DISTRIBUTION OF ASCORBIC ACID IN THE BLOOD AND ITS NUTRITIONAL SIGNIFICANCE

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This paper is concerned first, with an enquiry into methods of determining ascorbic acid in whole blood and blood cells; second, with the partition of ascorbic acid between the plasma and the formed elements; and third, with the significance of the apparent ascorbic acid concentration of, respectively, the whole blood, the plasma, the red cells, and the white cells and platelets as an index of vitamin C deficiency.

The rapid changes in plasma ascorbic acid concentrations following the addition to or withdrawal of ascorbic acid from the diet (1, 2, 3) render a single determination of the plasma level a very limited index of vitamin C nutrition. While relatively high fasting plasma concentrations, *i.e.* values between 0.8 and 1.4 mgm. per 100 cc., indicate, according to present knowledge, satisfactory nutrition (4), low plasma concentrations do not provide a reliable index of either deficiency or unsaturation (1, 4, 5). Van Eekelen, Emmerie and Wolff (6), Neuweiler (7) and Heinemann (8) have presented evidence that whole blood concentrations, which include the ascorbic acid content of the blood cells as well as the plasma, reflect vitamin C nutrition to a greater extent than do plasma concentrations and actually indicate the degree of saturation or depletion. However, failing the general acceptance of methods of whole blood analysis, various ascorbic acid tolerance tests have been developed.

Determinations of the ascorbic acid content of whole blood, as reported in the literature, present conflicting evidence concerning the analytical procedures. Stephens and Hawley (9) and Cuttle (10) analyzed whole blood filtrates obtained after trichloracetic acid precipitation. Kellie and Zilva (11), van Eekelen (12), Emmerie and van Eekelen (13), Borsook, Davenport, Jeffreys and Warner (14), Bessey (15), Butler and Cushman (16), and Fujita, Ebihara, and Numata (17) have found that hemolysis decreases the concentration of ascorbic acid in plasma and that precipitation of whole blood by hemolyzing reagents such as trichloracetic or metaphosphoric acid oxidizes ascorbic acid and invalidates results. This oxidation can be prevented by saturating the whole blood with CO or CO_2 (11, 13, 16, 17). Pijoan and Eddy (18) added a large amount of potassium cyanide to whole blood to prevent oxidation during acid precipitation. Friedman, Rubin and Kees (19), Farmer and Abt (20) and Cushman and Butler (21) have shown that the addition of such amounts of cyanide may be a source of error. Emmerie and van Eekelen (22) advocated the treatment of such whole blood filtrates with mercuric acetate to remove non-ascorbic acid reducing substances and with H_2S to recover the ascorbic acid reversibly oxidized to dehydroascorbic acid during the precipitations. The validity of this procedure has been questioned (14, 16, 23, 24, 25, 26). Heinemann (8), using this procedure, observed that the hemolysis of red cells increased the apparent ascorbic acid concentration in plasma or serum samples.

There has been little agreement concerning the distribution of ascorbic acid between plasma and red cells calculated from plasma and whole blood analyses and hematocrit determinations. Stephens and Hawley (9) found the ascorbic acid concentrations of plasma and red cells from subjects with normal white cell counts to be approximately equal. Cuttle (10), and Pijoan and Eddy (18) found the plasma concentration to exceed those of the red cells. Heinemann (8, 27) and Mirsky, Swadesh and Soskin (28) found the concentration in the plasma to be less than the concentration in the red cells. Borsook, Davenport, Jeffreys and Warner (14) observed that ascorbic acid added to whole blood *in vitro* remained in the plasma and concluded that the red cells were nearly, if not absolutely, impermeable to added ascorbic acid. From *in vitro* and *in vivo* experiments Heinemann (8) concluded that added ascorbic acid passed from the plasma into the red cells.

The work reported here was undertaken in order to provide methods of analysis of whole blood and blood cells satisfactory for clinical use. It was hoped that the application of these methods might afford a more reliable index of vitamin C nutrition than is given by fasting plasma levels or by whole blood levels determined by procedures previously used in clinical investigations. The evidence for the validity of the methods we have used and data obtained by them are presented. The data show that the apparent ascorbic acid content of the whole blood and of the white cells and platelets may provide indices of vitamin C nutrition which fulfill with a fair degree of satisfaction the hope expressed above.

PART I

Examination of methods of whole blood analysis

The more commonly used indicator, 2–6 dichlorophenolindophenol, is not entirely satisfactory because it is reduced by thiosulfate, cysteine, and other substances containing the sulfhydryl group. However, under the conditions prescribed by the analytical procedures, ascorbic acid reduces the dye more rapidly than other known reducing substances (24, 29). Therefore, the rate of reduction can to some extent be used as a criterion of specificity. For this reason Meunier (30), Mindlin and Butler (31), Evelyn, Malloy and Rosen (24) and Bessey (32) have suggested the use of the photoelectric colorimeter in the determination of ascorbic acid so that the rapid reduction of the dye can be distinguished from a slow reduction. Such colorimetry, therefore, has been applied in the present study to the analysis of various filtrates.

In the photoelectric procedures the concentration of ascorbic acid in the sample solution C was calculated in the manner previously described (31), except that the procedure and calculation included, where indicated, a correction for turbidity (32).

$C = K$ (log. G_s — log. G_b + log. 100 — log. G_T), where G_s is the galvanometer reading of the unknown filtrate, G_b the reading of the dye-blank solution, and G_T is the reading of the dye-unknown filtrate solution after complete decolorization following the addition of a small crystal of ascorbic acid.

Photoelectric colorimetry has also been applied to the analysis of filtrates by a modification of Martini and Bonsignore's methylene blue method (33). We have used this method in the analysis of whole blood for comparison with analyses in which indophenol is used and in the analysis of red blood cells where the reduction of indophenol by substances other than ascorbic acid makes that indicator unsatisfactory. Under the conditions specified in the modified procedure, methylene blue appears to be a more specific and sensitive indicator of reduction due to ascorbic acid than any other oxidation-reduction indicator. Thiosulfate, cysteine, and glutathione do not reduce the dye. The sensitivity of the procedure, as measured by change in color and galvanometer deflection, is about threefold that of 2–6 dichlorophenolindophenol. A brief outline of the method is appended to this paper. A fuller description with its application to a microprocedure will be reported later.

Using such photometry, the ascorbic acid concentrations in whole blood and plasma have been measured in filtrates obtained by the following procedures:

(*1*) Filtrates from plasma prepared according to the method of Mindlin and Butler (31).

(*2*) Filtrates from whole blood precipitated by 20 per cent trichloracetic (9) or 10 per cent metaphosphoric acid.

(*3*) Filtrates from plasma and whole blood prepared by the method of Emmerie and van Eekelen as described by Heinemann (8). For the photoelectric procedure the final dye-unknown filtrate, as well as the dye-blank solutions, was adjusted to pH 3.0 so that the dye did not fade as a result of the acidity (31).

(*4*) Filtrates from whole blood which were saturated with CO, precipitated with metaphosphoric acid and filtered in an atmosphere of CO as described in the Appendix.

Table I gives the ascorbic acid concentrations in whole blood using filtrates prepared by the methods of Stephens and Hawley (9), of Emmerie and van Eekelen (8, 22), and of CO saturation during HPO_3 precipitation. From the plasma values in the table it is seen that whole blood values by the trichloracetic acid precipitation of Stephens and Hawley do not reflect the ascorbic acid known to be present in the plasma. In addition, known amounts of ascorbic acid added to the blood samples are not recovered after such precipitations. These results support the findings of

TABLE I

Apparent ascorbic acid concentration in whole blood found in filtrates prepared by different methods together with the plasma concentrations and data on the recovery of known amounts of ascorbic acid added to whole blood

Sample	Filtrate by method of	Mgm. ascorbic acid equivalents per 100 cc.				Per cent recovery
		Before addition of ascorbic acid		Mgm. ascorbic acid added per 100 cc.	After addition of ascorbic acid	
		Whole blood	Plasma*		Whole blood	
N.T.	Stephens and Hawley †	0.0	(1.1)	0.6	0.0	0
N.T.	Emmerie and van Eekelen	1.6	(1.1)			
N.T.	CO saturation plus Mindlin and Butler	1.4	(1.1)	1.0	2.4	100
A.B.	Stephens and Hawley †	0.0	(0.9)	1.3	0.0	0
A.B.	Emmerie and van Eekelen	1.2	(0.9)	0.4	1.6	100
A.B.	CO saturation plus Mindlin and Butler	0.9	(0.9)	1.0	1.9	100
E.M.	Stephens and Hawley †	0.2	(1.4)	1.3	0.7	48
E.M.	Emmerie and van Eekelen	1.5	(1.4)	0.4	2.1	110
E.M.	CO saturation plus Mindlin and Butler	1.3	(1.4)	1.0	2.2	96

* The plasma concentrations were determined by method of Mindlin and Butler (31). The values so obtained are in close agreement with values obtained from plasma filtrates by the three methods specified in the table.

† When 10 per cent metaphosphoric acid is used instead of 20 per cent trichloracetic acid similar losses of ascorbic acid occur.

other workers referred to above (11, 12, 13, 14; 15, 16, 17). There is complete recovery of added ascorbic acid when filtrates are prepared by the methods of Emmerie and van Eekelen and of CO saturation during HPO_3 precipitation. Such recovery, however, does not prove the validity of either method, for it does not show that the reduction in excess of that due to added ascorbic acid is a measure of ascorbic acid originally present in the blood. The data of the table show that the apparent ascorbic acid concentrations obtained by the Emmerie and van Eekelen procedure are greater than those obtained by the CO saturation procedure.

Observation of the rate of reduction in the latter two procedures provides an explanation of the difference in results. In Figure 1 the rates of reduction of the dye by a pure ascorbic acid solution, by a plasma filtrate (31), and by whole blood filtrates prepared by the Emmerie and van Eekelen and the CO saturation procedures are shown. The reducing capacity expressed as mgm. of ascorbic acid equivalents per 100 cc. of solution, plasma or blood is plotted against time in seconds. The rate of reduction of the dye by ascorbic acid varies

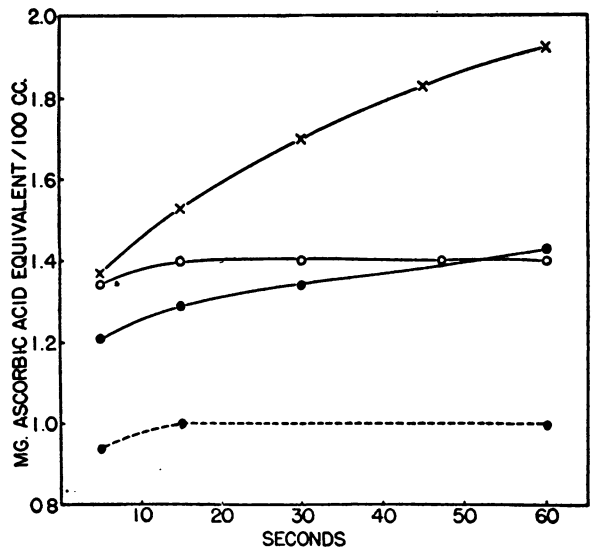

FIG. 1. RATE OF REDUCTION OF INDOPHENOL BY ASCORBIC ACID SOLUTION, PLASMA FILTRATE, AND WHOLE BLOOD FILTRATES

●----● ascorbic acid solution.
○——○ plasma filtrate.
●——● whole blood filtrate prepared by CO saturation procedure.
×——× whole blood filtrate prepared by Emmerie and van Eekelen procedure.

with the acidity of the solution (24). At pH values less than 3.0 the acidity becomes a factor in decolorizing the dye (31). Therefore, the solutions analyzed in these experiments were adjusted to pH 3. At this pH the curve for the pure ascorbic acid solution (Figure 1) reaches its height at 15 seconds and that time interval has been chosen for estimating the apparent ascorbic acid concentrations in these experiments. The plasma curve (Figure 1) is similar to that of pure ascorbic acid. Curves on plasma filtrates obtained by the Mindlin and Butler, the CO saturation and the Emmerie and van Eekelen procedures vary but little. On the other hand, as shown in Figure 1, curves on filtrates of whole blood prepared by the latter two procedures continue to rise during the 60-second period, the Emmerie and van Eekelen procedure always having a steeper slope. This continued fading of the dye beyond the time interval characteristic of ascorbic acid reduction suggests reduction due to some other substance or substances. The difference in the slopes of these whole blood curves shows that the filtrate of the CO saturation procedure includes much less non-

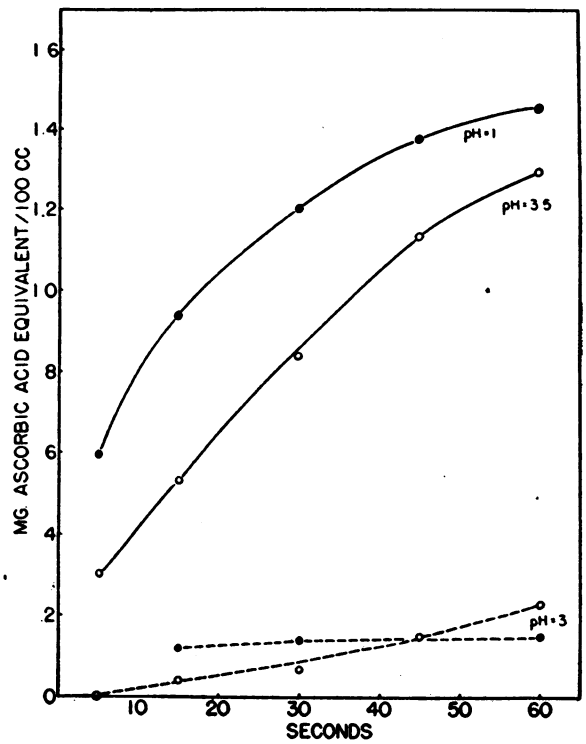

FIG. 2. RATE OF REDUCTION OF INDOPHENOL BY FILTRATES OF WHOLE BLOOD FROM SCORBUTIC PATIENTS

O----O, ●----● filtrates prepared by CO saturation procedure.

O——O, ●——● filtrates prepared by Emmerie and van Eekelen procedure.

ascorbic acid reduction than does the filtrate of the Emmerie and van Eekelen procedure.

It has been suggested (23, 25, 26) that the H_2S treatment of the Emmerie and van Eekelen procedure reduces substances other than ascorbic acid, which then in turn reduce the indophenol. The data of Figure 2 give further support to such a premise. They show analyses of whole blood filtrates from four scorbutic patients whose plasmas contained no ascorbic acid. The solid line curves represent analyses of filtrates obtained by the Emmerie and van Eekelen procedure, whereas the broken line curves represent analyses of filtrates prepared by the CO saturation procedure. The difference between the slopes of the two upper curves and the slopes of the two lower curves could hardly be due to ascorbic acid but must result from substances reduced in the course of the Emmerie and van Eekelen procedure.

The data of Table II compare the apparent ascorbic acid concentrations of CO saturated whole blood determined by the indophenol and the methylene blue procedures. Both procedures give values that are in close agreement. Because the procedures give complete recovery of added ascorbic acid, it seems likely that the apparent ascorbic acid values obtained include the true ascorbic acid and are not too low. Available evidence indicates that the amount of dehydroascorbic acid in whole blood is not significant (14, 23). The use of methylene blue with filtrates of whole blood obtained by the Emmerie and van Eekelen procedure is not altogether satisfactory as there is a continued fading of this dye also.

TABLE II

Comparison of apparent ascorbic acid concentrations in plasma and whole blood by the indophenol and methylene blue procedures, before and after addition of known amounts of ascorbic acid

Sample		Mgm. ascorbic acid equivalents per 100 cc.			
		Before addition		After addition of 1 mgm. ascorbic acid per 100 cc.	
		Methylene blue	Indophenol	Methylene blue	Indophenol
R.M.	Plasma.....	0.7	0.8	1.6	1.7
J.M.	Plasma.....	1.1	1.2	2.0	2.1
M.C.	Plasma.....	1.5	1.5	2.5	2.5
M.C.	Whole blood...	1.3	1.4	2.3	2.5
M.C.	Whole blood...	1.5	1.7	2.4	2.5
N.T.	Whole blood...	1.5	1.4	2.6	2.4

TABLE III

Apparent ascorbic acid content of whole blood in terms of the plasma, red cells and white cells-platelets; normal subjects

Sample		Volume per 100 cc. blood		Mgm. ascorbic acid equivalents per 100 cc. by analysis				Calculated mgm. ascorbic acid equivalents per 100 cc. whole blood			
		Red cells	White layer	Plasma	Red cells	White layer	Whole blood	In the plasma	In the red cells	In the white layer	Total by addition
		1	2	3	4	5	6	7	8	9	10
1	A.B.	39.8	0.6	0.8	0.4*	25	0.8	0.47	0.16*	0.15	(0.8)
2	F.	42.5	0.8	1.0	0.9*	32	1.3	0.57	0.37*	0.26	(1.3)
3	B.	43.1	0.4	1.1	0.6	38	1.1	0.62	0.26	0.15	1.0
4	M.C.	41.5	0.7	1.5	0.7	36	1.4	0.87	0.29	0.25	1.4
5	M.C.†	41.0	0.5	1.2	1.1	36	1.6	0.70	0.45	0.18	1.3
6	M.C.†	40.5	0.5	1.4	1.4	35	1.6	0.83	0.57	0.18	1.6
7	R.M.	51.4	0.7	0.8	0.7	34	1.1	0.39	0.36	0.24	1.0
8	R.M.‡			2.5	1.0		1.9	1.20	0.51		2.0
9	R.M.‡			1.9	1.5		1.8	0.91	0.77		1.9
10	R.M.‡	51.0		1.2	1.0		1.2	0.58	0.51		1.3

* Calculated from plasma, whole blood, and hematocrit values.

† On a diet high in ascorbic acid. See text.

‡ A saturation test following 700 mgm. ascorbic acid. See text.

Though satisfactory criteria for the validity of whole blood ascorbic acid analyses are lacking, the data presented demonstrate that precipitation of whole blood by the CO saturation procedure outlined here is not accompanied by loss of ascorbic acid by oxidation and that filtrates obtained by this procedure contain less interfering reducing substances than filtrates obtained by the Emmerie and van Eekelen procedure.

Therefore, the whole blood and red blood cells analyses presented in the following sections have been carried out on filtrates obtained by the CO saturation procedure.

PART II

Whole blood ascorbic acid partition between the plasma, the red cells, and the white cells and platelets

Table III shows the mgm. of apparent ascorbic acid per 100 cc. of plasma, of red cells, of white cells and platelets [1] and of whole blood determined by direct analyses in samples of blood from normal subjects. The plasma and white cell plus platelet analyses were made according to the procedure of Mindlin and Butler (31), the whole blood analyses by the indophenol or methylene blue procedure applied to filtrates obtained by CO saturation and metaphosphoric acid precipitation, and the red cell analyses by the methylene blue procedure applied to filtrates similarly obtained. Actually the concentrations in the white cells plus platelets were determined per 100 grams of cells. However, for convenience of tabulation and without significant error the results are recorded as per 100 cc. From the concentrations in the three phases and the volumes of red cells and of white layer per 100 cc. of whole blood, the mgm. of apparent ascorbic acid in the plasma, the red cells, and the white cells plus platelets in 100 cc. of whole blood were calculated and are given in the table. Finally, the sum of these values is given for comparison with the whole blood content found by direct analysis.

By comparing the concentrations of apparent ascorbic acid in the plasma and red cells in Tables III and IV, it is seen that the ratio of plasma ascorbic acid concentration to red cell ascorbic acid concentration varies from 2.6 to 0.3. The first sample from Subject M. C., Table III, was taken in the mid-morning after a vitamin C-free breakfast when on a routine diet without added ascorbic acid. The second and third samples were taken at similar times after one week's daily ingestion of 250 mgm. and 500 mgm., respectively, of ascorbic acid. The ascorbic acid concentrations of the red blood cells rose from 0.7 to 1.4 mgm. per 100 cc. as the ingestion of ascorbic acid increased, while the plasma and the white cell-platelet ascorbic acid concentrations showed no significant change. The first sample of blood from Subject R. M. was a fasting specimen taken immediately before the ingestion of a single dose of 700 mgm. of ascorbic acid. Samples were then taken 3, 7, and 24 hours later. The plasma ascorbic acid concentration rose over the first 3 hours and fell during the next two periods. The ascorbic acid concentration of the red blood cells rose less rapidly, the concentration at 7 hours being the highest value. The data of the tables thus show the importance of the nutritional state and the time interval between the ingestion of ascorbic acid and the taking of the blood sample in respect to the plasma and red blood cell ascorbic acid concentrations. They also show the slow penetration of ascorbic acid into the red cells as reported by Heinemann (8).

Taking the average white cell-platelet apparent ascorbic acid concentration of 34 mgm. per 100 cc. of white layer as a normal value (Table III), and taking the average volume of white layer per 100 cc. of blood as 0.6 cc., the white layer accounts for approximately 0.2 mgm. of ascorbic acid per 100 cc. of blood. Indeed, the amount of ascorbic acid that the white layer contributes to several of the whole blood ascorbic acid concentrations of Table III is approximately equal to that contributed by the red blood cells. That the apparent ascorbic acid concentration of the white layer in abnormal states may be the major factor in whole blood values is shown in the following section.

[1] By microscopic examination the upper pure white portion of the white layer consists of platelets with a few white cells, while the lower buffy portion consists largely of white cells with some platelets and a few red cells (35 b). Analyses of these two portions, however, have given within the error of the method the same reduction or apparent ascorbic acid concentration.

PART III

The apparent ascorbic acid content of the white cells and platelets

Stephens and Hawley (9) and Cuttle (10) observed a high concentration of indophenol-reducing substance in the white blood cells of human subjects. Kellie and Zilva (34) found that the white blood cells of guinea pigs had a high reducing power. This increased comparatively little after the intravenous administration of large quantities of ascorbic acid. Its concentration fell when the animals were placed on a scorbutic diet, but there was a residual reducing substance or substances which could not be identified by these authors.

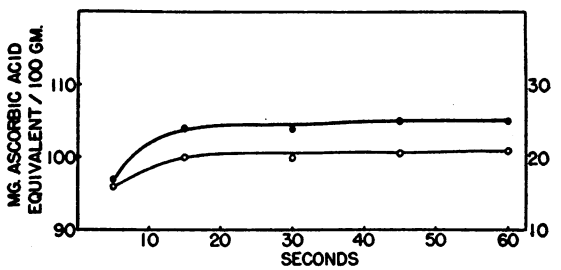

FIG. 3. RATE OF REDUCTION OF INDOPHENOL BY FILTRATES FROM METAPHOSPHORIC ACID PRECIPITATION OF NORMAL AND LEUKEMIC WHITE BLOOD CELLS

○——○ leukemic cells ordinate on left.
●——● normal cells ordinate on right.

Figure 3 shows the time curve of reduction of indophenol by metaphosphoric acid filtrates of the white layer of centrifuged blood from normal and leukemic subjects. It will be seen that the curve corresponds to that of pure ascorbic acid in Figure 1. The reaction of the reducing substance with methylene blue also corresponds to that of ascorbic acid. Because neither plasma nor white cell-platelet filtrates give delayed reduction, such reduction by filtrates from whole blood obtained by the CO saturation procedure must be due to substances within the red cells. This conclusion is further substantiated by analyses on such filtrates from samples of red blood cells in which, as already remarked, slowly acting reducing substances make analyses with the indophenol dye unsatisfactory.

Table IV presents analyses of whole blood ascorbic acid equivalents in terms of the plasma,

TABLE IV

Apparent ascorbic acid content of whole blood in terms of the plasma, red cells, and white cells-platelets from subjects on diets deficient in vitamin C

Subject	Type of deficiency	Volume per 100 cc. blood		Mgm. ascorbic acid equivalents per 100 cc. by analysis				Calculated mgm. ascorbic acid equivalents per 100 cc. whole blood			
		Red cells	White layer	Plasma	Red cells	White layer	Whole blood	In the plasma	In the red cells	In the white layer	Total by addition
C	Acute scurvy	35.0	1.0	0.0	0.0	3	0.0	0.00	0.00	0.03	0.03
	After vitamin C	35.0	1.3	0.3	0.6	22	0.8	0.20	0.21	0.29	0.70
Y	Acute scurvy	33.7	1.1	0.0	0.0	0	0.0	0.00	0.00	0.00	0.00
	After vitamin C	32.4	1.1	0.1	0.3	12	0.4	0.07	0.10	0.13	0.30
Ly	Acute scurvy	46.0	0.3	0.0	0.0	2	0.0	0.00	0.00	0.01	0.01
	After vitamin C	51.0	0.4	0.0	0.0	12	0.0	0.00	0.00	0.05	0.05
	After vitamin C	49.0	0.4	0.1	0.3	23	0.3	0.05	0.15	0.09	0.29
	After vitamin C	50.0	0.3	1.0	1.1	38	1.3	0.50	0.55	0.11	1.16
Bl	3 weeks' experiment	51.0	0.9	0.2	0.3	29	0.4	0.10	0.15	0.25	0.51
	4 weeks' experiment	46.0	0.6	0.1	0.1	22	0.2	0.05	0.05	0.13	0.23
Cr	3 weeks' experiment	49.0	0.6	0.2	0.2	28	0.3	0.10	0.10	0.16	0.36
	4 weeks' experiment	49.0	0.5	0.1	0.1	26	0.1	0.05	0.05	0.13	0.23
	6 weeks' experiment	47.0	0.4	0.0	0.0	10	0.1	0.00	0.00	0.04	0.04
	12 weeks' experiment	48.0	0.6	0.0		3	0.0				
G.	Diet by history		0.5	0.0		21	0.3	0.00		0.11	
Cer	Diet by history	37.0	1.1	0.0	0.2	12	0.2	0.00	0.07	0.13	0.20

the red cells and the white cells and platelets from subjects receiving diets deficient in vitamin C. In the untreated scorbutic patients the apparent ascorbic acid in the white cells and platelets varied from 0.0 to 3.0 mgm. per cent compared to a variation in the normal individual from 25 to 38 mgm. per cent (Table III). Following vitamin C therapy there was a significant rise in the reducing substance in the white layer and in the whole blood of the scorbutic patients before such a rise occurred in the plasma. The data for Subject Ly show the return of the white layer concentration to a low normal value during a period when the fasting concentration of the plasma rose to but 0.1 mgm. per cent. One infant, not reported in the table, recovered from all the symptoms of acute scurvy and by a saturation test [2] showed fair saturation without the fasting plasma level prior to the test going above 0.2 mgm. per cent.

Subjects Bl and Cr [3] were on a self-prescribed deficient diet. The data show the fall in plasma concentrations to very low levels before there was an appreciable drop in the white cell-platelet concentrations. The fall in the apparent ascorbic acid content of the red cells with the drop in plasma concentration resulted in a decrease in the

[2] Performed by Dr. R. L. Mindlin.

[3] The samples of blood from these two subjects were provided through the kindness and cooperation of Dr. John Crandon.

whole blood level independent of and earlier than the decrease in white cell-platelet concentration. At the end of 12 weeks on the deficient diet the apparent ascorbic acid concentration of subject Cr's white blood cells plus platelets had fallen to 3 mgm. per 100 grams of white layer. Though the subject felt below par, no definite signs of scurvy had appeared.

Infants G and Cer had no ascorbic acid in the plasma and 21 and 12 mgm. per cent, respectively, in the white layer of centrifuged blood. Each had a dietary history suggesting vitamin C deficiency but, as might be expected from the preceding data, neither presented any roentgenological or clinical signs of scurvy.

These findings indicate that the whole blood and the white layer of centrifuged blood of subjects whose vitamin C nutrition is relatively poor may contain measurable amounts of apparent ascorbic acid after the plasma level has become zero. *Therefore, the apparent ascorbic acid content of the whole blood or of the white blood cells plus platelets of individuals not suffering from infection or leukemia provides an index of vitamin C deficiency that extends beyond the limits defined by plasma values.* Because the white cell-platelet concentration is less dependent upon fluctuations in plasma concentration than is the whole blood content, the former appears to be the best index of physiologically significant deficiency. On the other hand, it is clear from the data of Tables III and IV that white cell-platelet analyses will not provide maximal information concerning the saturation of subjects whose vitamin C nutrition is relatively good, because high white cell-platelet concentrations are found early in the recovery from, and late in the development of, deficiency and therefore are constantly high in relatively well nourished subjects. In this sense the data support Heinemann's conclusion that high concentrations " in both cells and serum seem to be the phenomena in blood which really characterize saturation in the strictest sense."

Although the milligrams of ascorbic acid in the white cells and platelets per 100 cc. of whole blood are proportional in each patient with the vitamin C nutrition, these values are not so clearly informative as the white cell-platelet ascorbic acid concentrations. Further data from subjects with infection and shifting white cell and platelet counts should provide information on this point.

TABLE V

Apparent ascorbic acid content of whole blood in terms of the plasma, red cells, and white cells-platelets from patients with leukemia

Patient	Type of eukemia and therapy	Volume per 100 cc. blood		Mgm. ascorbic acid per 100 cc. by analysis			Calculated mgm. ascorbic acid per 100 cc. whole blood	
		Red cells	White layer	Plasma	White layer	Whole blood	In the plasma	In the white layer
I	Lymphatic leukemia	32.5	6.5	0.3	100	7.6	0.2	6.5
St	Myelogenous leukemia	30.7	6.5	0.3	47	4.3	0.2	3.1
Yo	Myelogenous leukemia	9.0	13.0	0.3	47		0.3	6.1
Hi	Lymphatic leukemia	33.0	6.0	0.1	48	2.8	0.1	2.9
Hi	Lymphatic+vitamin C	33.0	6.0	0.3	95	6.0	0.2	5.7
Pet	Lymphatic leukemia	15.0	8.7	0.0	30		0.0	2.6
Pet	Lymphatic+vitamin C	19.2	9.1	0.2	70	7.0	0.2	6.4
Sm	Lymphatic leukemia	30.1	3.7	0.2	90	4.0	0.1	3.3
Sm	Lymphatic+vitamin C	29.9	3.7	0.3	139	5.3	0.2	5.2

Stephens and Hawley (9), Cuttle (10), and Butler and Cushman (16) have reported high concentrations of an indophenol reducing substance in leukemic white cells. The rate of reduction of indophenol (Figure 3), and the reduction of methylene blue by the reducing substance of the white layer, is similar to that of ascorbic acid. Table V presents examples of the high reducing capacity of the white layer of centrifuged blood from leukemic patients. From the plasma, white layer and whole blood values it is seen that the relation between the ascorbic acid concentrations of the plasma and the apparent ascorbic acid concentration of these leukemic cells is very different from that in normal subjects. The white cell-platelet values exceed the maximum normal values even though the plasma levels are low. It is of interest that the reducing power of the white layer of the last three patients of Table V increased with an increase in vitamin C intake. However, though the relative reducing power of leukemic cells and platelets may be proportional to the vitamin C intake, their absolute reducing capacity does not reflect vitamin C nutrition as do the values reported in Table IV. It is for this reason that an exception has been made of leukemic subjects when concluding that the apparent ascorbic acid concentration of white cells plus platelets is an index of deficiency. A subsequent paper will deal with the identity of the reducing substance or substances found in the blood of leukemic subjects.

SUMMARY

Errors involved in the determination of the ascorbic acid concentration of whole blood are discussed.

Procedures for the analysis of the ascorbic acid content of red blood cells, white blood cells plus platelets, and whole blood are described.

Because the reducing power of the white cells plus platelets varies from an average normal of 34 mgm. of ascorbic acid equivalents per 100 grams of white layer to a level of approximately 0.0 in scorbutic subjects, we conclude that this reducing substance is ascorbic acid or some substance metabolically associated with it.

The data show that ascorbic acid passes from the plasma to the red cells and that the distribution ratio of the plasma concentration to apparent red cell concentration varies with the state of vitamin C nutrition.

They show that the apparent ascorbic acid concentrations of the white cells plus platelets and of the whole blood of individuals not suffering from infection or leukemia provide indices of vitamin C deficiency which extend beyond the limit of the index furnished by fasting plasma concentrations. They suggest that the apparent ascorbic acid concentration of the white cells plus platelets of such individuals is the best index of physiologically significant deficiency. They also indicate that the apparent ascorbic acid content of red blood cells or of whole blood is a better index of saturation, as differentiated from unsaturation or deficiency, than plasma or white cell-platelet concentrations.

The occurrence of very high concentrations of an ascorbic acid-like reducing substance in the white layer of centrifuged blood from leukemic patients is confirmed.

APPENDIX

1. CO saturation and precipitation of whole blood

Add 1 drop of caprylic alcohol to 2 cc. of whole blood and bubble CO through this for 10 minutes. While the CO is still passing through the sample, add 12 cc. of water and, after 5 minutes, 2 cc. of 32 per cent HPO_3. Thoroughly mix by means of the bubbles of CO for 30 seconds. Then immediately deliver the precipitated blood to a filter in a glass container through which CO is passing and collect the filtrate. The precipitate on the filter paper should be bright red. Darkening of the precipitate means loss of ascorbic acid by oxidation.

2. White blood cell-platelet analysis

Twelve to 15 cc. of whole blood are collected in a flask containing ammonium and potassium oxalate as described by Wintrobe (35). Determine the hematocrit on 0.5 cc. of the oxalated blood. Depending upon the hematocrit, 10 or 12 cc. of blood are transferred to a centrifuge tube constricted near the middle to a capillary 3 mm. diameter and 10 mm. length in which the white cells and platelets after centrifugation will be packed above the red cells. After centrifuging an hour or more at high speed the plasma is pipetted off. By means of a capillary pipette 10 to 20 milligrams of white layer are transferred to a centrifuge tube containing 0.5 cc. of 4.5 per cent HPO_3 which has been weighed with the HPO_3 before addition of white cells. A second weighing gives the weight of white cells and platelets. The cells are pressed with a glass rod, 1 cc. of 4.5 per cent HPO_3 is added, and the contents are shaken thoroughly.

After centrifugation, pipette 0.8 cc. off the supernatant fluid to a micro-colorimeter cell and add 0.2 cc. indophenol-acetate solution, containing equal parts of 5 per cent 2–6 dichlorophenolindophenol and 5/3 M acetate buffer. This gives the desired pH as described by Mindlin and Butler (31). The galvanometer reading of this mixture and of a metaphosphoric acid and indophenol-acetate solution dye blank is recorded and the results are calculated as stated in the body of this paper.

3. Methylene blue procedure

To 4 cc. of filtrate from whole blood treated by the CO saturation and metaphosphoric acid precipitation procedure described above are added 4 cc. of a 0.5 mgm. per cent buffered methylene blue solution. This buffered dye solution contains equal parts of 1.0 mgm. per cent methylene blue and the acetate buffer solution referred to (31). The resulting pH of the filtrate-dye-solution mixture should be about 4. A similar mixture containing no ascorbic acid is prepared for the blank dye solution. Place the colorimeter tubes containing the unknown-dye and blank-dye solutions in a beaker of water and place this before a 500 watt electric light with reflectors focused on the tubes in the beaker. After a 1-minute exposure, add 2 cc. 0.5 N HCl to each tube, immediately discontinue the illumination and mix the contents of each tube. Using a filter that transmits light from approximately 600 to 700 mμ, take the galvanometer reading in the photometer with the center setting adjusted to read 100 with the dye completely decolorized. Calculate the result as described, using a K value determined by analyses of known ascorbic acid solutions.

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