CCLXV. THE DETERMINATION OF ASCORBIC ACID IN URINE WITH PHOSPHO-18-TUNGSTIC ACID.

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THE use of 2:6-dichlorophenolindophenol as an indicator in the quantitative estimation of ascorbic acid is open to the objection that the reaction is non-specific in that phenols and thiol compounds (especially cysteine) reduce the reagent. Birch [1933] has shown that in acid solutions glutathione does not interfere. At p_H 3 the action of phenols is suppressed or delayed [Johnson and Zilva, 1934] though cysteine reacts. Emmerie and van Eekelen [1934] removed cysteine by precipitation with mercuric acetate. The method, as worked out by them, whilst yielding more highly accurate results requires several hours (overnight) for completion. In all cases the end-point of the reaction is far from clear and varies somewhat with the speed of titration.

The reduction of phospho-18-tungstic acid by ascorbic acid is the basis of the method proposed here. It offers the advantages of simplicity, speed and accuracy. By carrying out the reaction in acid solution none of the other reducing substances of urine except thiol compounds interfere [Lugg, 1934]. Since the action of thiol compounds can be prevented by addition of formaldehyde [Mason, 1930] or of mercuric salts [Shinohara, 1935] the reaction becomes highly specific for ascorbic acid.

Method.

Reagents. *M* formaldehyde.

Sodium acetate-acetic acid buffer at p_H 5 [Shinohara, 1935] made in the proportion of 100 ml. of 2 *M* sodium acetate to 30 ml. of 2 *M* acetic acid.

Folin's uric acid reagent [Folin, 1934].

Ascorbic acid standard, 0.001 *M*. This solution is unstable and oxidation sets in immediately. It may be preserved for a number of days if saturated with H_2S and kept in an ice-box. H_2S must be blown out with nitrogen or carbon dioxide before using. The colour standards are stable for a period of several days if kept in the cold and dark.

Procedure. To 5 (1-5) ml. of urine in a 25 ml. volumetric flask add 1 ml. of formaldehyde solution. Into three other 25 ml. volumetric flasks pipette 1, 2 and 4 ml. of the ascorbic acid standard respectively. To each flask add 6.5 ml. of the buffer solution followed by 1 ml. of the uric acid reagent. Adjust the volume and read in the colorimeter after 20 min.

Calculation. $0.001 \times \frac{N}{n} \times \frac{20}{R} \times 0.1 = g.$ mols. ascorbic acid per 100 ml. of urine, where *N* represents ml. of standard, *n* represents ml. of urine and *R* reading of colorimeter with standard set at 20.

Results.

Rate of colour development. The blue colour produced by ascorbic acid with phospho-18-tungstic acid reaches full intensity in about 3 min. at room temperatures ($26^\circ \pm 4^\circ$). Addition of formaldehyde inhibits the rate of colour development though it does not prevent full intensity from being attained. Fig. 1

shows the effect of addition of 1 ml. of 1, 5, 10 and 37 % formaldehyde respectively. In all cases the reaction was completed within 20 min. When ascorbic acid is added to urine in the presence of 1 ml. of *M* formaldehyde in a final volume of 25 ml., the time required for the colour to reach a constant level varies with the sample of urine from 8 to 15 min.

Fig. 1. Increase in colour intensity *I* with time (min.) of solutions of ascorbic acid in the presence of varying amounts of formaldehyde. 1 ml. of 0.001 *M* ascorbic acid, 6.5 ml. of sodium acetate buffer, 1 ml. of phospho-18-tungstic acid reagent and 1 ml. of formaldehyde of varying concentrations in 25 ml. of solution. *A*, no formaldehyde; *B*, 1 % formaldehyde; *C*, 5 %; *D* 10 %; and *E*, 37 %.

Table I. *Proportionality between colour intensity and concentration of ascorbic acid in pure solutions and in urine. The determinations were made in each case in a total volume of 25 ml.*

Standard ascorbic acid 0.001 <i>M</i> ml.	Test sol. ascorbic acid 0.001 <i>M</i> ml.	Urine ml.	<i>R</i> Standard	<i>R</i> Test sol.	Found test sol. <i>M</i>	Recovered %
1	0.5	—	20	39.5	—	99
	1	—	20	20.0	—	100
	2	—	20	10.0	—	100
	4	—	20	5.0	—	100
	6	—	20	3.3	—	100
	8	—	20	2.6	—	96
(per 100 ml.)						
4	—	2	10	29.0	4.14	—
	—	5	20	23.3	4.12	—
	—	10	20	11.7	4.10	—
(per sample)						
4	—	5	20	15.8	2.53	—
	2	5	20	8.9	4.49	98
	4	5	20	6.1	6.55	100
	6	5	20	4.7	8.51	100

Ascorbic acid concentration and colour intensity. A series of standards is recommended in the procedure given above although the proportionality between concentration and colour holds over such a wide range that fewer standards may be employed as shown in Table I. In the first group of data it may be seen that in pure solutions of ascorbic acid the unknown may contain as much as six times the standard solution and yet be determined accurately. In the second group the ascorbic acid in 2, 5 and 10 ml. samples of urine was determined with an error of 0.5% using a single standard colour mixture. In the third group, 2, 4 and 6 ml. of 0.001 *M* ascorbic acid added to 5 ml. of urine were determined with one standard solution within an error of 2%.

Relative colour intensities produced by ascorbic acid and cysteine. A comparison of colour produced by ascorbic acid with those produced by cysteine reduced by tin powder in HCl and by cysteine standardised by the method of Shinohara [1935] indicates that cystine and ascorbic acid in equimolar concentrations produce the same colour intensity (*M* ascorbic acid = 2 *M* cysteine). This interchangeability of standards in cysteine and ascorbic acid determinations may prove convenient, especially in determinations of the former, since pure ascorbic acid is more readily obtainable than is pure cysteine.

Stability of colour standards. If the colour mixtures are kept in the cold, the intensity remains constant over a period of several days. Six tests on mixtures made up 5 days previously and kept in a refrigerator at 0–2° showed 100% colour intensity.

Oxidation of ascorbic acid. Ascorbic acid in urine oxidises rapidly, the rate depending upon temperature, intensity of light and p_H . Johnson and Zilva [1934] state that when urine is immediately acidified with sulphuric acid the ascorbic acid remains stable for several days. Emmerie and van Eekelen [1934] reduced the reversibly oxidised compound with H_2S . If freshly voided urine is acidified with acetic acid and saturated with H_2S the ascorbic acid remains unchanged for at least 24 hours.

Procedure. Pipette 5 (1–5) ml. of urine into a test-tube, add 1.5 ml. of 2 *M* acetic acid and saturate with H_2S , washing off the tip of the H_2S tube with a few drops of water. When ready to complete the determination bubble carbon dioxide or nitrogen through the solution for 20–30 min. Wash the tip of the generator tube, add 5 ml. of 2 *M* sodium acetate, 1 ml. of *M* H.CHO solution and 1 ml. of the uric acid reagent. Adjust the volume and read in the colorimeter after 20 min.

Fig. 2 depicts three curves (*A*, *B* and *C*) representing the rate of oxidation of ascorbic acid in three samples of the same urine adjusted to p_H 5.0, 6.5 and 8.0 respectively and kept in the light at $27^\circ \pm 3^\circ$. After various intervals of time portions were removed, treated as above and kept with the H_2S for 12 hours. The broken lines indicate the subsequent increases in their reducing ability.

As may be seen, rates of oxidation were approximately the same at p_H 5.0 and 6.5. At p_H 8.0 oxidation occurred rapidly, about 40% having been lost after 4 hours. At this time the reaction was completely reversible as shown by the broken line. After 8 hours, 66% of the ascorbic acid was oxidised in the alkaline medium, about 10% subsequently failing to become reduced with H_2S . After 12 hours only 18% remained unoxidised. At this time very little reversibility of the reaction was shown. At the end of 24 hours no unoxidised ascorbic acid could be detected in the alkaline medium and no reversibility of the reaction could be demonstrated. On the other hand, the ascorbic acid in the acid solutions had lost only about 40% of its reducing ability and regained the latter completely after treatment with H_2S .

Comparison of results with the phospho-18-tungstic acid and dichlorophenol-indophenol methods. Series of determinations made by these two methods agreed within $\pm 8\%$. Repeated determinations on the same sample of urine

Fig. 2. Rate of oxidation of ascorbic acid in urine at various pH . Abscissa represents time in hours and ordinate, I , intensity of colour developed. The broken lines indicate degree of reduction of the oxidised ascorbic acid after different stages of oxidation. 5 ml. of urine, 6.5 ml. of acetic acid-acetate buffer, 1 ml. of M formaldehyde and 1 ml. of uric acid reagent per 25 ml. of mixture.

by the method proposed here showed variations of less than 2%, whereas in our hands the variations with the indicator method showed much wider discrepancies between repeated determinations on a single sample.

Inhibition of colour production by cysteine by means of mercuric chloride. Mercuric chloride added to a solution of cysteine completely inhibits the action of the latter upon phospho-18-tungstic acid. Mercuric chloride also prevents the development of colour by ascorbic acid unless sulphite is present in the reaction mixture. Cysteine in the presence of mercuric chloride and sulphite gives no colour with the uric acid reagent.

Procedure. To 5 (1–5) ml. of urine in a 25 ml. volumetric flask, add 1.5 ml. of M $NaHSO_3$ followed by 6.5 ml. of sodium acetate buffer, 2 ml. of 0.1 $HgCl_2$ and 1 ml. of the uric acid reagent. Adjust the volume and read after 20 min.

In this procedure it is important that the bisulphite be added before the mercury salt since otherwise the latter partially oxidises the ascorbic acid and bisulphite fails to reduce it again. The degree of oxidation depends upon the time interval between the addition of these two components of the reaction mixture, and may be only 5% if the bisulphite is added immediately after the mercury salt.

Sodium sulphite may be substituted for the sodium bisulphite with observation of the same precaution as to order of addition of the various constituents of the mixture. Sodium sulphite produces an appreciable colour with the uric acid reagent and theoretically a blank should be subtracted. However, the error in determining the blank is so great that deducting it fails to reduce the error.

Table II gives a series of comparative determinations by the (a) formaldehyde, (b) mercuric chloride and bisulphite, (c) mercuric chloride and sulphite, and (d) indicator methods. As may be seen, the results by the two first methods are in almost perfect agreement. The other two methods show wider variation.

Table II. *Ascorbic acid excreted during 3-hour periods by one individual on diets of varying ascorbic acid content, as determined by the formaldehyde, mercuric chloride and bisulphite, mercuric chloride and sulphite and indicator methods.*

$M \cdot 10^{-4}$			
H.CHO	HgCl ₂ and bisulphite	HgCl ₂ and sulphite	Titration
2.00	2.00	1.94	2.15
3.20	3.20	3.25	3.40
7.61	7.57	7.58	7.34
1.07	1.07	0.99	1.29
5.32	5.32	5.37	5.63

Reduction of oxidised ascorbic acid. The only one of the reducing agents employed here which is able to reduce the reversibly oxidised compound is H₂S. Sulphite, even after 12 hours, reduces it only slightly. Tin dust and HCl also produce only slight reduction. In one instance a 0.002 *M* solution of ascorbic acid, on standing in the laboratory for 5 hours had lost 30% of its reducing ability. It could not be reduced with tin powder or sulphite, but after treatment overnight with H₂S all of its original reducing power was recovered.

SUMMARY.

A method is described for the determination of ascorbic acid in urine by use of Folin's phospho-18-tungstic acid reagent.

Phenols and thiol compounds do not interfere with the test.

A simple method is described for the preservation of urine for several hours. It may also be employed for reduction of the reversibly oxidised ascorbic acid.

An alternative method is offered with mercuric chloride and sodium bisulphite. With sodium sulphite the error is greater.

The findings by the formaldehyde method agree within about $\pm 8\%$ with those obtained by the dichlorophenolindophenol method.

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