MEDICAL EVALUATION OF NUTRITIONAL STATUS[1]

XIII. THE EXPERIMENTAL ERROR OF DETERMINATIONS OF ASCORBIC ACID IN PLASMA BY MICROMETHOD OF MINDLIN AND BUTLER

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INCREASING interest in the ascorbic acid content of blood plasma has raised the question of the accuracy of single determinations obtained according to the widely employed colorimetric procedures of Mindlin and Butler (1). A previous paper (2) from the Medical Evaluation of Nutritional Status Study, undertaken jointly by the New York City Department of Health, the United States Public Health Service, Cornell University Medical College, and the Milbank Memorial Fund, discussed the sources of experimental error in the *macromethod* of Mindlin and Butler and estimated their aggregate effect upon a single determination of the ascorbic acid content of plasma. The present report does this for the *micromethod*. The observations were made in the Study laboratories in order to provide a basis for estimating the reliability of a single determination of the ascorbic acid content of plasma. The possibility of *systematic* error is discussed briefly at the end of the paper.

Both the micromethod and the macromethod are based upon the same principles of physical chemistry, and these are discussed by Mindlin and Butler in their original communication (1). The reduction of 2.6 dichlorophenol-indophenol by ascorbic acid is objectively measured by means of the photoelectric micro-colorimeter.

[1] This paper is the thirteenth 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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The ascorbic acid content of a sample of plasma may be calculated from the expression

$$(1)\quad C = K(\log G_s - \log G_b)$$

where G_s is the galvanometer reading on the dye-acetate blank plus plasma filtrate, and G_b is a parallel reading on the dye-acetate blank. The factor K is the ratio of a standard concentration of ascorbic acid in metaphosphoric acid to the difference between the logs of the galvanometer readings on the dye blank plus the standard solution and on the dye blank. It is obtained by solving equation (1) when G_s is the galvanometer reading on the dye-acetate blank plus the standard solution of strength C.

For purposes of statistical analysis it is convenient to rewrite equation (1) as

$$(2)\quad C_p = \frac{C_s(L_b - L_p)}{(L_b - L_s)} = t = \frac{X_1 \cdot X_2}{X_3}$$

where

$C_p = t$ = estimated ascorbic acid content of the plasma, *e.g.*, .75 mg. per cent;

$C_s = X_1$ = standard concentration of ascorbic acid in metaphosphoric acid added to dye-acetate blank, and stated as mg. per cent;

$(L_b - L_p) = X_2$ = difference between logs of galvanometer readings on dye blank and dye blank plus plasma filtrate, where L_b may be the mean of several independent readings, and any L reading is $\log \left(\frac{100}{G}\right)$ or $2 - \log G$; and

$(L_b - L_s) = X_3$ = difference between logs of galvanometer readings on the dye blank and on the dye blank plus the standard solution, and where $(L_b - L_s)$ may be the difference between the means of several independent readings. An L reading, again, is $2 - \log G$.

Each element in the above series is subject to one or more kinds of experimental error. If the magnitude of each can be estimated, the resulting information can be combined so as to provide an estimate of the error of a single plasma determination.

SOURCES OF EXPERIMENTAL ERROR AND THEIR ESTIMATION

The major opportunities for error arise from

(1) the operations involved in preparing the solutions, especially the standard ascorbic acid solutions;

(2) the manipulation of the colorimeter and the galvanometer;

(3) whatever physical and chemical differences or changes there may be in the properties of the dye-stocks, dye solutions, etc.;

(4) the arbitrary standard concentration of ascorbic acid employed in finding K, as shown by Wiehl and Kantorovitz (2).

From multiple, independent galvanometer readings (L_b, L_s, and L_p in 2-log form) on the same day with a given dye-acetate solution, estimates of the error of each of these three factors and of the differences ($L_b - L_p$) and ($L_b - L_s$) may be found as the average within-the-day variances computed from a considerable number of replicate experiments done over a period of time, with different dye-stocks, and with many samples of plasma filtrate. The error made in preparing standard ascorbic acid solutions (error of C_s, or S_1^2) must, however, be estimated indirectly. Wiehl and Kantorovitz (2) showed that the error of K among days, for a given C_s level, should be calculable from the formula for the variance of a ratio as

$$(3) \quad S_K^2 = S^2_{\left(\frac{x_1}{x_3}\right)} = \left(\frac{\overline{X}_1}{\overline{X}_3}\right)^2 \left[\frac{S_1^2}{\overline{X}_1^2} + \frac{S_3^2}{\overline{X}_3^2} - 2r\frac{S_1 S_3}{\overline{X}_1 \overline{X}_3}\right]$$

where the variances are the error variances about the means $\overline{X}_1$ (the value of C_s, and $\overline{X}_3$ (the average value of $L_b - L_s$). The term involving the correlation coefficient vanishes under the assumption that the error made in preparing C_s solutions is independent of the error of $L_b - L_s$. It is further assumed that the error of K among days depends upon the error of C_s and upon the experimental error of the difference ($L_b - L_s$), the latter being best approximated as the within-the-day variance of replications. The

among-days variance of ($L_b - L_s$) is increased by the errors of C_s and is thus a poor estimate of S_3^2. Then the error of C_s is found by solving equation (3), shorn of the third term within the brackets, for S_1^2. Should it be found that the error of C_s depends upon the C_s level itself, a set of estimates could be obtained which would be appropriate to the different C_s levels. Finally, from estimated values of $\overline{X}_1$, $\overline{X}_2$, $\overline{X}_3$, S_1^2, S_2^2, and S_3^2, and from known formulas for the variance of a product and of a ratio (3), the error variance of C_p may be computed from the formula

$$(4)\quad S_{c_p}^2 = S_t^2 = \left(\frac{\overline{X}_1 \cdot \overline{X}_2}{\overline{X}_3}\right)^2 \left[\frac{S_1^2}{\overline{X}_1^2} + \frac{S_2^2}{\overline{X}_2^2} + \frac{S_3^2}{\overline{X}_3^2} + \frac{S_1^2 S_2^2}{\overline{X}_1^2 \overline{X}_2^2} - 2rS_3\sqrt{S_1^2\overline{X}_2^2 + S_2^2\overline{X}_1^2 + S_1^2 S_2^2}\right]$$

where r is the coefficient of correlation between X_3 and the product $X_1 \cdot X_2$. It can be shown that, for this particular problem, no matter what value r may take within its range $-1 \leq r \leq 1$, the term in which it appears must be negligible in size.

The Laboratory Observations

The Study laboratory provided three sets of observations:

(1) *The first test series.* On each of six days, independent, triplicate readings were made for L_b and also for L_s using separate aliquots of .1, .3, .5, .75, 1.0, 1.5, and 2.0 mg. per cent standard solutions (fresh daily) of ascorbic acid which were added to a single dye-acetate solution which itself was unchanged over the six-day period. Two or three independent observations were also made on six different dye blank plus plasma filtrate samples on two different days.

(2) *The second test series.* On each of five days quadruplicate galvanometer readings were taken for L_b and also for L_s using .1, .3, .5, .75, 1.0, 1.5, and 2.0 mg. per cent standard solutions of ascorbic acid added to a second dye-acetate solution prepared from a second dye-stock. No blood samples were studied.

(3) *The third test series.* On each of five to nine days triplicate

(rarely, duplicate) observations were made on L_b and also on L_s using 1.25 and 1.88 mg. per cent standard solutions of ascorbic acid added to a single dye-acetate solution from a single stock, and for four different dye-stocks. On most days duplicate (rarely, multiple) L_p readings were taken on many dye blank plus plasma filtrate samples.

A. For dye-stock dated 10/10, for 13 plasma samples, and for seven work-days during a nine-day interval.

B. For dye-stock dated 2/19, for 82 plasma samples, and for nine work-days during a fourteen-day interval.

C. For dye-stock dated 3/11, for 68 plasma samples, and for six work-days during a twelve-day interval.

D. For dye-stock dated 4/1, for 51 plasma samples, and for five work-days during a five-day interval.

A small amount of information pertaining to the work of one or two days on each of several different dye-acetate solutions was sacrificed in view of the marked variation among dye-stocks found by Wiehl and Kantorovitz. Only runs of five or more days on a single dye-acetate solution, therefore, are included in the statistical analysis which follows, and each run of observations was first treated as a separate set of data. Throughout the statistical analysis to be described the observations were first processed as single determinations, and account of replication was taken later by dividing the error variances of single readings by the appropriate constants.

Experimental Error of Individual Laboratory Operations

Reading on Dye Blank (L_b). The error of L_b may be gauged from the six independent estimates of variance which appear in Table 1. Each estimate gives the experimental error of a single L_b reading and was found as the within-the-day variance of replicated (usually triplicated) readings. The estimate of 0 for series III-D is derived from only five pairs of duplicates and represents an almost

negligible amount of information. It has been omitted in what follows because the approximate test for homogeneity which was adopted (4) makes use of log S^2 and log 0 is meaningless. As a set the five remaining individual estimates differ only insignificantly (.50 < P < .70) and have been combined to yield the average variance of 108.775 x 10^{-8}* which appears on the total line of the table. For subsequent computations, therefore, 108.775 x 10^{-8} will serve as the best available estimate of the error variance of L_b. Hence, one-third of this quantity, or 36.258 x 10^{-8}, is the estimated error of L_b found as the mean of three independent readings upon the same dye-blank.

Table 1. Summary of information on the error of a single galvanometer reading upon a dye blank, in log units.

Series	Degrees of Freedom (n)	Error Variance[1] (S^2 x 10^8)
I	12	68.72
II	15	76.07
III-A	13	121.33
III-B	16	144.81
III-C	10	131.92
III-D	4	0
ALL[2]	66	108.775

[1] Computed as average within-the-day variance for replicated readings, since the much larger variance among days reflects not only the error made in taking a galvanometer reading, but also any changes in the reaction of the dye with time or changes in the calibration of the instruments.

[2] Omitting III-D. The value of S^2 was found as: $\frac{n_1S_1^2+n_2S_2^2+\ldots+n_5S_5^2}{n_1+n_2+\ldots+n_5}$.
For this set of variances $\chi^2=2.86$, and .70> P> .50.

Reading on Dye Blank Plus Plasma (L_p). The error of L_p, the reading on the dye blank plus the plasma filtrate, is found almost as readily. In the expectation that the error of L_p might depend upon the concentration of ascorbic acid in the plasma, three levels were recognized. These are: under .50, .50 to .99, and 1.00 or more mg. ascorbic acid per 100 cc. of plasma. Table 2 presents the available information on the magnitude of this error. For series I and III-A it is the within variance for triplicate or quadruplicate readings which is tabled. For the other series the error variance has been estimated as one-half the variance of the difference between

* Powers of 10 are employed to avoid unwieldy numbers. The reader will recall that multiplication by 10^{-2}, for example, amounts to division by 100, or to moving the decimal point two places to the left. Thus 108 x 10^{-8} is equivalent to .000 001 08.

independent duplicates.[2] At the lowest ascorbic acid level there is evidence of heterogeneity, the value of 726 being well in excess of those of 118 and 117, but the estimates within each of the other two levels are reasonably homogeneous. More important, however, is the absence of any suggestion that the error of L_p depends upon the ascorbic acid level. A weighted average variance of all twelve estimates, therefore, has been computed directly in the usual fashion and appears on the total line of Table 2. The value 246.300×10^{-8} is well above the error variance of 108.775×10^{-8} estimated for single L_b readings. This result is entirely expected, for the handling of micro quantities of plasma in addition to the dye should introduce new errors. Whenever an estimate of the error of a single L_p reading is needed, therefore, use will be made of the figure 246.300×10^{-8}.

Reading on Dye Blank Plus Standard Solution (L_s). In similar fashion Table 3 summarizes the various estimates of the error of a single galvanometer reading on a dye-acetate blank to which a standard concentration of ascorbic acid has been added. Although some of the paired comparisons from series III differ significantly, there is no convincing evidence that the error of L_s depends upon the strength of the ascorbic acid solution added to the dye.[3]

[2] Given a series of duplicate observations on X,

$$X'_1, X'_2, \ldots X'_n \text{ and } X''_1, X''_2, \ldots X''_n,$$

where $X'_i = X_i + e'_i$ and $X''_i = X_i + e''_i$,

the difference $d_i = X'_i - X''_i = e'_i - e''_i$

and the variance of the d's may be written

$$S^2_d = S^2_{e'} + S^2_{e''} - 2rS_{e'}S_{e''}$$

where r is the coefficient of correlation between the errors e′ and e″. Since e′ and e″ may be assumed to be independent when the duplicate observations are independent, and since $S^2_{e'}$ and $S^2_{e''}$ are both equally good estimates of S^2_e, the error of X,

$$S^2_e = \tfrac{1}{2} S^2_d.$$

[3] The information at each level of ascorbic acid solution was pooled to provide an estimate of error at each of the 9 levels. As standard deviations multiplied by 10^4 these es-

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SERIES	DEGREES OF FREEDOM (n)	ERROR VARIANCE (S^2 x 10^8)
Plasma Ascorbic Acid Under .50 Mg. Per Cent		
III-A[1]	17	726
III-C[2]	24	118
III-D[2]	22	117
Plasma Ascorbic Acid .50–.99 Mg. Per Cent		
III-A[1]	12	241
III-B[2]	32	123
III-C[2]	23	279
III-D[2]	14	466
Plasma Ascorbic Acid 1.0 or More Mg. Per Cent		
III-A[1]	6	614
III-B[2]	48	197
III-C[2]	18	273
III-D[2]	12	94
Undifferentiated Plasma Ascorbic Acid Content		
I[1]	9	189
ALL	237	246.300

[1] Computed as within-the-day variance among independent triplicates or quadruplicates done on the same day.
[2] Computed as ½ the variance of the difference between independent duplicates done on the same day.

Table 2. Summary of data on the error of a single galvanometer reading on a dye blank plus plasma filtrate, in log units, by dye-stock, and by ascorbic acid content of plasma.

The average level in series I, the first work with the micromethod which was done in the Study laboratory, is higher than in series II and III. Series II should represent optimum performance for the Study laboratory. The values for the four series which provide the bulk of the information on L_p lie between those for series I and II, in the main, and average 134.72 x 10^{-8}. If all the information of Table 3 is pooled, the average variance is 158.389 x 10^{-8}. Despite the evidence of heterogeneity among estimates in Table 3, this average variance is considered to be the best single estimate which the data

timates are 8.9, 10.3, 10.0, 16.3, 12.1, 11.3, 15.1, 11.9, and 15.1 for the nine levels from .10 to 2.00 per cent. The regression coefficient is 2.22, and t=1.89, which is well within the range of chance variation for 7 degrees of freedom.

Strength of Standard Ascorbic Acid Solution Mg. Per Cent	Degrees of Freedom (n)	Error Variance[1] (S^2 x 10^8)	Degrees of Freedom (n)	Error Variance[1] (S^2 x 10^8)
	Series I		Series II	
.10	10	124	15	51
.30	12	138	15	82
.50	12	82	15	115
.75	12	399	15	158
1.00	12	156	15	137
1.50	15	299	15	159
2.00	15	369	15	88
All	88	233.73	105	112.83
	χ^2=12.65, n=6, P=.05		χ^2=6.95, n=6, .30 <P <.50	
	Series III-A		Series III-B	
1.25	10	124	15	89
1.88	12	118	14	245
	F=1.05, P> .05		F=2.76, P <.05	
	Series III-C		Series III-D	
1.25	8	221	5	101
1.88	9	80	5	17
	F=2.76, P> .05		F=6.00, P <.05	

[1] Computed as within-the-day variance for replicated independent readings.

Table 3. Summary of data on error of a single galvanometer reading on a dye blank plus a standard solution of ascorbic acid, in log units, by strength of standard solution, and by dye-stock.

afford[4]. It compares with 108.775 x 10^{-8} for L_b and 246.300 x 10^{-8} for L_p.

Differences $(L_b - L_p)$ *and* $(L_b - L_s)$. From these three variances may be derived the error variances of X_2 or $(L_b - L_p)$ and X_3 or $(L_b - L_s)$, assuming L_b and L_s to be triplicated, and the errors in readings on L_b and L_p, and on L_b and L_s, to be independent in the statistical sense. Thus, S_2^2, the error variance of X_2, may be found

[4] High, low, and medium estimates might be used, but they would all be of the same general order of magnitude, 100 x 10^{-8} to 200 x 10^{-8}.

as $[1/3\ (108.775) + (246.300)] \times 10^{-8}$ or 282.558×10^{-8}. If L_p were duplicated, and the mean of the two independent duplicates taken as the L_p reading, the error variance of $(L_b - L_p)$ would be reduced to 159.408×10^{-8}. Similarly, the error variance S_3^2 may be found as $1/3\ (108.775 + 158.389) \times 10^{-8}$, or 89.054×10^{-8}.

In order to test the assumption that the individual errors are uncorrelated and thus the advisability of adding the separate error variances to estimate the error variance of a difference, a special study was made of the error of $(L_b - L_s)$. In each series the daily triplicate readings of L_b were paired randomly with the triplicate readings on that day for L_s for a particular standard ascorbic acid concentration, *e.g.*, one per cent, and the three independent differences $(L_b - L_s)$ were found. Thus, a series of six days furnishes eighteen $(L_b - L_s)$ differences for a given standard concentration of ascorbic acid. Their variance within days, accordingly, constitutes an estimate of the experimental error of $(L_b - L_s)$ for single readings. A series having L_s readings at six C_s levels provides six different estimates of the error variance of $(L_b - L_s)$[5]. For comparison, the within variance of L_b for that series may be added to the within variance of L_s for the particular standard concentration of ascorbic acid to yield another estimate of the error variance of $(L_b - L_s)$. The laboratory observations permit twenty-two such comparisons. In every instance the F-ratio of the two variances is insignificant and close to unity. Eleven of the estimates based on sums exceed those found directly, nine fall below, and two are identical because the within error of L_b was zero. Hence, the assumption of zero correlation between the errors of corresponding duplicates is amply supported for $(L_b - L_s)$ and may likewise be extended to the parallel $(L_b - L_p)$ difference.

Standard Concentration (C_s). The error variance of C_s, or S_1^2, may be found indirectly by the device described by Wiehl and

[5] Since, however, they all share common L_b values, they may not be considered independent estimates.

Kantorovitz (2). It will be recalled that C_s is the standard ascorbic acid concentration (in metaphosphoric acid) added to the dye-acetate blank. The standard solutions were freshly prepared each day, and it is most reasonable to regard the errors involved in their preparation as normally distributed about zero means. Since the same C_s solution was used for all experiments on a given day, any error in C_s carried through the day without affecting the variation among replicate readings. Only the variation among days is increased by the error of C_s. Wiehl and Kantorovitz suggested that the error of K, where

$$K = \frac{C_s}{L_b - L_s} = \frac{X_1}{X_3},$$

should be calculated directly both within and among days for a single dye-acetate solution and standard ascorbic acid concentration. Then, since there is no error of C_s within days, it should be possible to compute the error of K within days from estimates of the error of $(L_b - L_s)$, using formula (3) above. This equation reduces to

$$(5)\quad S_K^2 = \left[\frac{\overline{X}_1}{\overline{X}_3}\right]^2 \cdot \left[\frac{S_3}{\overline{X}_3}\right]^2$$

for $r = 0$, $S_1 = 0$.

Such estimates of the within-the-day error of K are, in fact, in close agreement with those obtained directly from the series of random $(L_b - L_s)$ differences mentioned above. Moreover, from the study of duplicate L_p readings on blood samples, many of which were done on a single day, it is clear that the experimental error of L_p readings among days is of the same order as that within days. A similar conclusion should follow for the L_b and L_s readings. Hence, it seems entirely fair to employ the within-the-day variance of $(L_b - L_s)$ as the error variance of $(L_b - L_s)$ among days, freed of

any influence which errors in C_s might have upon it if it were taken as the among-days variance.[6] Then the variance of K among days may also be written as the variance of a ratio, or

$$(6)\quad S_K^2 = \left[\frac{\overline{X}_1}{\overline{X}_3}\right]^2 \cdot \left[\frac{S_1^2}{\overline{X}_1^2} + \frac{S_3^2}{\overline{X}_3^2}\right]$$

and S_1^2 may be found by substituting for all other factors and solving directly. Formula (6) is, of course, the same as formula (3) except for the omission of the third term within the brackets, which may be assumed to vanish. By this method twenty-two estimates of the error of C_s were derived for the nine different C_s levels. Because each estimate is based upon rather few degrees of freedom, the twenty-two estimates are highly variable but suggestive of a marked relationship to the C_s level. When all the information pertaining to each C_s level is combined,[7] regression analysis reveals a highly significant trend of error with increasing C_s. Figure 1 shows the nine error estimates as standard deviations and the regression line

$$Y = .03718 + .03939\ X$$

which fits the points rather well. From the regression equation, therefore, it is possible to obtain S_1^2 appropriate to the strength of any standard solution employed in the laboratory. Such estimates appear in Table 4. The relative error of 41 per cent for .10 per cent solutions may seem high, but the corresponding standard deviation of .041 is less striking and represents a degree of accuracy within the range of toleration. All these estimates are, however, well above those of 1.5 to 3 per cent reported by Wiehl and Kantorovitz for the macromethod. The increase in error is one price of the economy of plasma which the micromethod makes possible.

[6] Errors in C_s should materially affect the difference ($L_b - L_s$) among days, and analysis shows that the variance of ($L_b - L_s$) among days usually exceeds that within days by a significant margin.

[7] The two or four estimates at each C_s level do not differ significantly.

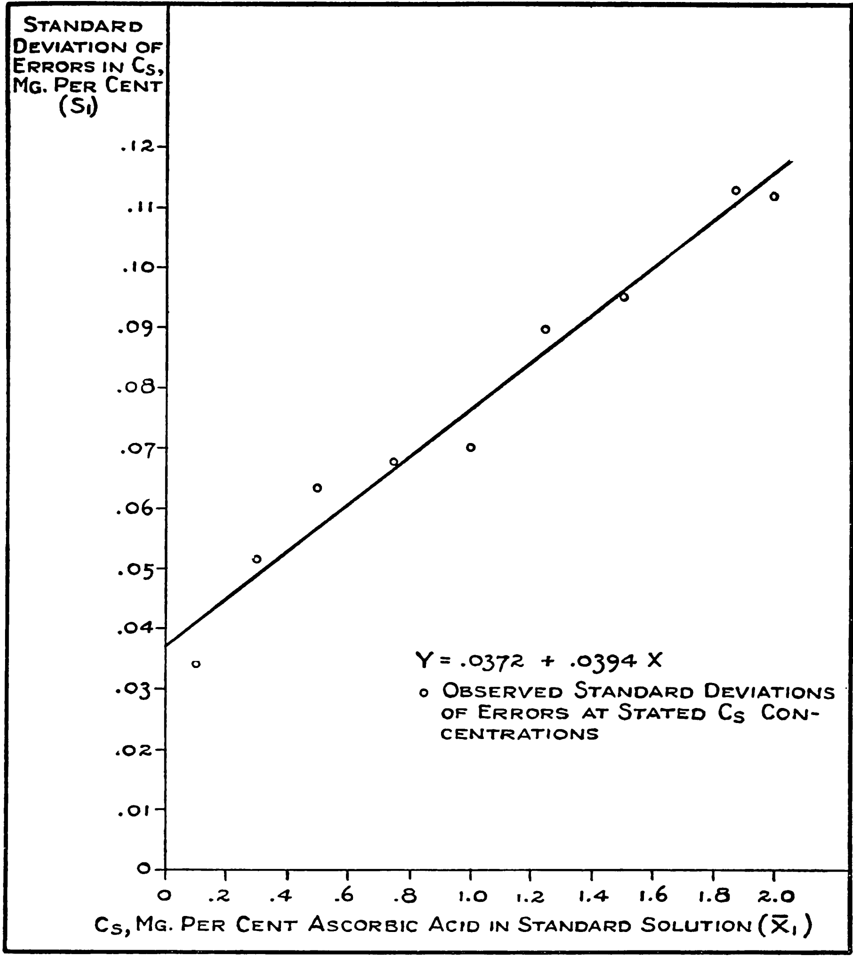

Fig. 1. Relation between error made in preparing standard ascorbic acid solutions and the concentration of ascorbic acid employed for the standard solution.

Estimates of Mean Values for C_s, $(L_b - L_s)$, and $(L_b - L_p)$

Before the three estimates of error may be utilized in formula (4) to yield the error of a determination upon a sample of plasma, the values of $\overline{X}_1$, $\overline{X}_2$, and $\overline{X}_3$ must be obtained. Evidently $\overline{X}_1$ is the standard concentration of ascorbic acid in the metaphosphoric acid solution added to the dye-acetate blank. The six series yield twenty-two estimates of $\overline{X}_3$, the mean of $(L_b - L_s)$. The average values for

each of the nine C_s levels fall along a straight line of equation

$$Y = .00087 + .0204\,X$$

where X is the C_s level (mg. per cent ascorbic acid in standard solution). Theoretical considerations suggest a regression line passing through the origin, whereas the observed line cuts the Y axis at +.00087. Such a discrepancy, of course, is entirely too small to be taken as evidence against theoretical expectation. Forced through the origin, the line would have the approximate equation $Y = .021X$. Although values of $\bar{X}_3$ calculated from such an equation could accord more nicely with theoretical expectation than those obtained from the observed regression equation, the differences between the estimates would be small. The values of $\bar{X}_3$ in Table 5, and shown in Figure 2, have been computed from the observed regression equation.

Table 4. Estimates of the error made in preparing standard ascorbic acid solutions, by strength of solution.

Concentration of Ascorbic Acid in Standard Solution Added to Dye Blank, in Mg. Per Cent	Error Variance[1] ($S^2 \times 10^3$)	Coefficient of Variation Per Cent
.10	1.690	41.1
.30	2.400	16.3
.50	3.234	11.4
.75	4.451	8.9
1.00	5.862	7.7
1.25	7.467	6.9
1.50	9.265	6.4
1.88	12.371	5.9
2.00	13.445	5.8

[1] Estimated from the regression equation $Y = .03718 + .03939\,X$, where X is the concentration of ascorbic acid in standard solution added to the dye blank, and Y is the standard deviation of the errors of Cs.

The laboratory observations provide no direct means of estimating $\bar{X}_2$, the mean $(L_b - L_p)$, for a given concentration of ascorbic acid in the plasma, since the "true" content of any sample is, of course, unknown. However, the relationship between C_p and $(L_b - L_p)$ is theoretically the same as that between C_s and $(L_b - L_s)$, and the same regression equation should be applicable to both systems. Although it may involve some systematic error, especially in the region below .5 mg. per cent ascorbic acid, the observed regres-

Estimates for C_S the Strength of the Prepared Ascorbic Acid Solutions in Mg. Per Cent		Estimates for (L_b-L_s) at Each C_S Level	
$\overline{X}_1$	$S_1^2 \times 10^8$	$\overline{X}_3$	$S_3^2 \times 10^8$
.10	1.690	.0029	89.054
.30	2.400	.0070	89.054
.50	3.234	.0111	89.054
.75	4.451	.0162	89.054
1.00	5.862	.0213	89.054
1.25	7.467	.0264	89.054
1.50	9.265	.0315	89.054
1.88	12.371	.0392	89.054
2.00	13.445	.0417	89.054

Estimates for (L_b-L_p) at All C_S Levels, According to C_p Level Stated as Mg. Per Cent and Number of L_p Readings Made

C_p	$\overline{X}_2$	Error Variance $S_2^2 \times 10^8$ by Number of Readings		
		One L_p	Duplicate L_p	Triplicate L_p
.10	.0029	282.558	159.408	118.358
.25	.0060	282.558	159.408	118.358
.50	.0111	282.558	159.408	118.358
.75	.0162	282.558	159.408	118.358
1.25	.0264	282.558	159.408	118.358
2.00	.0417	282.558	159.408	118.358

Table 5. Summary of estimates of means and standard deviations required for computation of the error of a determination of the ascorbic acid content of plasma.

sion equation was employed in computing the values of $\overline{X}_2$ in Table 5.

Experimental Error of Estimated Ascorbic Acid Content of Plasma

The estimates derived thus far may be put together by means of formula (4) which substitutes the formula for the variance of a product $(Z=X_1 \cdot X_2)$ in the formula for the variance of a ratio (Z/X_3) on the assumption, justified above, that the errors of X_1 and X_2 are uncorrelated. The correlation coefficient in formula (4) pertains to Z and X_3, and is of unknown magnitude. It is not worth

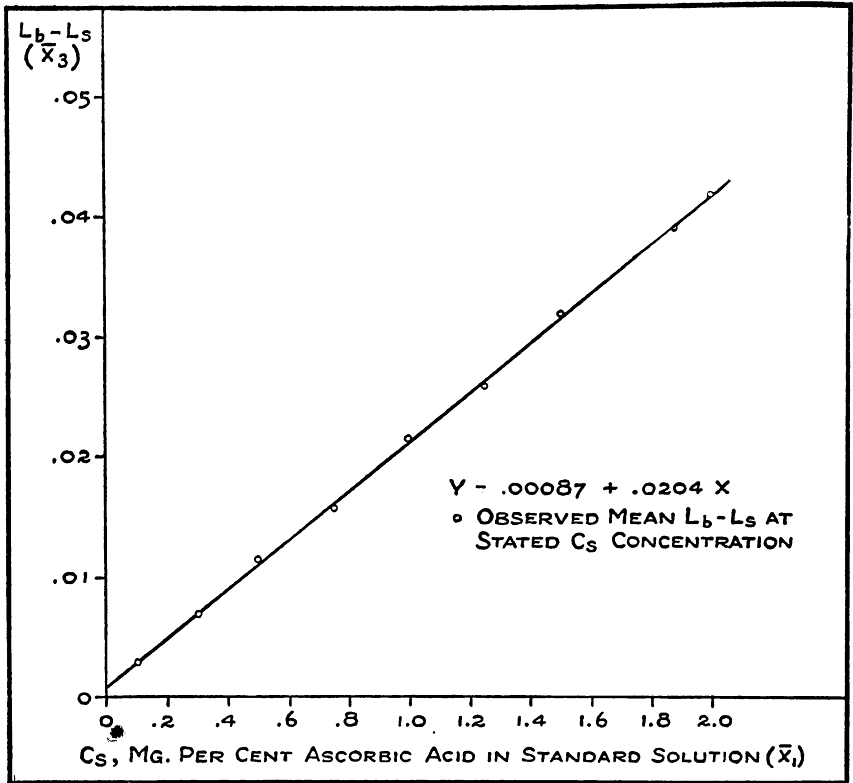

Fig. 2. Relation between difference in log readings on dye-blank and on dye-blank plus standard solution and the ascorbic acid content of the standard solution.

the labor of estimation, however, for it can be shown that, no matter what value r may take within its possible range from −1 to +1, the term in which it appears must be negligible in size. Then it is an easy matter to compute the variance of an estimate of the ascorbic acid content of a sample of plasma by means of the shortened formula

$$(7)\quad S_t^2 = \left[\frac{\bar{X}_1 \cdot \bar{X}_2}{\bar{X}_3}\right]^2 \cdot \left[\frac{S_1^2}{\bar{X}_1^2} + \frac{S_2^2}{\bar{X}_2^2} + \frac{S_3^2}{\bar{X}_3^2} + \frac{S_1^2 S_2^2}{\bar{X}_1^2 \bar{X}_2^2}\right]$$

Estimates of S_t for the 9 C_s levels and for 6 ascorbic acid levels are given in Table 6. Figure 3 gives a plot of these estimated standard

Strength of Standard Solution Per Cent	Error of Determination According to Amount of Ascorbic Acid Found in Plasma					
	.10	.25	.50	.75	1.25	2.00
.10	.082	.125	.210	.299	.481	.756
.30	.078	.091	.124	.164	.250	.385
.50	.078	.085	.104	.128	.185	.277
.75	.079	.083	.095	.112	.152	.220
1.00	.080	.083	.091	.104	.135	.190
1.25	.080	.083	.090	.100	.126	.173
1.50	.081	.083	.088	.097	.120	.162
1.88	.081	.083	.088	.095	.114	.151
2.00	.081	.083	.087	.094	.113	.148

Table 6. Estimates of the error of single determinations of plasma ascorbic acid, according to strength of standard solution and the estimated ascorbic content of plasma.

deviations and permits one to read off the estimated standard deviation appropriate to any C_s and C_p within the range .10 to 2.0 mg. per cent.

The error curve for any C_s concentration above .10 per cent rises only slowly with increasing C_p, and declines fairly rapidly as C_s is increased. Evidently the accuracy obtainable with prepared dye solutions containing as little as .10 per cent ascorbic acid is not high, entirely apart from any question of systematic bias. The use of standard solutions containing one or more per cent ascorbic acid is clearly indicated. The lowest line in Figure 3 gives the curve of error reported by Wiehl and Kantorovitz for C_p determinations obtained by means of the macromethod described by Mindlin and Butler. A significant disparity exists between this curve and the lowest curve of error for the micromethod. For maximum accuracy, therefore, the macromethod seems indicated if the available quantities of plasma are sufficiently large. The reliability of the micromethod seems rather high in the light of the fact that it requires about 1/20th of the amount of plasma needed for the macromethod. When it is imperative to economize on blood, therefore, the micromethod may be utilized with only slight sacrifice of accuracy.

The practical utility of these results stems from the ready way

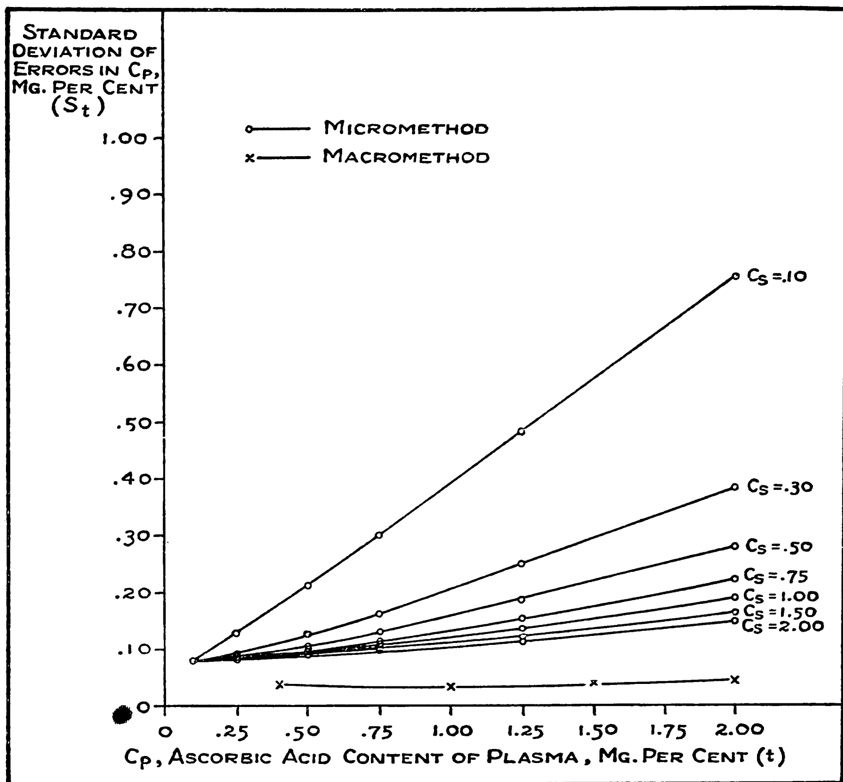

Fig. 3. Estimates of experimental error for single determinations of the ascorbic acid content of plasma by micromethod, for different standard concentrations, with comparative estimates for the macromethod.

in which errors may be read from the curves of Figure 3. Other biochemical laboratories may operate with more or less efficiency than the Study laboratory upon the work of which this report is based, but the differences might not be large. Assuming a laboratory routine of comparable efficiency, and employing triplicate L_b and L_s readings, the error of any ascorbic acid determination may be read directly from the appropriate curve in Figure 3. If the standard solution should contain 2.0 mg. per cent ascorbic acid, for example, the curve for C_s equal to 2.0 per cent would be the appropriate error curve to consult. A plasma determination of, say, .60 mg. per cent of ascorbic acid could then be placed within its 95 per cent confidence limits ($t \pm 1.96\ S_t$) of $.60 \pm .18$, or .42 to

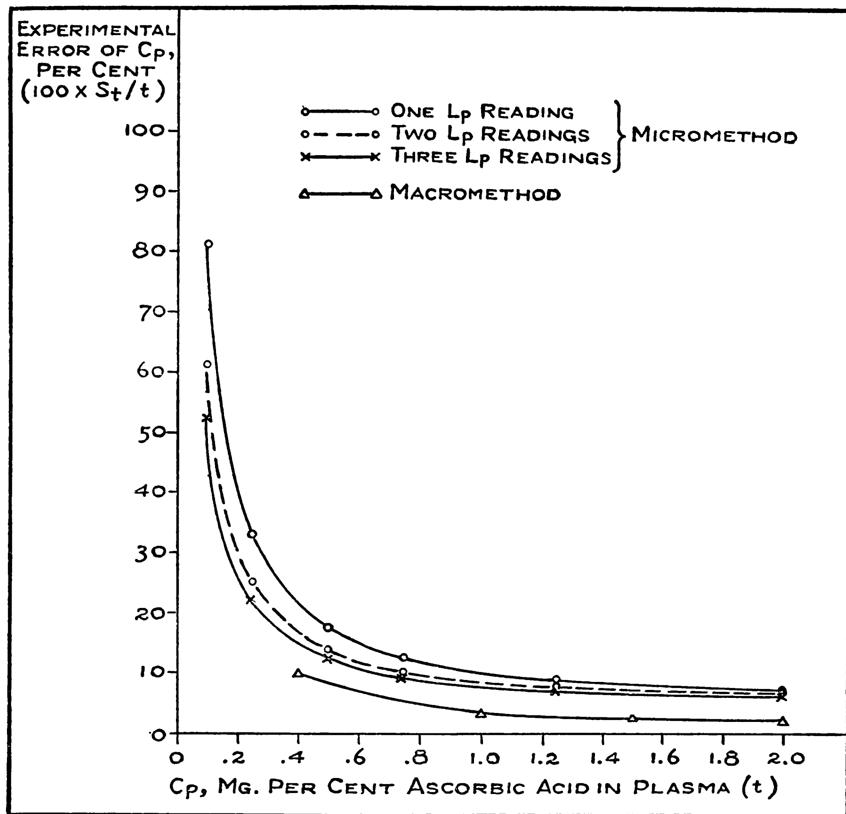

Fig. 4. Effect of duplication and triplication of galvanometer reading on dye-blank plus plasma filtrate in reducing the experimental error of C_p, in percentage terms.

.78. For a plasma determination of 2.0 mg. per cent the limits would be 2.0 ± .29, or 1.71 to 2.29 mg. per cent.

The reliability of micro determinations can, of course, be somewhat increased by independent replication of the L_p reading. Since the micromethod requires such small quantities of plasma, replication demands only a small additional amount. Figure 4 shows the extent to which the error curve for C_s equal to 2.0 mg. per cent may be reduced by duplication and by triplication of the L_p reading, and also permits comparison with the error of the macromethod using one L_p reading. The standard deviations are presented in relative form in this figure, in contrast to Figure 3, as

percentages of the estimated ascorbic acid contents of the blood samples, in order to bring out the rapid change in relative accuracy with increasing C_p. Even triplication does not suffice to bring the micromethod curve down to that for the macromethod, and triplication has little advantage over duplication. It also seems clear that replication has little effect upon the relative error of an estimated C_p above 1.25 per cent.

Systematic Error From Choice of Standard Concentration

The foregoing statistical analysis is concerned with *random* errors of measurement and does not touch upon the question of *systematic* or biased errors. It is, in fact, quite possible that the micro determinations are subject to appreciable bias. One point in the preceding discussion gives a clue to a minor bias to which the method was subject in the Study laboratory.

In the course of estimating mean values of $(L_b - L_s)$ for different standard concentrations (C_s), it was noted that the regression line failed to pass through the origin as required by the theory of the transmission of monochromatic light through a homogeneous solution. Although the departure from expectation is too small to affect the estimates of *random* error, it is sufficient to introduce some *systematic* error, especially if low standard concentrations are employed in the derivation of K. The observed regression line yields the following set of K values for the standard concentrations (C_s) .1, .3, .5, .75, 1.0, 1.25, 1.5, and 2.0 mg. per cent: 34.5, 42.9, 45.0, 46.3, 46.9, 47.3, 47.6, and 48.0. Since K is employed as a multiplier of $(L_b - L_p)$ or the log of the ratio of galvanometer readings on dye blank plus plasma and on dye blank alone, clearly the estimated ascorbic acid content of the plasma depends upon the choice of standard concentration.

By assuming fixed values for $(L_b - L_p)$ for particular "true" C_p values, it is possible to compute the estimates which would follow from the use of several different standard concentrations. Such

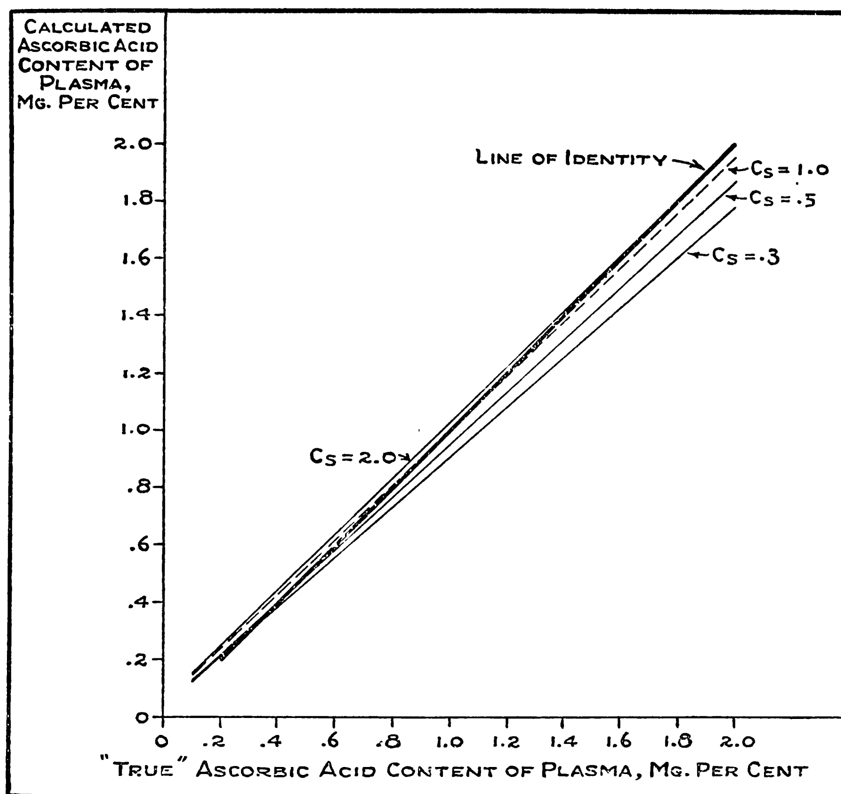

Fig. 5. Systematic errors in estimated ascorbic acid content of plasma arising from choice of standard concentration.

estimates have been plotted in Figure 5, the ordinate being the calculated value of C_p, and the abcissa its "true" value. The heavy line there represents the region of accurate estimation. The lighter lines for several different standard concentrations tend to approach this line as C_s is increased to 2.0 mg. per cent. They also cross the heavy line at the points where $C_s = C_p$. There is no bias from this source if the standard concentration is identical with the estimate for the plasma filtrate sample. The use of standard concentrations below 1.0 mg. per cent involves considerable error in the region above 1.0 mg. per cent, but such low standard concentrations are not generally considered for routine work. For standard concentrations between 1.0 and 2.0 mg. per cent this particular type of error is

negligible, although it involves errors of .03 to .04 in the region below .5 mg. per cent ascorbic acid in plasma.

The Turbidity Correction

One possible source of error in photelometric determinations of ascorbic acid is the presence, in solutions introduced into the colorimeter, of particles which reduce the transmission of light. The turbidity correction is advocated (5, 6) to compensate for this error, which operates by depressing galvanometer readings, especially on test solutions. An imperceptible degree of turbidity may lower a galvanometer reading enough to make a marked reduction in the estimated ascorbic acid content of the plasma filtrate.

The procedure followed in the Study laboratory is based upon that given by Butler and Cushman (6). In lieu of their expression

$$C = K (\log G_s - \log G_b + \log 100 - \log G_{t_s}),$$

where G_{t_s} is the galvanometer reading on the completely decolorized test solution, the Study laboratory employed

$$C = K (\log G_s - \log G_b + \log G_{t_b} - \log G_{t_s})$$

where G_{t_b} is the galvanometer reading on the decolorized dye blank. Since either G_t reading should be 100 in the absence of any turbidity, and only the test solution (containing plasma) is subject to appreciable turbidity, the difference between the two procedures is minor. Most G_{t_b} readings were either 100 or very close to it. However, the inclusion of both turbidity readings does introduce the possibility of a *reduction* in the final estimate of C, whereas correction for turbidity in the test solution only must lead to an *increase* in C if $G_{t_s} < 100$. A reduction could arise only if $G_{t_s} > G_{t_b}$, or when, in other words, the dye blank was more turbid than the test solution. The preparation of the solutions makes this very unlikely. Hence, the presence of negative corrections in C should index the experimental error implicit in the turbidity correction.

If it were found that the turbidity corrections in C values con-

centrate about 0 with relatively little scatter, it would follow that the solutions employed were clear enough to permit accurate determinations by the Mindlin and Butler method. On the other hand, large positive deviations from 0 would reveal the presence of particles interfering with light transmission. Turbidity of this degree might be introduced occasionally into the mixture of dye blank plus plasma filtrate because of insufficient or improper centrifuging. It is instructive, therefore, to study the corrections actually made in the Study laboratory. Parenthetically it may be noted that the correction does not alter the order of magnitude of the final estimates of experimental error presented above in Table 6 and in Figure 3.

The turbidity correction was performed for 222 different blood samples in series III-B, C, and D. In 82, or 36.9 per cent of the cases, the correction was zero. A complete distribution of the changes appears in Table 7 in both relative and absolute form, and separately for parts B, C, and D of series III. Large changes are

Table 7. Distributions of turbidity corrections made in several series of micro determinations of the ascorbic acid content of plasma.

Turbidity Correction Mg. Per Cent Ascorbic Acid	Series						Total	
	B		C		D			
	Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Per Cent
−.150 to −.101			1	1.3			1	.5
−.100 " −.051	7	7.7	9	12.0	11	19.6	27	12.2
−.050 " −.001	10	11.0	22	29.3	10	17.9	42	18.9
0 " +.049	50	54.9	29	38.7	19	33.9	98	44.1
+.050 " +.099	8	8.8	7	9.3	5	8.9	20	9.0
+.100 " +.149	5	5.5	5	6.7	8	14.3	18	8.1
+.150 " +.199	4	4.4	1	1.3	2	3.6	7	3.2
+.200 " +.249	5	5.5	1	1.3			6	2.7
+.250 " +.299								
+.300 " +.349	1	1.1					1	.5
+.350 " +.399								
+.400 " +.449								
+.450 " +.499	1	1.1					1	.5
+.500 " +.549					1	1.8	1	.5
Total	91	100.0	75	99.9	56	100.0	222	100.2

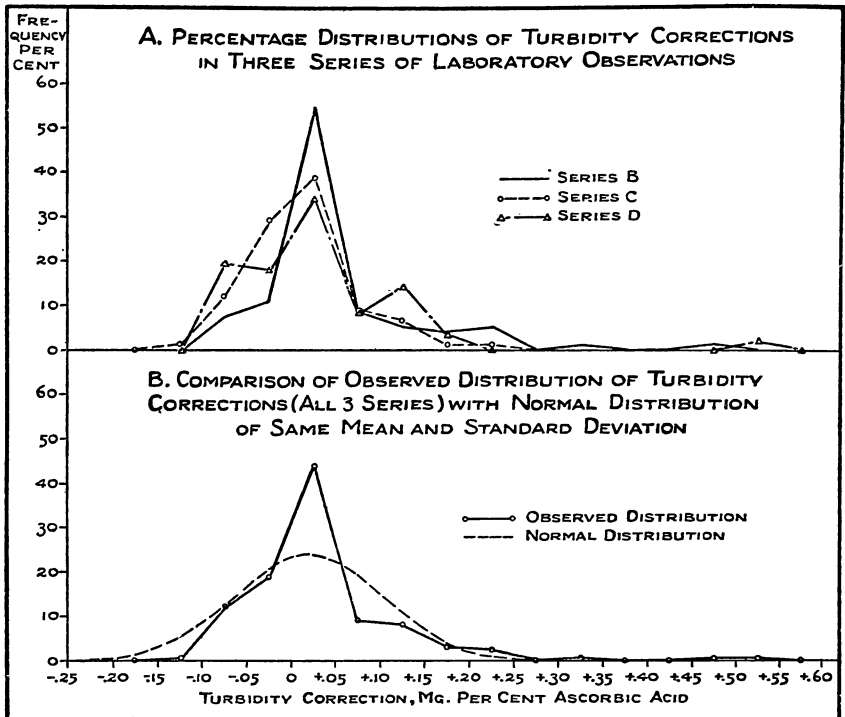

Fig. 6. Distributions of turbidity corrections made in estimates of ascorbic acid content of plasma samples.

clearly infrequent, 44.1 per cent lying between 0 and +.049, 72.0 per cent between -.050 and +.099, and 92.3 per cent between -.100 and +.149 mg. per cent ascorbic acid. The three changes of some magnitude are +.315, +.459, and +.530. The mean is +.0198.

Refined statistical treatment is somewhat hampered by the fact that, in any series, the corrections made on a given day are not completely independent, since the same dye-acetate solution was used, and a single turbidity factor for the dye blank was also employed. However, the various statistical tests which may be readily applied, such as the F test of means, the likelihood ratio test of variances, and the contingency-table test for independence, all unite in suggesting that the three parts of series III differ by a wider margin than chance would be expected to produce. Furthermore, within each part there is significant heterogeneity from day to day, al-

though the corrections themselves are independent of the uncorrected C_p values. The correlation coefficients between the corrections and the uncorrected C values are -.11, +.08, and -.08 for parts B, C, and D, respectively. Each distribution of corrections departs very significantly from the normal probability distribution, being too peaked and skewed in the direction of higher + changes. Figure 6 contains a plot of each distribution and also a comparison of the total distribution with a normal distribution having the observed mean and variance. The following hypothesis may be ventured in explanation of the distribution of corrections: to a chance distribution of zero mean there is added a fairly small set of + changes belonging to a different system; this set represents the determinations on plasma samples having some turbidity, and only for them does the correction assume any real importance. The fact that 30 per cent of the turbidity corrections are reductions indicates that the method of adjusting for turbidity is subject to some experimental error. However, this error is rather small, since only one among more than 100 exceeds .1 mg. per cent ascorbic acid.

Summary

Laboratory observations from the Medical Evaluation of Nutritional Status Study provide a means of estimating the experimental error of single determinations of plasma ascorbic acid by the micromethod of Mindlin and Butler. The sources of error are analyzed and estimates of each component made on the basis of independent duplicate or replicate readings. The experimental error is shown to depend not only upon the amount of ascorbic acid judged to be present in the plasma, but also upon the strength of the known concentration of ascorbic acid employed for comparison. The resulting standard deviations of error are shown graphically and contrasted with the lower estimates of Wiehl and Kantorovitz for the macromethod. With standard (known) concentrations of 1.0 to 2.0 mg. per cent ascorbic acid, the standard deviations are of the

order of .08 to .20 mg. per cent if the galvanometer readings on dye blank and on dye blank plus standard solution are triplicated. Duplication or triplication of the galvanometer reading on dye blank plus plasma filtrate fails to lower the experimental error by appreciable margins.

The errors of .08 to .20 mg. per cent ascorbic acid compare with errors of about .04 mg. per cent estimated for the macromethod. The sacrifice of accuracy which the micromethod entails, therefore, is slight in view of its economical blood requirements, and apart from the question of *systematic* bias.

Some systematic error is involved in the choice of standard concentrations. However, the use of high standard concentrations (*e.g.,* 1.0 to 2.0 mg. per cent) leads to far less error in the region of low plasma values than does the use of low standard concentrations for the region of high plasma values. Both systematic and random (experimental) error, therefore, are minimized if standard concentrations of 1.0 or more mg. per cent are employed.

Routine turbidity corrections made for more than 200 plasma determinations indicate that only infrequently does this refinement sensibly disturb the estimated ascorbic acid content of plasma samples. In a few instances, however, the corrections were appreciable.

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