0 mg. per cent concentration					
Dye Lot I						
Total	9.16	45	0.1435	.379		
Among D.A. Means		12	0.2057		1.70	>.05
Within D.A. Sol.—Days		33	0.1209	.348		
Dye Lot III						
Total	9.29	25	0.1693	.411		
Among D.A. Means		3	0.1669		1.02*	>.05
Within D.A. Sol.—Days		22	0.1696	.412		
Dye Lot V						
Total	9.35	12	0.1252	.354		
Among D.A. Means		2	0.1915		1.71	>.05
Within D.A. Sol.—Days		10	0.1120	.333		
Dye Lot VI						
Total	9.45	25	0.2873	.536		
Among D.A. Means		5	0.6411		3.47	.01–.05
Within D.A. Sol.—Days		20	0.1845	.430		
Dye Lot VII						
Total	9.70	11	0.4454	.667		
Among D.A. Means		2	0.2535		1.93*	>.05
Within D.A. Sol.—Days		9	0.4881	.699		
	1.5 mg. per cent concentration					
Dye Lot I						
Total	9.24	46	0.0822	.287		
Among D.A. Means		12	0.1435		2.37	.01–.05
Within D.A. Sol.—Days		34	0.0606	.246		
Dye Lot III						
Total	9.40	24	0.0986	.314		
Among D.A. Means		3	0.2417		3.09	.01–.05
Within D.A. Sol.—Days		21	0.0782	.280		
Dye Lot V						
Total	9.37	13	0.0530	.230		
Among D.A. Means		2	0.0436		1.25*	>.05
Within D.A. Sol.—Days		11	0.0547	.234		
Dye Lot VI						
Total	9.58	25	0.1773	.421		
Among D.A. Means		5	0.5651		7.90	<.01
Within D.A. Sol.—Days		20	0.0715	.267		
Dye Lot VII						
Total	9.70	11	0.2006	.448		
Among D.A. Means		2	0.0824		2.75*	>.05
Within D.A. Sol.—Days		9	0.2268	.476		
	2.0 mg. per cent concentration					
Dye Lot I						
Total	9.34	41	0.0444	.211		
Among D.A. Means		11	0.0884		3.13	<.01
Within D.A. Sol.—Days		30	0.0282	.168		
Dye Lot III						
Total	9.39	25	0.0507	.225		
Among D.A. Means		3	0.1468		3.91	.01–.05
Within D.A. Sol.—Days		22	0.0376	.194		
Dye Lot V						
Total	9.49	9	0.0260	.161		
Among D.A. Means		2	0.0350		1.49	>.05
Within D.A. Sol.—Days		7	0.0235	.153		
Dye Lot VI						
Total	9.59	24	0.1582	.398		
Among D.A. Means		5	0.4281		5.17	<.01
Within D.A. Sol.—Days		19	0.0828	.288		
Dye Lot VII						
Total	9.77	11	0.0927	.304		
Among D.A. Means		2	0.0750		1.29*	>.05
Within D.A. Sol.—Days		9	0.0967	.311		

* The greater mean square is that *within* dye acetate solutions.

degrees of freedom). When K was derived from solutions of 1.5 and 2.0 mg. per cent, the variance among dye solutions was significantly greater than that within solutions for each of the three dye lots from which four to twelve solutions had been prepared. In general, it can be said that there is a definite tendency for K values for different dye acetate solutions of the same dye lot to differ significantly.

It seemed, *a priori*, that the variance of K for days when the same dye solution was used should be approximately that which would result from the accidental errors of measurement of L_b and L_s, and of the concentration (C). Thus, the standard deviations shown in Table 5 for K *within* dye solutions should be similar to the estimates given in Table 4. However, in nearly every instance, the observed standard deviation within dye solutions was higher than the estimated expected standard deviation from accidental errors. The magnitude and consistency of a variation for K within dye acetate solutions in excess of that from measured accidental errors suggested that some other factor affected the K on different days.

The only new factor introduced in the determination of K on different days for which the same dye acetate solution was used was that of time. As previously mentioned, the dye blank values from day to day on the same dye acetate solution varied more than would be expected from the accidental error of L_b. The accidental error of L_b was found to be .0030, and the standard deviations for L_b within dye solutions for several dye stocks were as follows: Stock I, .0050; Stock III, .0063; Stock V, .0040; Stock VI, .0041; and Stock VII, .0047. In order to test whether K changed if the L_b changed for the same dye solution after several days of use, the K values obtained on the first and last day for the same dye acetate solution were compared and the difference in K was then related to the difference in L_b on the first and last day. The *differences* between K on the first and last day (last day – first day) for the same dye acetate solution are plotted in Figure 1, for twenty-five

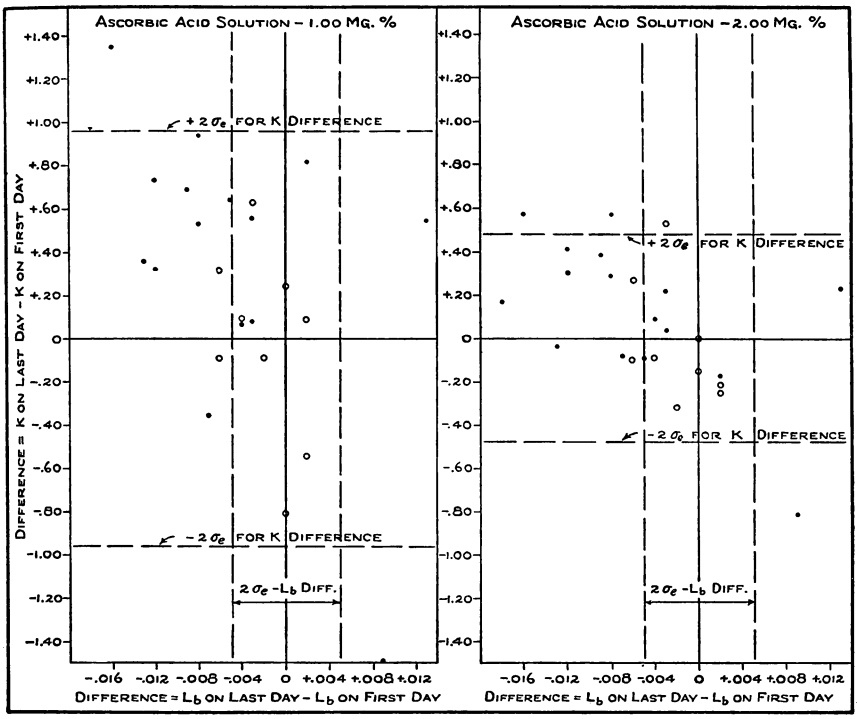

Fig. 1. Change in K values derived from same dye acetate solution after three or more days of use and the associated change in L value for dye solution. Approximate limits for twice the standard deviation expected on the basis of accidental errors of measurement are shown for a difference between two K values and for a difference between two L values for a dye blank.

dye acetate solutions and the associated difference between L_b on the first and last day also is shown. One dye solution was used for sixteen days; seventeen solutions were used from five to eight days, and the other seven were used only three or four days.

It is evident in Figure 1, that there was a definite tendency for K to increase after the dye solution stood for several days, and this increase obviously was correlated with a decrease in the L_b value.[9] If the differences in K were the result of random association of the accidental errors and the reaction of the dye blank to ascorbic acid

[9] Open circles in Figure 1, indicate K differences and L_b differences for the nine dye acetate solutions used to determine the percentage error of C. The differences in K shown for these solutions are not skewed toward high positive values and therefore do not seem to be affected by a change in the dye reaction to ascorbic acid.

had been constant regardless of its L value, we should expect about an equal number of plus and minus K differences, with all but one or two values within the limits of the dotted horizontal lines. Minus differences, with one exception, were less than twice the expected standard deviation of a difference,[10] that is, above the lower horizontal dotted line; but plus differences for K, both at 1.0 and 2.0 mg. concentration, were skewed toward the upper horizontal line and two differences were above the line for 1.0 mg. solutions and three were above the line for 2.0 mg. solutions. Although the change in K with time was not consistent for all dye acetate solutions, the data strongly suggest a tendency for the dye solution to diminish in density and to show a small change in the reaction to ascorbic acid when kept for a number of days.

It is outside the scope of this report to attempt any interpretation of this change in dye reaction to ascorbic acid after the dye solution stood for several days. Time alone may not have been the real cause. However, the change was a significant factor in increasing the variation in K values for some dye solutions. In order to eliminate this factor as a source of error in K, a K value derived for the specific day on which determinations are to be made seems essential.

Measurement of the reduction in density of a specific dye by a given amount of ascorbic acid is the critical value for determining a K which will furnish an accurate estimate of ascorbic acid in a plasma specimen. Therefore, the accuracy of the plasma determination depends on eliminating as far as possible the effect of sources of change in the K value. Different dye solutions have been found to react quite differently to ascorbic acid and even the same solution sometimes was not constant over a period of several days. From the

[10] The approximate expected standard deviation of a difference between two daily K values is estimated from the total accidental error of a daily K value shown in Table 4, and is $\sqrt{2}$ times σ_{e_K}.

The σ_{e_K} used was intermediate between those given for K equals 9.2 and 9.7. A more precise test of the significance of each difference based on data specific for each dye solution probably would show a greater number of differences to be larger than twice their standard deviation.

foregoing analysis, it is possible to generalize that, unless K is obtained for each day, large errors may be introduced into the plasma determinations.

Since dye solutions from the same dye stock frequently gave different K values, it is not surprising that different dye stocks were characterized by quite different K values. The mean K values at three concentrations for five different dye stocks are shown in Table 5. The range for mean K values on 1.00 mg. per cent solutions was 9.16 to 9.70; on 1.50 mg. per cent solutions was 9.24 to 9.70; and on 2.00 mg. per cent solutions 9.34 to 9.77. It is significant to note that changes in the mean K values with use of a different dye stock were approximately the same for K at each concentration. The variation among means for these different dye stocks is beyond the statistical limits of likelihood for random samples drawn from a homogeneous population of dye stocks. For some reason, these dye stocks did not react similarly to the addition of ascorbic acid, or, in other words, the reduction in dye density was not constant for a given amount of ascorbic acid. Furthermore, the variation in K values differed for these dye stocks and the reaction of some stocks apparently was much more variable than that of others.

Differences in K Derived from Different Concentrations. The mean K values for each dye stock derived from ascorbic acid solutions of three different concentrations, shown in Table 5, indicate a definite tendency for K to increase slightly as the concentration was increased. If K changes significantly at different concentrations, it obviously is important to know the magnitude of the difference in K for the range of concentrations to be expected for plasma or other materials being assayed. Present use of the method indicates acceptance of K as a constant value for all levels of ascorbic acid or as differing so slightly as to have no important effect on the accuracy of the determination.

The mean K values for the entire routine series are given in

Concentration of Ascorbic Acid in Prepared Solution	Routine Laboratory Series		Initial Series	
	Number of Days	Mean K	Number of Days	Mean K
0.10 Mg. Per Cent			20	10.02
0.30 Mg. Per Cent			20	9.38
0.50 Mg. Per Cent			20	9.35
0.75 Mg. Per Cent			20	9.60
1.00 Mg. Per Cent	127	9.31	20	9.56
1.50 Mg. Per Cent	127	9.40	20	9.62
2.00 Mg. Per Cent	127	9.45	20	9.67

Table 6. Comparison of mean K values derived from prepared ascorbic acid solutions of different concentrations.

Table 6 for K at 2.0 mg., K at 1.5 mg., and K at 1.0 mg. per cent. From these means, differences between any two levels are readily obtained. The method used to test whether the difference between the mean K values derived from two solutions of different concentrations is statistically significant was to obtain the standard error of this difference from the differences between K values for two concentrations on the same day. The two values compared are based on the same dye blank and the concentrations are from the same solution of weighed crystalline ascorbic acid, although a slight error for separate additional dilution is involved, and the differences, therefore, are not affected by the various sources of large variability in K which have been discussed. Each of the mean differences between K values at two concentration levels is significant statistically for the routine series. For concentration 2.0 mg. per cent, the K value was, on the average, 0.14 higher than for concentration 1.0 mg. per cent. In view of the large amount of data on which this finding is based, it can be concluded that K is not constant at all levels of ascorbic acid and that the use of K derived from one concentration, or the average of several concentrations, introduces a constant error in specimen determinations with concentrations differing from that on which K was derived. It is important, therefore, to determine the magnitude of the differences between the true K values for various concentrations, especially between

the low concentrations of interest in plasma determinations and higher concentrations customarily used for deriving K.

From the initial experimental data, previously mentioned, a relatively small amount of data are available for concentrations of 0.1 mg., 0.3 mg., 0.5 mg., and 0.75 mg. per cent, in addition to 1.0, 1.5, and 2.0 mg. per cent. Two determinations on each level were made on twenty days, and from these data, the mean K values at the various levels are given in Table 6. The K values for the seven concentrations show a definite tendency to increase as the ascorbic acid concentration is increased, with the exception that on 0.1 mg. per 100 cc. the K value is highest and therefore entirely out of line. At 0.75, 1.00, and 1.50 mg. per cent in this initial series, the K values do not differ significantly; but they are very significantly higher than those at 0.3 and 0.5 mg. per 100 cc. and at 1.5 mg. per cent, K is significantly lower than at 2.0 mg. per 100 cc. These data show the same trend in differences as that found for the routine daily records, but the difference between K at 1.0 and at 1.50 mg. is somewhat greater for the routine series than in the initial series. At 0.1 mg., the variability of K from accidental error of L_s is very large and the likelihood of a high percentage error in the solution is great; therefore, a larger number of carefully made observations is needed to obtain an accurate K at this level. For the remainder of the range of ascorbic acid solutions, these data strongly support a conclusion that a constant K relationship between the measured reduction of dye density and the amount of ascorbic acid does not hold precisely for all levels of ascorbic acid concentration.

The practical importance of the constant bias in the determination of ascorbic acid in a specimen with ascorbic acid content different from that of the solutions from which K was derived depends on the magnitude of the error introduced and the accuracy desired. The maximum difference in mean K values was 0.32 for 0.5 mg. and 2.0 mg. solutions. This is about 3 per cent of the K value and a K derived from 2.0 mg. solutions would overestimate

ascorbic acid content at 0.5 mg. by this per cent, or .015 mg. An error of this size, on the average, is not important in routine determinations. For some experimental work, any overestimate or underestimate in a determination may have significance as, for example, in comparison of determinations by two different methods.

The differences in K derived from solutions with different concentrations have a bearing on the choice of which concentrations furnish the best K for plasma determinations. The bias of overestimation of low plasma values if K from high concentrations is used is small in absolute amounts of ascorbic acid and this error is more than offset by the much smaller accidental error of K at high levels. Furthermore, K from high concentrations results in a conservative estimate of low plasma values.

Summary Discussion of K as a Source of Error in Plasma Determination

The foregoing analysis of sources of variation in K values has provided evidence that, for the greatest accuracy, K should be determined each day. Data presented have shown that the mean K values derived from five different dye stocks varied from 9.16 to 9.77; and the characteristic K for any stock must be determined. Furthermore, for different dye acetate solutions from the same dye stock, the mean K values frequently varied more than would be expected if they were means of homogeneous samples affected only by the factors which cause variability in K when the same dye acetate solution is used for several days. Finally, even when the same dye acetate solution was used, the K values obtained for different days showed a tendency for K to be significantly higher after the dye solution had been used for several days than it was on the day on which a fresh dye solution was prepared. These sources of variation in K, namely, different dye stocks, different dye acetate solutions, and the effect of time on a dye solution, can be controlled if K is determined each day from prepared solutions of ascorbic acid.

For a K value determined for a dye solution at a given time and from a single ascorbic acid solution, four sources of error have been discussed, as follows:

1. Dye blank reading, L_b. The standard deviation of a single observation on the dye blank was found to be, in L values, 0.00300.

2. Dye density reading, L_s, after addition of ascorbic acid solution to dye blank. The error in L_s readings varied somewhat for different concentrations; at 1.00 mg. per cent, the standard deviation of L_s for a single observation was 0.00264; at 1.50 mg. per cent, it was 0.00245; and at 2.00 mg. per cent, it was 0.00233.

3. Percentage error in ascorbic acid solutions. The estimated error in the concentration of prepared solutions, C, is 2.94 per cent for 1.00 mg. solutions, 1.90 per cent for 1.50 mg. solutions, and 1.46 per cent for 2.00 mg. solutions.

4. Differences between K values derived from solutions with different concentrations. K values were highest if derived from 2.00 mg. per cent solutions and consistently decreased as the concentration was reduced. K at 2.00 mg. was, on the average, 0.14 higher than at 1.00 mg. Insufficient data are available for reliable estimates at lower concentrations, but K continued to decrease.

The first three sources of error contribute to a total accidental error of K on any one day for a specific concentration. The fourth source of error is effective only as K for a specific concentration is applied in determinations for specimens with a different concentration.

The standard deviation of K from all accidental sources for one day decreases as the concentration from which it is derived increases and it increases slightly as the K value increases. With three readings on the dye blank and three readings on the measurement of dye reduction by the ascorbic acid solution, the error of measurement of K with a value 9.2 is estimated to be approximately 0.334 on solutions of 1.00 mg. per cent, 0.216 on solutions of 1.50 mg., and 0.163 on solutions of 2.00 mg. per cent. About two-thirds of the variance, or square of this standard error of K, is contributed by the error in preparing the solutions of ascorbic acid.

The use of an average K derived each day from several solutions with different concentrations does *not* reduce the error of K for several reasons. In the first place, if each solution is a dilution of the same solution of a single weighed amount of crystalline ascorbic acid, as it was in this Study, error in the master solution is carried over into the dilutions and is not averaged. By the use of two or more independently prepared master solutions for one day, the error of K could be reduced; but this does not seem necessary since the error is not large. In the second place, the accidental error of K increases as the concentration is reduced, and the average error of K from several concentrations will be intermediate between that for the highest concentration and that for the lowest concentration. Finally, since K is not constant at each concentration, the mean K from several concentrations is accurate only for some specific intermediate concentration, approximately the average of the concentrations used. Furthermore, the observed variability of the K values from which the mean is derived does not afford a measure of the true error of the mean K.

The true error of a mean K based on several ascorbic acid solutions of different concentrations is the mean of the variances of the measurement errors at each concentration. This eliminates the effect of differences between K values for different concentrations. The standard error of mean K when K is determined at concentrations of 1.00 mg., 1.50 mg., and 2.00 mg. is shown below, and this error is compared with that for mean K derived only on the two higher concentrations. Since K differs slightly at each concentration, L_d also is not exactly proportional to the difference in concentrations used. With adjustment for the mean trend in K with the concentration and n equals 3 for L_b and for L_s, the standard error of mean K becomes:[11]

$$\text{If } C = 1.00 \text{ and } K = 9.31;\ \sigma_{e_K} = 9.31 \times .0364 = 0.339$$

[11] The calculation is from the formula on p. 190 for σ_{e_K}. The L_d values at each concentration are given in Table 7.

If C = 1.50 and K = 9.40; $\sigma_{e_K} = 9.40 \times .0236 = 0.222$
If C = 2.00 and K = 9.45; $\sigma_{e_K} = 9.45 \times .0179 = 0.169$
Mean K centering at about 1.50, $\sigma_{e_K} = 9.39 \times .0271 = 0.254$
Mean K centering at about 1.75, $\sigma_{e_K} = 9.425 \times .0210 = 0.198$

Thus, the standard error of mean K for one day from accidental sources is 0.254 if solutions of 1.00, 1.50, and 2.00 mg. per cent are used, and it is 0.198 if the two higher solutions are used. The percentage error in K derived from solutions with 2.00 mg. per 100 cc. was only half as great as that for solutions of 1.00 mg., and the standard error of a mean K based only on solutions of 1.50 and 2.00 mg. per cent is appreciably lower than that for a mean K including the 1.00 mg. solution.

Error in Determination of Ascorbic Acid in Plasma

The concentration of ascorbic acid in the plasma filtrate is equal to K ($L_b - L_s$), where L_b is the L value for the dye blank and L_s is the L value for the galvanometer reading of the dye solution with filtrate added. The error of the determined concentration in the filtrate is that attributable to the error of K plus that due to the error of measurement of reduction in dye density ($L_b - L_s$) by the plasma filtrate. The standard deviation of a determined concentration of ascorbic acid in plasma which is expected from total errors of measurement[12] is given in Table 7. The only new value needed for estimating the error of a plasma concentration (C_2) is the accidental error of L_s for filtrates. Data are available for duplicate readings on plasma filtrates from which an estimate of the standard deviation of a single observation was made. The standard deviations of L_s at four levels for plasma are shown in Table 7. The error of measurement of L_s when plasma filtrate is added to the dye is somewhat higher than the error of measurement of L_s when prepared solutions are used.

[12] The standard deviation for the ascorbic acid concentration in plasma, C_2, derives from

(Continued on page 204)

Concentration True C to be Determined Mg. Per 100 cc.	K Value For Specific C	L Difference Expected for C	Error of Single Galvanometer Reading on Dye + Filtrate σ_{e_S}	C Mean Value Obtained for K = 9.425	Percentage Error of C	Standard Deviation of C Value, About Mean C
0.40	9.15[1]	.0437	.0038[2]	.412	.097	.040
1.00	9.31	.1074	.0029[3]	1.012	.035	.035
1.50	9.40	.1596	.0027[4]	1.504	.026	.040
2.00	9.45	.2116	.0026[4]	1.994	.022	.044

[1] Estimated from difference in K values in initial series between mean at 0.30 and 0.50 mg. per cent and 1.50 mg. per cent; other K values from routine data.
[2] From fifty-one pairs of duplicate observations on plasma with C of .20–.59 mg. per cent.
[3] From thirty-seven pairs of duplicate observations on plasma with C of .80–1.19 mg. per cent.
[4] Estimated as 20 per cent higher variance than for prepared ascorbic acid solutions (Table 2) on basis of ratio at 1.00 mg. per cent.

Table 7. Reliability of concentration values of ascorbic acid in plasma determined on the Evelyn colorimeter by the macromethod with K derived from solutions of 1.50 mg. and 2.00 mg. per cent and based on three galvanometer readings for the dye blank and for each solution and one reading on the dye filtrate.

The error estimated for a determination of ascorbic acid in plasma is given in Table 7 for concentrations of 0.40 mg. per cent, 1.00 mg., 1.50 mg., and 2.00 mg. The error is that to be expected when K is derived from standard solutions of 1.50 mg. and 2.00 mg. per cent, or an average concentration of 1.75 mg. per cent. If lower concentrations are used for K, the error will be larger. The standard deviations for the determined ascorbic acid content indicate the expected variability of determinations for plasmas with equal ascorbic acid content, but this is the variability around a mean

both a product and a quotient value, as is seen if $K(L_b - L_s)$ is expanded for the K factor to read:

$$C_2 = \frac{C_1}{Lb_1 - Ls_1}(Lb_1 - Ls_2)$$

where C_1 is the concentration of the standard solution used. The standard deviation for the product-quotient value (C_2) was obtained from the formulae for a product and a quotient (3) and was adjusted for an approximate correlation value that results from a constant L_b value for the standard solution and the plasma specimen. The correlation reduces the standard deviation very little because of the relatively large influence of the standard deviation of C_1 and of L_{s_2}.

value which is somewhat too high for concentrations below the concentration at which K centers and is somewhat too low at higher concentrations. The mean concentration about which this variability occurs is also shown in Table 7.

The standard deviation of a plasma determination at a level of 1.5 mg. per 100 cc. is shown to be .040 mg. per 100 cc. and is 2.6 per cent of the determined amount, if one reading is taken on dye plus filtrate. As the K used centers at 1.75 mg. per cent, the mean value of a series of determinations is 1.504, and in about 95 per cent of determinations the derived values would be between 1.584 mg. per cent and 1.424 mg. per cent. This is shown in Figure 2.

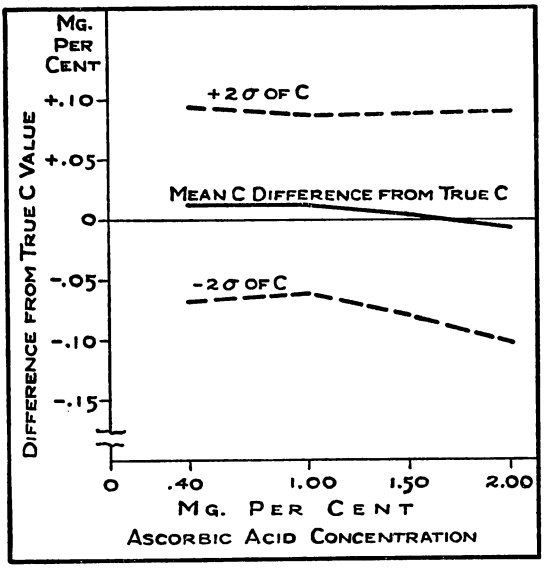

Fig. 2. Variation of ascorbic acid values for plasmas of four different concentrations to be expected from all errors of measurement about the mean difference from the true concentration when K is derived from solutions of 1.50 mg. and 2.00 mg. per cent.

The standard deviation of the ascorbic acid determination on plasma with a concentration as low as 0.40 mg. per cent is estimated as .040, and is the same as the standard deviation at 1.50 mg. per cent. Thus, although the percentage error increases steadily as the concentration decreases, the absolute error changes very little. If allowance is made for the mean overestimate at 0.40 mg. per cent, when K is at 1.75 mg., the probability is 95 in 100 that the determined value for a true value of 0.40 will be between 0.332 and 0.492 mg. per cent, and the mean of a series of determinations will be about 0.412 mg. per cent.

The errors estimated here for plasma determinations are, of course, those found for a single colorimeter and a specific group of laboratory workers. They do not represent the minimum error possible, but it is believed they are typical of what should be expected in routine work in a busy laboratory. The tendency for K to increase slightly at higher concentrations introduces a bias which is of little significance for diagnostic determinations, and the greater reliability of K derived from concentrations above 1.00 mg. per cent improves the accuracy of determinations. The major source of error in low plasma values is that associated with the accidental error in the measurement of dye reduction, and if greater accuracy is desired, independent readings should be made on two or three aliquots of the filtrate.

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