CCLVIII. THE DETERMINATION OF ASCORBIC ACID IN BLOOD

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Titration with 2:6-dichlorophenolindophenol [Tillmans *et al.* 1932] and titration with methylene blue in bright light [Martini and Bonsignore, 1934] are the most important methods for the determination of ascorbic acid. The method most widely used is that of titration against 2:6-dichlorophenolindophenol in acid medium. Titration with methylene blue, however, is more specific because some sulphur-compounds (cysteine, ergothioneine and thiosulphate) which interfere with the titration with 2:6-dichlorophenolindophenol do not influence the titration of ascorbic acid with methylene blue. These interfering sulphur-compounds can be eliminated by precipitation with mercuric acetate [Emmerie, 1934; Emmerie & van Eekelen, 1934].

In our laboratory the determination of ascorbic acid in blood, which is of great importance for the diagnosis of vitamin C deficiency, is carried out by titration with 2:6-dichlorophenolindophenol after treatment of the trichloroacetic acid extract with mercuric acetate and H_2S [van Eekelen *et al.* 1937]. Recently Lund & Lieck [1937] have described a method for the estimation of ascorbic acid in blood serum by titration with methylene blue at *p*H 2–3. In this way only the reduced ascorbic acid present in serum can be detected; the ascorbic acid content of whole blood and of serum containing oxyhaemoglobin cannot be estimated by this method, for in the presence of oxyhaemoglobin ascorbic acid passes into the reversibly oxidized state when the proteins are precipitated [Kellie & Zilva, 1935; 1936; van Eekelen, 1936; Borsook *et al.* 1937; Johnson & Zilva, 1937].

According to Gabbe [1937] and Fischer [1937] ascorbic acid is not oxidized but adsorbed by oxyhaemoglobin during the precipitation of oxyhaemoglobin. If this is true, a quantitative determination of ascorbic acid in whole blood is impossible in the presence of oxyhaemoglobin [Gabbe, 1937]. We do not at all agree with this opinion. It appears from our earlier investigations [van Eekelen, 1936] that a definite quantity of ascorbic acid added to whole blood is recovered completely as well in the presence of oxyhaemoglobin as of reduced haemoglobin and of carboxyhaemoglobin. The following experiment shows that the loss of ascorbic acid in the presence of oxyhaemoglobin is only apparent because ascorbic acid is partly reversibly oxidized and can be regenerated by reduction with H_2S.

20 ml. of whole human blood (containing 20 mg. of potassium oxalate to prevent clotting) are aerated and 1 ml. of a solution of crystalline ascorbic acid (Hoffmann-La Roche) in 0·85% NaCl (containing 4·03 mg. ascorbic acid per ml.) is added. 20 ml. of the same blood are saturated with CO_2 and the same quantity of ascorbic acid is added. The ascorbic acid content of the blood being 0·17 mg. per 20 ml., the total quantity in both samples amounts to 4·20 mg. To both samples 20 ml. of a 10% solution of trichloroacetic acid are added, the mixture is centrifuged and the ascorbic acid content of the supernatant liquid determined. 10 ml. of the supernatant liquid are neutralized with $CaCO_3$ and 2 ml. of a 20% solution of mercuric acetate are added to remove interfering reducing substances. The precipitate is centrifuged, the supernatant liquid is treated with H_2S, filtered and, after standing overnight, titrated after removal of H_2S.

	mg. ascorbic acid per 10 ml. trichloroacetic acid extract	
	After precipitation with CCl_3COOH	After reduction with H_2S
Aerated blood	0·55	1·03
Blood saturated with CO_2	0·99	1·03

These observations demonstrate that in the presence of oxyhaemoglobin a considerable part of the ascorbic acid is reversibly oxidized. The quantity of ascorbic acid in both samples is recovered completely after reduction with H_2S (10 ml. of the blood extract contain 1·03 mg., the total amount of the extract, 41 ml., contains $4·1 \times 1·03 = 4·22$ mg.).

We prefer the determination of ascorbic acid in whole blood since the ratio of the ascorbic acid content of erythrocytes to that of plasma varies under abnormal conditions (e.g. vitamin C deficiency or anaemia) [Heinemann, 1936].

To investigate if our extracts of whole blood still contain interfering reducing substances after treatment with mercuric acetate and H_2S, parallel determinations have been carried out with 2:6-dichlorophenolindophenol and with methylene blue.

Experimental

The extracts of the blood are prepared by the method described by us [van Eekelen *et al.* 1937] and titrated (1) according to our method with 2:6-dichlorophenolindophenol and (2) with methylene blue after Lund & Lieck [1937]. The quantity of methylene blue reduced by a given amount of ascorbic acid depends besides other factors on the *p*H of the medium [Neuweiler, 1936]; therefore we have standardized the methylene blue solution by titrating 2 ml. of the blood extract with and without addition of a given quantity of pure ascorbic acid. Blood extract and methylene blue solution have nearly the same *p*H (4·5). Under our experimental conditions, 1 ml. of methylene blue solution corresponds to 14·8γ of ascorbic acid. The quantity of methylene blue used for titration of 2 ml. extract of ten different specimens of whole human blood was observed to vary from 0·13 to 0·88 ml. In the following table results of parallel determinations are given.

	mg. ascorbic acid per litre blood		
Sample	Methylene blue method	2:6-Dichlorophenol-indophenol method	Difference
1	2·4	2·8	+0·4
2	3·8	3·7	−0·1
3	6·7	6·4	−0·3
4	7·4	6·9	−0·5
5	9·8	9·2	−0·6
6	10·6	10·6	0
7	12	11	−1
8	12·6	12·4	−0·2
9	13	12·8	−0·2
10	16·3	16·1	−0·2
		Average	−0·27

From this table it will be seen that the values by both these methods closely agree with each other. Substances other than ascorbic acid which reduce both methylene blue and 2:6-dichlorophenolindophenol may still occur in the blood filtrate but cannot at present be detected.

SUMMARY

The ascorbic acid content of extracts of whole human blood prepared according to our method has been determined by titration against 2:6-dichlorophenolindophenol and methylene blue. The same values having been found, we may conclude that all the interfering substances which reduce 2:6-dichlorophenolindophenol and which do not reduce methylene blue are removed by precipitation with mercuric acetate.

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