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THE DETERMINATION OF ASCORBIC ACID IN WHITE BLOOD CELLS

A COMPARISON OF W.B.C. ASCORBIC ACID AND PHENOLIC ACID EXCRETION IN ELDERLY PATIENTS

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INTRODUCTION

THE white blood cell (W.B.C.) ascorbic acid has been shown to be the most sensitive index of ascorbic acid deficiency, and onset of clinical scurvy occurs more quickly following white cell depletion than following plasma depletion (Bartley, Krebs and O'Brien, 1953 ; Crandon, Lund and Dill, 1940). Results of saturation tests following a loading dose of ascorbic acid are equivocal whether estimations are performed on 24 hour urine specimens, or 2 hour urine specimens obtained during peak excretion, and do not differentiate between cases of frank scurvy and patients on a low dietary intake of ascorbic acid (Bartley *et al.*, 1953).

Dobriner, Lavin and Rhoads (1942) have shown that patients with clinical scurvy excrete abnormally high amounts of phenolic acids. Boscott (1954) later identified the phenolic acids by chromatography and showed the presence of p.hydroxy phenyl acetic acid (P.H.P.A.) in the urine of cases of steatorrhœa, whose dietary intake of ascorbic acid was adequate but in whom an abnormally high consumption of ascorbic acid was necessary for normal tyrosine metabolism.

The present work was prompted because of the need for a simple white blood cell ascorbic acid method, and in the belief that because of the low excretions of ascorbic acid obtained in preliminary saturation tests in elderly people, admitted as patients, a correlation might exist between ascorbic acid levels and the excretion of abnormal phenolic acids.

METHODS

W.B.C. ascorbic acid

The method of Bessey, Lowry and Brock (1947) was modified to permit of easier separation of the white cells. Counting of the cells was adopted as an alternative to the tedium and inaccuracy of weighing a small sample with adherent fluid and red cells, or to an assessment of weight indirectly by an additional biochemical estimation of a cell constituent, for example acid insoluble phosphorus.

Reagents

(1) Diluent for blood. 200 ml. physiological saline
50 ml. 6% dextran
2·0 ml. 10% sequestrene.

This was prepared in bulk and distributed in 12·5 ml. amounts.

(2) Reagent 100 vols. 2·2% 2·4 dinitrophenylhydrazine in 10N H_2SO_4
5 vols. 5% thiourea
5 vols. 0·6% Cu SO_4 $5H_2O$

(3) 5% W/V trichloracetic acid A.R.

(4) 65% H_2SO_4

The method routinely adopted was the following.

Approximately, 3 ml. of blood was delivered into 12·4 ml. of diluent. The diluted blood was allowed to stand at room temperature for half an hour, during which time the majority of the red cells sedimented by gravity. The supernatant fluid containing the white cells and platelets was removed, thoroughly mixed and an aliquot of 0·2 ml. added to 0·8 ml. of W.B.C. diluting fluid and a white cell count performed on this. A measured 10–12 ml. volume of the leucocyte containing fluid was centrifuged at 3,000 r.p.m. for 15 minutes, and the supernatant discarded by inverting the tube and allowing it to drain for 30 seconds. To the compact button of white cells and platelets 1·3 ml. of 5% trichloracetic acid was added and the deposit thoroughly homogenised. The tubes were left at this stage at 2–4°C., and the estimations performed in batches within 3 to 4 days. The homogenised deposit was centrifuged and 1·0 ml. of the trichloracetic acid supernatant removed to a 3″ × ½″ test tube. 0·3 ml. of reagent was added and the tubes incubated at 37°C. for 4 hours. They were then cooled in ice-water and 1·5 ml. of 65% H_2SO_4 added. The extinctions were read in a Unicam absorptiometer at 520 mμ against a reagent blank. A standard curve consisting of 0·4, 0·8, 1·6, 4·0, and 10·0 μg. of ascorbic acid in 1 ml. volumes of 5% trichloracetic acid were similarly treated.

The ascorbic acid content of the white cells was expressed as μg/10^8 W.B.C.'s and calculated as follows :—

$$\frac{U}{S} \times 1{\cdot}3 \times \text{strength of standard} \times \frac{10^8}{\text{No. of W.B.C.'s in aliquot}} = \mu\text{g./}10^8 \text{ W.B.C.'s}$$

Plasma estimations were performed by the same colorimetric method on 1 ml. of supernatant obtained after centrifuging a mixture of 1 ml. plasma and 2 ml. of 5% trichloracetic acid.

Identification of urinary phenolic acids

Specimens of urine were collected between 2 and 4 p.m. without dietary restrictions, and the phenolic acids extracted by the method of Smith (1958). 20 ml. of urine was used for extraction and the extracted phenolic acids dissolved in a final volume of 1 ml. of ethyl alcohol. One way chromatograms using the solvent mixture isopropyl alcohol/ammonia/water were run overnight in an 8″ frame. These were run in batches and each paper was controlled with a normal urine, and two standards of 25 μg. and 10 μg. of p.hydroxyphenylacetic acid spotted on a normal phenolic acid free urine extract. 20 μl. of the phenolic acid extracts were spotted in a 3 to 4 mm. diameter area. The use of standards of known concentration, for comparison against the urine extracts rendered the method semi quantitative. The colours were developed in diazotised diethyl amino ethyl p. amino phenyl sulphone (I.C.I.5091).

Collection of specimens

Blood and urine specimens were obtained from 23 male and 27 female consecutive patients on routine admission to the geriatric unit. No selection of patients was made, but the patients mostly were old age pensioners, with little additional income. Specimens were again obtained from a further small group after the administration of ascorbic acid, no dietary restriction being imposed.

Normal blood and urine specimens were obtained from 17 male and 8 female members of the hospital staff.

RESULTS

Ascorbic acid content of W.B.C.'s.

Twenty-five normal persons aged 18–45 gave a W.B.C. ascorbic acid range of 21 to 53 μg./10^8 W.B.C.'s (average value 35 μg./10^3 W.B.C.'s). Fifty patients aged 65 to 91 gave a range of 2·0 to 36 μg./10^8 W.B.C.'s (average value 13·4 μg./10^8 W.B.C.'s). Fig. 1 shows the distribution of values for the patients and normals.

Distribution of ascorbic acid between W.B.C.'s and suspending media

During the course of routine ascorbic acid estimations, it was found that when white cells and plasma were allowed to stand in contact with the diluent at 2 to 4°C., the cell content of ascorbic acid increased and was quite marked after 4 to 6 hours.

TABLE 1

The uptake of ascorbic acid from plasma by white cells suspended in diluent

μg. of ascorbic acid/10^8 W.B.C.'s			% increase in W.B.C. ascorbic acid	Average % uptake
Time (hrs.)		Difference		
0	4			
53·8	75·8	22·0	41	
34·0	44·5	10·5	31	
42·3	50·0	7·7	18	
35·3	42·5	7·2	20	35·4
23·6	30·4	6·8	29	
31·6	48·9	17·3	55	
30·2	46·5	16·3	54	

This is shown in Table 1 the average increase after this time being 35·4%. The increase also occurred when the white cells were left in contact with plasma or whole blood. The process was accelerated by incubation at 37°C., and was completely inhibited by 1·6% potassium oxalate, the diluent used by Bessey *et al.* (1947). Uptake of ascorbic acid by white cells was of similar degree when the diluent was adjusted to pH 8·0 or 6·5, although lower values were obtained at the higher pH.

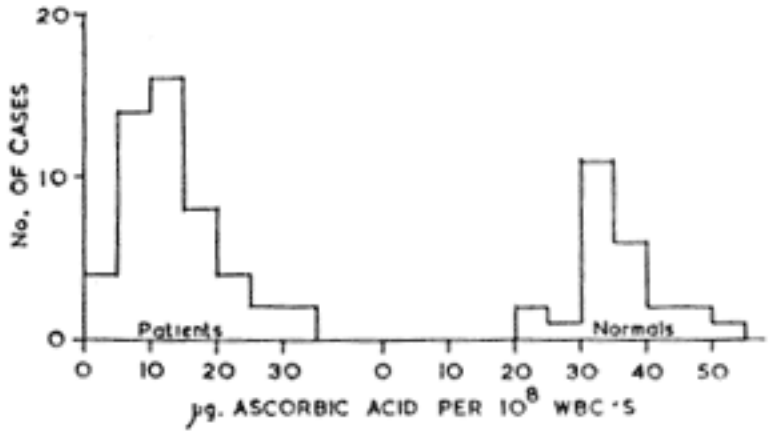

Fig. 1.

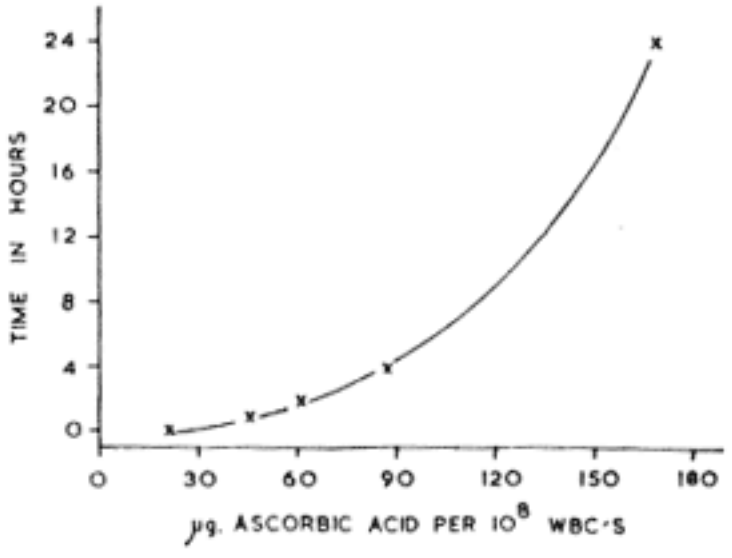

Fig. 2.

Fig. 1. Distribution of W.B.C. ascorbic acid levels in 50 patients and 25 normal persons.

Fig. 2. The uptake of ascorbic acid by white cells suspended in plasma + diluent with added ascorbic acid at a concentration of 2·5 mgs./100 ml. diluent.

The addition of 2·5 mgms. of ascorbic acid to 100 ml. of diluent plus white cells resulted in a marked increase in the white cell ascorbic acid uptake which continued for 24 hours as illustrated in Fig. 2. The renal threshold for ascorbic acid is about 0·8 mgms./100 ml. and maximum values for W.B.C. ascorbic acid at this plasma level are of the order of 40 to 50 μg./10^8 W.B.C.'s. The added ascorbic acid represents a total level of about 3·0 mgms./100 ml. and the corresponding W.B.C. ascorbic acid content was 170 μg. after 24 hours. Since the weight/volume ratio of ascorbic acid is much higher in the cells than plasma it can be seen that this concentration gradient is maintained and that the uptake is not a process of simple diffusion.

To demonstrate a parallel decrease in plasma ascorbic acid, plasma and white cell ascorbic acids were determined in the following manner :—25 ml. of blood was collected into 50 mgms. of sequestrene (sodium salt) and the cells sedimented by centrifuging at 500 r.p.m. for 2 to 5 minutes. 8 to 10 ml. of the supernatant plasma containing the white cells was removed and centrifuged at 3000 r.p.m. for 5 minutes. The plasma was removed by inverting the tube and the button of cells and platelets resuspended in 2·6 ml. of plasma, resulting in a three to four fold increase in the concentration of white cells. 0·05 ml. of a 1 mgm./ml. solution of ascorbic acid was then added to ensure an adequate plasma level, and 1·3 ml. of the plasma with suspended white cells was removed to another tube. This was centrifuged immediately, the plasma removed by inverting the tube, and the separate white cells and plasma allowed to stand at room temperature for 4 hours. The remaining 1·3 ml. of white cells suspended in plasma were similarly treated after standing at room temperature for 4 hours. 2·6 ml. of 5·0% trichloracetic acid was added to the cells and 2·0 ml. of trichloracetic acid to 1 ml. of the plasma. Duplicate estimations were then performed on 1 ml. volume of the trichloracetic acid supernatants. The results are shown in Table 2, from which it can be seen that the leucocyte gain was accompanied by a similar plasma loss of ascorbic acid.

TABLE 2

The uptake of ascorbic acid by white cells from 1 ml. of plasma and the corresponding decrease in plasma ascorbic acid

	μg. Ascorbic acid in white cells separated immediately	μg. Ascorbic acid in white cells separated after 4 hrs.	Difference μg.	μg. Ascorbic acid in plasma before absorption	μg. Ascorbic acid in plasma after 4 hours in contact with white cells	Difference μg.
1.	15·2	20·0	4·8	12·6	8·5	4·1
2.	6·8	11·4	4·6	15·8	11·4	4·4

The phenolic acid excretion in normal and geriatric patients

Of 25 normal adults, 4 excreted detectable amounts of up to 0·5 mgms. P.H.P.A./20 ml. of urine. One excreted 1·25 mgms./20 ml. of urine. Of 50 geriatric patients, 7 excreted amounts up to 0·5 mgms./20 ml. urine, whilst 4 excreted amounts grossly

TABLE 3

The W.B.C. ascorbic acid levels in 11 patients showing the presence of urinary p.hydroxy phenylacetic acid and the W.B.C. ascorbic acid levels in 7 selected patients showing absence of urinary P.H.P.A.

W.B.C. ascorbic acid μg./10^8 W.B.C.'s	P.H.P.A.	W.B.C. ascorbic acid μg./10^8 W.B.C.'s	P.H.P.A.
6·1	++	2·5	−ve
4·5	+	2·0	−ve
16·6	+	5·1	−ve
13·7	+	8·3	−ve
5·4	+	7·6	−ve
18·7	+	7·6	−ve
35·3	+	6·1	−ve
36·1	++		
7·4	+		
9·8	++		
13·9	++++		

in excess of 1·25 mgms. Table 3 shows the complete lack of correlation between the excretion of abnormal phenolic acids and white cell ascorbic acid levels. 7 patients were given 200 mgms. of ascorbic acid daily for 2 weeks and white cell ascorbic acid levels again determined. The results are shown in Table 4. Ascorbic acid levels in all cases rose after treatment to levels within the normal range. In one case showing the presence of P.H.P.A. before treatment the abnormal phenolic acids disappeared after treatment. In a second case they persisted after treatment. In a third case showing no P.H.P.A. before treatment the latter appeared following treatment.

TABLE 4

The W.B.C. asorbic acid levels and urinary P.H.P.A. in 7 patients before and after treatment with 200 mgms. ascorbic acid daily for 2 weeks

	Before treatment		After treatment	
Case	W.B.C. ascorbic acid μg./10^8 W.B.C.'s	P.H.P.A.	W.B.C. Asorbic acid μg./10^8 W.B.C.'s	P.H.P.A.
1.	7·3	−ve	38·1	−ve
2.	6·1	−ve	44·6	−ve
3.	12·8	−ve	32·6	+ve
4.	13·7	+ve	38·5	+ve
5.	4·5	+ve	31·8	−ve
6.	11·4	−ve	25·8	−ve
7.	12·3	−ve	56·8	−ve

Discussion

The low range of W.B.C. ascorbic acids levels found in the present group of patients is consistent with the low dietary intake of ascorbic acid, to be expected among poorer class old age pensioners. These low levels are not related to the excretion of abnormal phenolic acids which might be anticipated since it has been shown that abnormal phenolic acids are excreted in cases of frank scurvy, in premature infants on a high protein and low Vit. C intake, in mature infants given a tyrosine supplement, or in abnormal conditions such as steatorrhœa or megaloblastic anæmias in which an excessively high consumption of ascorbic acid is necessary for normal tyrosine metabolism. In the latter connection Zannoni (1960) has presented evidence to show that ascorbic acid protects P.H.P.A. oxidase from inhibition by its substrate. Boscott and Cook (1954) also found urinary P.H.P.A. in some cases of thyrotoxicosis, pernicious anæmia, liver disease and rheumatoid arthritis. A few normals also had small amounts present which disappeared after administration of ascorbic acid. In the present study the four cases showing abnormal amounts of urinary P.H.P.A. did not have minimal ascorbic acid levels, however the patients were routine hospital admissions and the above mentioned diseases or lack of dietary restrictions may have been responsible for the phenolic acid excretion.

The uptake of ascorbic acid from plasma by white cells against a concentration gradient is most interesting. This clearly indicates why the white cell level is the most sensitive index of depletion, since in the presence of such a concentration gradient even minimal amounts in the plasma are likely to be removed by the white cells. When the latter become depleted, body stores and circulating plasma levels are virtually nil.

The rate of uptake at different plasma levels has not been investigated, however it seems likely that in depleted subjects this will be rapid following a rise in plasma level and probably related to the degree of saturation.

SUMMARY

A simple method for determining the white cell ascorbic acid with enumeration of the cells is described. The normal range for 25 healthy adults was 20 to 50 μg./10^8 W.B.C.'s, whilst a range of 2·0 to 36 μg./10^8 W.B.C.'s was obtained for 50 geriatric patients. Low ascorbic acid levels in geriatrics are not related to the excretion of p.hydroxyphenylacetic acid.

White cells are capable of concentrating ascorbic acid from plasma.

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