THE QUANTITATIVE DETERMINATION OF ASCORBIC ACID IN SMALL AMOUNTS OF WHITE BLOOD CELLS AND PLATELETS

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(Received for publication, January 2, 1947)

A knowledge of the concentration of ascorbic acid in the white blood cells and blood platelets has been shown to be of value as an addition to blood plasma analysis in evaluating nutritional status, particularly at low levels of ascorbic acid intake (1–5). Furthermore, since it is probably more convenient to obtain specimens of white blood cells than of any other living cells of the body, it seems likely that analysis of these cells will become of increasing value in studying the physiology of ascorbic acid and other nutritive essentials in body fluids and tissues. Stephens and Hawley (6) determined the ascorbic acid content of white blood cells by an analysis of the buffy layer removed from 50 ml. blood specimens. This layer contains both white cells and blood platelets. It was later shown by Butler and Cushman (1) that the concentration of ascorbic acid is nearly the same in both of these fractions. (Throughout this communication the white cells plus blood platelets will be referred to collectively as "white cells.") Butler and Cushman (1) simplified the collection of the white cells by centrifuging oxalated blood in special tubes with a central narrowed portion which converted the buffy coat into a well defined accessible column of cells. The required amount of blood, 15 ml., was subsequently reduced by Lubschez (7) to 4 or 5 ml.

By a procedure which will be described below, it has proved feasible to isolate the white cells from as little as 0.1 ml. of blood and to analyze them for ascorbic acid. Blood from the finger tip is diluted with isotonic potassium oxalate and centrifuged slowly to precipitate the red cells. The white cells, which settle more slowly, remain in the supernatant and are centrifuged in a second tube and analyzed for both ascorbic acid and acid-insoluble phosphate. The measurement of this nearly constant phosphate fraction permits calculation of the amount of white cells present without the necessity of weighing the rather minute sample. The procedure is more rapid than with larger quantities of blood and venipuncture is not required. Two persons can collect and analyze 50 to 75 samples in 2 days.

EXPERIMENTAL

Method

Reagents and Equipment—

1. Beckman spectrophotometer fitted with a special diaphragm and cuvettes to permit the use of 0.05 ml. fluid volumes (8). (Diaphragm and cuvettes obtained from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28.)

2. 6 × 50 mm. serological tubes, *e.g.* Kimble, No. 45060. Square racks made of $\frac{3}{8}$ inch wire mesh are convenient for handling up to 100 tubes.

3. Pipette for transferring blood (9), 5 to 6 mm. in diameter with a short tip narrowed to 1 mm. in outside diameter and with the opening at least 0.5 mm. in diameter. A coarse constriction placed at 0.1 to 0.12 ml. volume aids in preventing the accidental sucking of blood too far into the pipette. This pipette, although uncalibrated, is similar in construction to the quantitative constriction pipettes listed below.

The inside is paraffined by heating the pipette, sucking up molten paraffin, blowing it out, and, while still blowing, cooling with water. This leaves a thin film of paraffin effective in delaying blood clotting without unduly constricting the tip.

4. Footed stirring rod made of glass or stainless steel wire. The shaft is 1 to 1.5 mm. in diameter and 75 mm. long; the foot is 2.5 to 3 mm. in diameter and is flattened.

5. Pasteur pipette of 1 to 1.5 ml. capacity with bent tip for transferring white cell suspension.

6. Constriction pipettes, 10, 30, 40, 50, and 200 c.mm. (9).

7. Device for removing the supernatant from white blood cells. A small glass tube with a bent narrow tip not over 0.5 mm. in outer diameter is connected with rubber tubes, through a 20 to 50 ml. bottle, to the mouth.

8. 1.6 per cent potassium oxalate. This is preserved at 4° to prevent the growth of microorganisms and must be centrifuged just before use to remove possible traces of suspended material.

9. 5 per cent trichloroacetic acid.

10. 2 per cent dinitrophenylhydrazine, 0.25 per cent thiourea, 0.03 per cent $CuSO_4 \cdot 5H_2O$ in 9 N H_2SO_4. This reagent is stable for at least a week at 4°, but should be centrifuged before use unless crystal-clear. It is prepared from the stable solution of 2.2 per cent dinitrophenylhydrazine in 10 N H_2SO_4 by the addition of 5 volumes per cent of 5 per cent thiourea and 5 volumes per cent of 0.6 per cent $CuSO_4 \cdot 5H_2O$.

11. 65 per cent H_2SO_4 (70 ml. of concentrated H_2SO_4 plus 30 ml. of H_2O).

12. 6.5 N sulfuric acid.

13. 10 per cent perchloric acid.

14. Phosphate reagent. In 45 ml. of water is dissolved 0.3 gm. of a powder consisting of 5 per cent Na_2SO_3, 94.3 per cent $NaHSO_3$, and 0.7 per cent 1,4-aminonaphtholsulfonic acid. To this are added 5 ml. of 2.5 per cent ammonium molybdate in water.

Isolation of White Cells—In a 6 × 50 mm. tube are placed 0.5 ml. of 1.6 per cent potassium oxalate and a footed stirring rod. A finger is lanced to give a free blood flow, *e.g.* with a Bard-Parker No. 11 blade held by projection from a cork. After any residual alcohol is wiped off, the area is lightly coated with vaseline and the first blood blotted off. As rapidly as possible the paraffined pipette is filled and the blood delivered into the tube, the contents of which are at once gently but thoroughly mixed with the rod. (The pipette is rinsed with fresh oxalate but is not dried before reuse.) The tube is centrifuged within an hour at a predetermined slow speed which settles the red cells without loss of too many white cells. A safe time of centrifuging is twice the interval required just to throw the bulk of the red cells to the bottom. The centrifuge must come smoothly to a stop to avoid remixing of the sample. The turbid supernatant which contains the white cells is transferred with a Pasteur pipette to another tube of the same size. The suspension close to the red cell layer should be avoided, since this usually contains some red cells. The second tube is centrifuged (within 2 hours) at 3000 R.P.M. for 15 minutes. The clear supernatant is sucked off with the device for removing the supernatant from white blood cells, described above, and discarded. Great care is taken to remove a maximum amount of fluid without loss of the sediment. At first, a magnifying glass may prove helpful. If 90 per cent of the fluid is removed and the tube allowed to stand 5 or 10 minutes for drainage, the last 10 per cent can be removed more completely.

Ascorbic Acid Analysis—The white cells are distributed as thinly as possible by tapping the tube, and 40 c.mm. of 5 per cent trichloroacetic acid are added. For adequate extraction of ascorbic acid and acid-soluble phosphorus, it is imperative that the precipitate be well dispersed. A mechanical vibrator device (10) has been found very useful for vigorous mixing or agitation of the contents of these small tubes. After centrifuging, a 30 c.mm. aliquot of the supernatant is transferred to another 6 × 50 mm. tube and 10 c.mm. of the dinitrophenylhydrazine reagent are added. (The residue is saved for measuring the acid-insoluble phosphorus (see below).) After mixing and capping with parafilm or a vial stopper, the tube is incubated for 4 hours at 38° (bacteriological incubator or air bath).

After chilling in ice water, 50 c.mm. of ice-cold 65 per cent H_2SO_4 are added. Because of the viscosity the pipette should be emptied slowly. *Vigorous and thorough mixing is required* after this addition. Also, it is

advisable to tap the tubes with the finger to mix again just before reading. The light absorption is measured at 520 mμ in the spectrophotometer any time within the first 3 or 4 hours. Blanks and standards are provided by treating 30 c.mm. aliquots of 5 per cent trichloroacetic acid and of fresh 0.4 mg. per cent ascorbic acid (0.004 γ per c.mm.) in 5 per cent trichloroacetic acid in the same manner as the white cell extracts.

The ascorbic acid analysis may be deferred indefinitely if desired by storing the 30 c.mm. aliquots in well stoppered tubes at −20° or colder.

Acid-Insoluble Phosphorus Analysis—After the aliquot is removed for ascorbic acid analysis, the residue is washed (within 1 hour at room temperature or 4 hours at 4°) by mixing with 0.2 to 0.3 ml. of 5 per cent trichloroacetic acid, centrifuging, and sucking off the supernatant. 30 c.mm. of 4.5 N H_2SO_4 are then added and the sample (with others in a rack) is heated for 1 or 2 hours in an oven at 95–98° to drive off excess moisture without spattering, after which ashing is accomplished by adding 10 c.mm. of 70 per cent perchloric acid and heating in an oven for 2 hours at 145–160°.

Color is developed by adding 0.2 ml. of the phosphate reagent. The samples are vigorously mixed and are mixed again by tapping with the finger just before reading.

Blanks are supplied by measuring into 6 × 50 mm. tubes 30 c.mm. of 4.5 N H_2SO_4. Standards are provided by substituting an equal volume of 0.5 mM of KH_2PO_4 in 4.5 N H_2SO_4 (0.015 micromole of P). These blanks and standards are carried through the same evaporation and ashing procedures as the white cell samples. The optical absorption is measured at 690 mμ in not less than 20 minutes after mixing. Subsequent color development is slow and occurs at a uniform rate. Some of the standards are read both before and after the unknowns to permit correction for any increase in optical density with time.

Calculation—The white cells have been found to contain an average of 3.34 mM of acid-insoluble phosphorus per 100 gm. (see the "Discussion"). Thus, the mg. of ascorbic acid per 3.34 mM of acid-insoluble phosphorus (or micrograms per 3.34 micromole) are numerically equal to the concentration per 100 gm. of white cells. Therefore

(1) $$\frac{\text{Micrograms ascorbic acid in sample}}{\text{micromoles P in sample}} \times 3.34 = \text{mg. ascorbic acid per 100 gm. white cells}$$

(2) $$\text{Micrograms ascorbic acid in sample} = 0.004 \times \frac{D_{AA}}{D_{\text{standard AA}}} \times 41.5 = K_1 \times D_{AA}$$

where D_{AA} and $D_{\text{standard AA}}$ are the optical densities of the unknown and standard respectively, corrected for the blank. 41.5 is the sum of the 40 c.mm. acid added plus approximately 1.5 c.mm. of fluid left behind with the original white cell sediment, and 0.004 equals the micrograms of ascorbic acid per c.mm. of standard.

(3) Micromoles P in sample = micromoles P in standard $\times$

$$0.004 \times \frac{D_P}{D_{\text{standard P}}} = K_2 \times D_P$$

In a series of analyses the standards and their respective optical densities are fixed, and hence Equations 1, 2, and 3 may be combined:

(4) $$\frac{K_1 D_{AA}}{K_2 D_P} \times 3.34 = K_3 \frac{D_{AA}}{D_P} = \text{mg. ascorbic acid per 100 gm. white cells}$$

(The volumes of all the pipettes, except the 40 and 30 c.mm., cancel out, since they are used for both standards and unknowns.)

DISCUSSION

In the development of the above method there were two major problems: (1) the isolation of white cells from finger blood and (2) the measurement of the size of the white cell sample. Various diluting agents and anticoagulants were investigated: heparin, citrate, heparin plus oxalate, and oxalate diluted with saline or Ringer's solution. None proved as satisfactory as isotonic oxalate for the prevention of clotting. The slightest degree of clotting will precipitate the white cells with the red blood cells. Since finger blood, even with the greatest care in collection, coagulates more rapidly than venous blood, this consideration is of first importance.

The method of isolation of the white cells is similar to that described by Gorham *et al.* (11), who allowed undiluted oxalated blood to stand for several hours until the red cells were partially settled and then centrifuged the supernatant. It was found expedient in adapting this principle to much smaller quantities of blood to dilute the sample with isotonic potassium oxalate. This would be expected to increase the yield of white cells and to permit more complete removal of serum without washing the cells. It also proved to be more satisfactory to remove the red cells by slow centrifugation rather than to wait for gravity to accomplish the same purpose. It is conceivable that the dilution might wash out ascorbic acid from the white cells. Actually, this does seem to occur if hypotonic oxalate is used. With isotonic oxalate, however, there appears to be no loss, as judged by the agreement recorded below, with analyses of white cells isolated on a larger scale from undiluted blood. Furthermore, the

same ascorbic acid values were found, whether the white cells were centrifuged immediately or were allowed to stand 2 or 3 hours suspended in oxalate solution.

It seemed impracticable to attempt to weigh the minute white cell samples isolated. It also proved on trial to be unsatisfactory to estimate the white cell volume from the length of the column of packed cells in a calibrated capillary tube. An attempt was, therefore, made to find a cell constituent that is relatively constant in concentration and which would not be unduly affected by the moderate contamination with red cells or fibrin which sometimes occurs. Such a substance should furnish a valid measure of the sample size. The total phosphorus or a phosphorus fraction appeared to be worthy of trial.

The total phosphorus proved to be rather constant in concentration in the white cell and as a consequence has been used as the basis for many hundreds of determinations. However, to measure the total phosphorus it was necessary to suspend the white cells evenly after isolation in order to take aliquots for both phosphate and ascorbic acid, and this was sometimes quite difficult. Both acid-soluble and acid-insoluble phosphorus were next investigated and the latter proved to be the more satisfactory. The acid-insoluble phosphorus contained in the acid-insoluble residue from the entire sample was available for analysis, whereas only part of the acid-soluble fraction could be utilized, since the rest of the acid extract would be required for the ascorbic acid determination. Furthermore, contamination of the sample with red cells would cause a greater distortion of the acid-soluble than of the acid-insoluble fraction. This reasoning is borne out by actual analyses, which proved to be more consistent when based on the acid-insoluble fraction.

Ten macro white cell samples from different individuals were isolated by centrifuging in tubes with a central narrow segment (1) and were analyzed for acid-insoluble phosphorus. An average of 3.34 mM per 100 gm. with a standard deviation of 0.25 mM per 100 gm. was found. (The individual values were 3.53, 3.16, 3.46, 2.94, 3.16, 3.36, 3.60, 3.44, 3.02, and 3.78 mM per 100 gm.)

Although the correlation between this phosphorus fraction and the wet weight is thus not perfect, it is conceivable that acid-insoluble phosphorus may be a more physiological basis of calculation than weight.

The ascorbic acid micromethod used is a modification of that previously developed for serum (12). In order to simplify the over-all procedure the conversion of ascorbic acid to dehydroascorbic acid was effected by copper instead of charcoal. The charcoal reagent is apt to cause difficulty due to floating, and its use would have necessitated several extra steps. When the charcoal is replaced, the results tend to be somewhat more uniform.

although the values observed are 2 or 3 mg. per cent higher. Apparently, charcoal removes some material (not ascorbic acid) which reacts with the reagent.

Comparative Analyses—Table I gives the data for ten white cell samples isolated from different bloods and analyzed on both a macro and micro scale for ascorbic acid. The macro samples were obtained from venous blood by the procedure described by Butler and Cushman (1). The micro samples were obtained from the finger, as described above. The ascorbic acid was determined in both cases by the dinitrophenylhydrazine procedure; the macrodeterminations were based on the weight of sample, and the microdeterminations were based on the acid-insoluble phosphorus.

The standard deviation between the two methods is 3.6 mg. per cent, as

TABLE I

Comparison of White Blood Cell Ascorbic Acid Values Measured by Macro- and Microprocedures

The values are recorded as mg. per cent.

	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8	Serum 9	Serum 10	Average
Macro	27	30	23	25	27	24	32	26	19	25	25.8
Micro	26	27	21	31	27	31	29	25	17	26	26.0
Difference*	−1	−3	−2	+6	0	+7	−3	−1	−2	+1	+0.2

* Standard deviation between the two methods, 3.6 mg. per cent.

judged by these data. Part of this difference is undoubtedly due to inaccuracies in the micromethod, which is based on two separate determinations, one for ascorbic acid and one for acid-insoluble phosphorus. The standard deviation of the individual microanalysis was found to be 2 mg. per cent in a series of thirty-five determinations on nine different individuals. Another source of discrepancy between the macro- and micromethods may be ascribed to differences in the acid-insoluble phosphorus content of the different white cell samples. Finally, it is difficult to isolate white cells on a macro scale and obtain entirely uniform samples. There is a possibility of different degrees of packing with variations in the accompanying fluid, and minute clots, if formed, will collect in the white cell layer and are often difficult to detect.

Reproducibility of Values for Same Individual—The white blood cells of two individuals were analyzed at intervals during the day (Table II). No significant changes were observed over a 6 hour period. Table II also

TABLE II

Hourly Measurements of Ascorbic Acid in White Blood Cells of Two Individuals

Subject A				Subject B			
Elapsed time	Isolated white blood cells (calculated)*	Ascorbic acid in white blood cells	Average	Elapsed time	Isolated white blood cells (calculated)*	Ascorbic acid in white blood cells	Average
hrs.	*mg.*	*mg. per cent*	*mg. per cent*	*hrs.*	*mg.*	*mg. per cent*	*mg. per cent*
0	0.23	35		0	0.34	34	
	0.24	32			0.36	33	
	0.26	30			0.27	33	
	0.33	33	32		0.33	27	32
0.05	0.36	32		0.05	0.24	34	
	0.30	32			0.28	33	
	0.31	31			0.24	33	
	0.24	32	32		0.32	32	33
0.10	0.38	30		0.1	0.31	33	
	0.28	31			0.31	33	
	0.26	32			0.36	33	
	0.22	29	30		0.29	33	33
0.15	0.27	32		0.15	0.26	32	
	0.39	27			0.28	32	
	0.28	33			0.28	34	33
	0.22	31	31	0.2	0.33	32	
0.20	0.23	33			0.32	32	
	0.30	30			0.32	32	32
	0.24	30		2	0.30	31	
	0.17	31	31		0.26	28	
2	0.35	31			0.28	29	
	0.31	31			0.13	24	
	0.33	32			0.14	24	
	0.32	29			0.14	24	27
	0.30	31		4	0.35	36	
	0.34	27	30		0.43	32	
4	0.40	26			0.31	29	
	0.46	30			0.31	26	
	0.47	30			0.31	27	
	0.35	21			0.32	33	30
	0.34	30		6	0.24	34	
	0.18	26	27		0.14	29	
6	0.29	29			0.19	29	30
	0.37	31					
	0.30	30					
	0.22	28	30				

* Calculated from acid-insoluble P, as described in the text.

indicates the mass of white cells usually isolated (0.2 to 0.3 mg.) from 0.1 to 0.15 ml. of blood.

SUMMARY

1. A method is described for the measurement of the ascorbic acid concentration in white blood cells and platelets of 0.1 ml. of blood. The cells are isolated by differential centrifugation from finger tip blood. The mass of white cells isolated are measured indirectly through a determination of the acid-insoluble phosphorus of the sample. The coefficient of variation of individual microanalyses was found to be about 8 per cent and the coefficient of variation between the micromethod described and a macromethod was about 13 per cent. Two analysts can measure 50 to 75 samples in 2 days.

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