CCXLV. OBSERVATIONS ON THE USE OF THE PHOSPHOTUNGSTIC ACID METHOD OF DETERMINING ASCORBIC ACID IN URINES WITH LOW ASCORBIC ACID CONTENT.

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SINCE publication of the method for the determination of ascorbic acid in urine with phospho-18-tungstic acid [Medes, 1935], special difficulties attendant upon its use in urines of extremely low ascorbic acid content have been pointed out to the author by Dr J. C. Andrews and by Dr G. A. Harrison, for whose suggestions she would like to express her appreciation.

1. *Colour.* Urines containing as little as approximately 4×10^{-4} g. mol. ascorbic acid per 100 ml. develop a blue colour with phospho-18-tungstic acid of such intensity that the yellow colour of the urine does not usually interfere in the colorimetric determinations. When, however, the ascorbic acid content falls greatly below this figure, the urinary pigments may predominate to such an extent that a pale yellowish green colour results. The difficulty may be met by a number of devices.

(a) When the interference is slight, the colour of the standard may be slightly altered by the introduction of a few drops of one of the indicators which are yellow in acid solution, such as bromophenol blue, as suggested by Shinohara & Padis [1936]. This method necessitates a series of standards containing graded amounts of the indicator. When the colour is a greenish yellow, such large amounts of the indicator must be added that an appreciable error is introduced, and this method cannot be employed.

(b) A colour filter (Monochromat Wratten No. 75, Eastman Kodak Company) in the ocular of the colorimeter, employed by Dr Andrews, proved satisfactory in a series of tests made by the author.

(c) Backing the standard by urine, diluted similarly to that in the test solution. In the author's laboratory, the McClendon compensating cups for hydrogen ion determinations are employed in routine determinations made directly on urine. The only precaution to be observed is that the standard and unknown must be of approximately the same intensity; otherwise satisfactory compensation is not attained.

2. *Cloudiness of the urine.* In urines extremely low in ascorbic acid a faint cloudiness frequently develops when the uric acid reagent is added (Andrews and Harrison, private communications). No explanation is offered for this phenomenon. Neither the author nor the investigators mentioned above have observed its occurrence in urines containing more than a trace of the vitamin. According to Harrison, some of these specimens fail to clear on centrifuging. Accurate readings cannot be secured in the colorimeter, values as high as ten times those determined by the titrimetric method being obtained. The colorimetric results in Table I are from determinations made by J. C. and K. C. Andrews on uncentrifuged specimens.

Table I. *Comparison of the determination of ascorbic acid in 4 samples of urine with phospho-18-tungstic acid and by titration with dichlorophenolindophenol.*

Colorimetric method			Titration method		
Av. value g. mol. $\times 10^{-6}$ per 100 ml.	Av. deviation %	Max. deviation %	Av. value g. mol. $\times 10^{-6}$ per 100 ml.	Av. deviation %	Max. deviation %
4.92	14.0	21.0	5.15	10.9	17.4
12.7	9.3	16.2	14.6	9.7	21.2
21.7	2.6	4.4	27.3	7.0	15.4
39.7	1.5	2.5	37.9	5.1	9.3

In the author's laboratory 100 specimens were investigated, all those giving more than a faint green colour with the reagents being rejected. About half of those selected showed faint cloudiness and 25 with appreciable cloud were retained for further study. To each one the reagents were added, the specimen was transferred immediately to the centrifuge and centrifuged at highest speed for 15 min. while the colour was developing. In all cases a clear specimen was obtained.

3. *Time element in development of the blue colour with the uric acid reagent.* In the original description of the phospho-18-tungstic acid method it was stated that readings in the colorimeter should be taken after 20 min. In many urines, colour continues to develop even after this period. This is not the case with pure solutions of ascorbic acid. In other urines, the colour remains constant from about 15 min. after the reagents are added up to several hours. It was concluded, therefore, that this persistent development of colour was not due to the ascorbic acid, but to some unknown factor and that the reading taken immediately at the close of the 20-min. period represents most nearly the true ascorbic acid value.

It is not known to what factors this gradual increase of colour is due. Shinohara [1935] investigated the effect on the uric acid reagent of a number of the normal constituents of urine, and although he found that several of them caused a slow appearance of colour, none produced an appreciable effect within 20 min. when present in physiological amounts. The author has extended these experiments, using varying combinations of these and other constituents, to determine how far their effects may be additive, but failed to account for the reaction. It seems possible that the urinary pigments, which probably have some reducing properties, may be at least partially responsible, since the phenomenon occurs most frequently in highly coloured urines. The precaution of taking the colorimeter readings at the close of the 20-min. period ± 5 min. should therefore be observed for all urines.

Accuracy of the method in urines containing small amounts of ascorbic acid. From a group of urines from the hospital wards, 4 were selected for comparison of the two methods, colorimetric and titration with dichlorophenolindophenol. The concentrations of ascorbic acid ranged from about 4.0×10^{-5} g. mol. per 100 ml., or approximately matching the 2 ml. standard, to 5.0×10^{-6} g. mol. per 100 ml., with which a standard containing 0.5 ml. of 0.001 *M* ascorbic acid should be employed against 10 ml. of urine. Table I gives the average values obtained in a series of 5 consecutive determinations by each method, with the average and maximum deviations in each group, expressed in percentages of the mean.

When about 5×10^{-6} g. mol. ascorbic acid was present, the maximum deviation from the mean was about 20% by the two methods, the average deviations being 14 and 11% respectively. As the concentration of ascorbic acid was

increased, the error of a single determination decreased much more rapidly by the colorimetric than by the titration method, until when about 4×10^{-5} g. mol. ascorbic acid was present, the maximum deviations from the mean became 2.5 and 9.3 % respectively.

A further test of the relative accuracy of the two methods was performed by adding increments of standardized ascorbic acid to the urines employed above and determining the percentage recovery. When 5 ml. of 0.001 *M* ascorbic acid were added to 100 ml. of the urines with lowest vitamin content, the average recovery ranged between 95 and 106 %, with average deviations approximately agreeing with those in the table for the corresponding urines. When 1 ml. of 0.01 *M* ascorbic acid was added to 100 ml. of the same urines, the recovery averaged 99 ± 5 % by the colorimetric and 104 ± 11 % by the titration method.

In other words, with extremely low concentrations of ascorbic acid the two methods are about equally accurate in the hands of the present investigator, and with concentrations of ascorbic acid which give rise to a blue colour with the phosphotungstic acid reagent, the colorimetric becomes the method of choice.

SUMMARY.

In using the phospho-18-tungstic acid method for the determination of ascorbic acid in urine, special precautions must be employed for urines with very low ascorbic acid content.

Suggestions are offered for compensation for the yellow colour developed when the urinary pigment interferes with comparison of standard and unknown.

The cloud which frequently develops in these urines may be removed by centrifuging.

Readings in the colorimeter should be taken within 20 ± 5 min.

In a series of tests on urines with low ascorbic acid content, the colorimetric and titration methods are of about equal accuracy. With urines of higher ascorbic acid content, the colorimetric method gives more highly accurate results.

REFERENCES.

- Medes (1935). *Biochem. J.* **29**, 2251.
Shinohara (1935). *J. biol. Chem.* **110**, 263.
—— & Padis (1936). *J. biol. Chem.* **112**, 709.