THE STABILITY AND STATE OF ASCORBIC ACID IN URINE.

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The stability of ascorbic acid in urine has been investigated in presence of different acids. The presence of combined ascorbic acid in urine is shown.

Recently vitamin C sub-nutrition has been associated with the etiology of various diseases. This vitamin C sub-nutrition is diagnosed by urine analysis. As the rate of excretion of ascorbic acid in urine is not uniform throughout the day and night, ascorbic acid content of 24 hours' urine should be determined. But ascorbic acid in urine is gradually lost on standing. Borsook *et al* (*J. Biol. Chem.*, 1937, **117**, 237) have shown that in an alkaline medium ascorbic acid may be oxidised to dehydroascorbic acid and 2 : 3-diketo-*l*-gulonic acid. The latter reaction being irreversible, only dehydroascorbic acid would be reduced to ascorbic acid when sulphuretted hydrogen is passed into the urine. It is, therefore, important to know under what conditions vitamin C in urine collected over 24 hours would be most stable. Although some work has been carried out on the subject, the position is by no means clear. As we also propose to investigate the state in which ascorbic acid is present in urine, it is necessary to know the factors concerned with the stability of vitamin C in urine in our conditions of experiment.

Freshly passed urine was treated separately with acetic acid, metaphosphoric acid, hydrochloric acid, sulphuric acid and diethyl dithiocarbamate. The last reagent was used in order to prevent any possible oxidation of vitamin C by means of traces of copper. The concentration of acids in urine was 5% by volume in each case ; the concentration of diethyl dithiocarbamate was 0·06 g. in 100 c.c. of urine. Samples of freshly voided urine were treated as above. Some samples were then titrated immediately with 2 : 6-dichlorophenol-indophenol (1 c.c. dye≡0·104 mg. of ascorbic acid) and others were kept overnight and titrated after 24 hours. The average results with the different treatments are shown in Table I.

Table I.

Urine kept with	1 C.c. of the dye was decolorised by: Freshly voided urine.	Urine preserved for 24 hours.
Acetic acid	1·45 c.c.	6·70 c.c.
Metaphosphoric acid	,,	1·90
Hydrochloric ,,	,,	1·45
Sulphuric ,,	,,	1·45
Diethy dithiocarbamate	,,	3·10

It is clear from Table I that hydrochloric and sulphuric acids preserve the vitamin best. Metaphosphoric acid is next best, while acetic acid, which has been most commonly used for preservation, is the least effective. Diethyl dithiocarbamate is also largely ineffective. Acetic acid and metaphosphoric acid impart no colour to the urine but sulphuric acid and hydrochloric acid impart colour to the urine. Hydrochloric acid gives a deeper colour to the urine than sulphuric acid, so that sulphuric acid is considered to be the best preservative under our conditions of experiment.

In order to find how far this stability produced by sulphuric acid is specifically related to ascorbic acid, different samples of urine treated with sulphuric acid were subjected to treatment with ascorbic acid oxidase. Results are shown in Table II. The fact that figures in columns 3 and 5 are substantially in agreement shows that sulphuric acid has not produced any non-specific reducing substances *de novo*. On the other hand very substantial differences between values obtained before and after oxidase treatment indicate that titrations of urine without such treatment would lead to erroneous results.

TABLE II.

Urine sample No	C.c. of urine required to reduce 0·5 c.c. of the dye: Urine (with H_2SO_4, 5%) freshly voided. Before oxidase action.	Urine (with H_2SO_4, 5%) freshly voided. After oxidase action.	Urine (with H_2SO_4, 5%) after 24 hours. Before oxidase action.	Urine (with H_2SO_4, 5%) after 24 hours. After oxidase action.
1	2·40	7·35	2·40	7·40
2	1·25	5·60	1·25	5·55
3	0·75	3·50	0·75	3·60
4	1·30	3·46	1·30	3·50
5	1·50	5·80	1·50	5·70
6	2·15	5·00	2·15	5·00

In order to see whether ascorbic acid added to urine containing 5% sulphuric acid can be correctly estimated or not as a further test of the reliability of this method, 5 mg. of ascorbic acid were added to 100 c.c. of urine containing 5% sulphuric acid. Titrations were carried out immediately and after 24 hours. Table III shows that the added ascorbic acid could be correctly estimated, as the values obtained by immediate titration and by titration after 24 hours are identical.

TABLE III.

Urine sample No.	C.c. of urine required to reduce 1 c.c. of the dye: Immediate titration.	Titration after 24 hours.
1	1·35	1·35
2	1·25	1·25
3	0·80	0·80
4	1·00	1·00

The State of Ascorbic Acid in Urine.

Investigations from this laboratory have shown that certain plant foodstuffs contain ascorbic acid in a combined state (Guha and Pal, *Nature*, 1936, **137**, 946; Guha and Sen-Gupta, *ibid.*, 1938, **141**, 947; Sen-Gupta and Guha, *J. Indian Chem. Soc.*, 1939, **16**, 496). That urine also contains a part of ascorbic acid in a combined state was indicated by them (Guha and Sen-Gupta, *Nature*, 1938, **141**, 947) Scarborough and Stewart (*Biochem. J.*, 1937, **31**, 2231) have also made similar observations. In the present paper a more detailed study of the subject is presented, as it was important for us to know which method would give reliable values of the free, combined and dehydroascorbic acid of urine. This information was necessary in connection with researches undertaken in this laboratory on the rôle of vitamin C in the normal condition of the body and in infections.

All urine used was freshly passed. The titrations were carried out using 2 : 6-dichlorophenol-indophenol as indicator. A 50 c.c. portion of urine was immediately titrated. Another aliquot of 50 c.c. of urine was taken in a conical flask into which H_2S was passed for 20 minutes. Excess of H_2S was removed by a current of carbon dioxide. This was then titrated with the dye. Into another 50 c.c. of urine cold H_2S was passed for 5 minutes to chase out the air. The flask was then immersed in a bath of boiling water and H_2S was passed for 10 minutes. The flask was then placed in a beaker of cold water and H_2S was passed for 5 minutes, by which time the flask cooled. Excess of H_2S was removed by a current of carbon dioxide and the urine then titrated as before.

Ascorbic acid oxidase was prepared from cucumber, approximately according to the method of Tauber *et al* (*J. Biol. Chem.*, 1935, **110**, 559). 600 G. of cucumber were chopped in a mincer and treated with 1800 c.c. of 30% alcohol. The mixture was shaken for 5 minutes and quickly filtered through glass wool, 1800 c.c. of acetone were added to the filtrate to precipitate the oxidase. The precipitate was quickly centrifuged off, dissolved in 240 c.c. of water, precipitated by 240 c.c. of acetone and centrifuged.

The precipitate was dried in a vacuum desiccator, and dissolved in 240 c.c. of water and a drop or two of toluene was added. 1 C.c. of this enzyme preparation could oxidise 0·5 mg. of ascorbic acid. The stability of the preparation has been investigated by Sen-Gupta and Guha (*J. Indian Chem. Soc.*, 1939, **16**, 549). The activity of the enzyme was determined by the amount of ascorbic acid solution oxidised by the method previously described (Ghosh and Guha, *J. Indian Chem. Soc.*, 1937, **14**, 721). 5 C.c. of untreated urine, cold H_2S-treated urine and hot H_2S-treated urine were taken in three 50 c.c. flasks. The p_H was brought to 5·6. 3 C.c. of the oxidase solution and 2 c.c. of *M*. acetate buffer (p_H 5·6) were added. These flasks were incubated at 37° for half an hour after which they were titrated. The difference in the titration values before and after oxidase treatment gives the true value of ascorbic acid. The results are shown in Table IV.

TABLE IV.

Mg. of indophenol reducing substances in 1 c.c. of urine calculated as ascorbic acid.

Sample No.	Immediate direct titration.		Cold H_2S titration.		Hot H_2S titration.	
	Apparent ascorbic acid.	True ascorbic acid.	Apparent ascorbic acid	True* ascorbic acid.	Apparent ascorbic acid.	True* ascorbic acid.
1	0·0866	0·0715	0·1038	0·0771	0·1600	0·0844
2	0·1223	0·0877	0·1600	0·1105	0·2080	0·1509
3	0·0385	0·0218	0·1300	0·0828	0·1890	0·1147
4	0·1040	0·0640	0·1320	0·0660	0·1400	0·1400
5	0·0217	0·0035	0·0397	0·0194	0·0643	0·0226
6	0·0288	0·0264	0·0399	0·0275	0·0495	0·0329
7	0·0086	0·0038	0·0152	0·0040	0·0169	0·0041
8	0·0252	0·0122	0·0346	0·0212	0·0371	0·0223

In order to investigate the nature of the non-specific reducing substances in the urine, the fresh urine and hot H_2S-treated urine were treated respectively with barium acetate, so that its final concentration in the urine was 5%. The filtrate was treated with concentrated H_2SO_4 to remove the excess of barium and the p_H was brought to about 3. The titrations were then carried out as usual. The results are shown in Table V.

* as determined by oxidase treatment.

TABLE V.

Mg. of indophenol reducing substance in 1 c.c. of urine calculated as ascorbic acid.

Sample No.	Original as such.	Original urine after oxidase treatment.	Original urine after Ba-acetate treatment.	Hot H_2S-treated urine.	Hot H_2S-treated urine after oxidase action.	Hot H_2S-treated urine after acetate treatment.
1	0·0260	0·0104	0·0144	0·0500	0·0160	0·0200
2	0·0192	0·0099	0·0140	0·0290	0·0100	0·0150
3	0·0081	0·0044	0·0052	0·0169	0·0129	0·0099
4	0·0081	0·0038	0·0057	0·0169	0·0041	0·0099
5	0·0288	0·0264	0·0273	0·0495	0·0329	0·0400

DISCUSSION.

These results indicate that urine contains besides ascorbic acid other substance or substances which decolourise 2 : 6 dichlorophenol-indophenol. After reduction with cold H_2S there is an increase of the dye-reducing substance. This increase is partly due to the increase in the ascorbic acid content as evidenced by treatment with ascorbic acid oxidase and partly due to other non-specific reducing substances. The higher ascorbic acid value is accounted for by the presence of dehydroascorbic acid which is reduced by sulphuretted hydrogen in the cold condition to ascorbic acid. Heating in presence of sulphuretted hydrogen gives rise to a dye-reducing value much higher than by reduction in the cold. Both ascorbic acid and some other reducing substances present are increased. Extra ascorbic acid liberated apparently comes from ascorbic acid present in the urine in a combined form, which as such has got no dye-reducing properties. It is interesting to note that along with combined ascorbic acid some non-specific reducing substance is also present in combined form which is also simultaneously split up by heating.

Results of treatment with barium acetate, which removes thiosulphate and some thiol bodies, indicate that this kind of non-specific reducing substances is present in ordinary urine and is also produced by treatment with hot sulphuretted hydrogen.

CONCLUSIONS.

Treatment with acetic, meta-phosphoric, hydrochloric, sulphuric acids and with diethyl dithiocarbamate shows that sulphuric acid is the

best preservative for vitamin C in urine. This has been confirmed also by treatment of the urine with ascorbic acid oxidase.

Treatment of urine with sulphuretted hydrogen in the cold and hot conditions shows that urine contains ascorbic acid, dehydroascorbic acid, combined ascorbic acid and non-specific reducing substances both in the free and combined states. This has been shown by treatment with ascorbic acid oxidase as well as with barium acetate.

My thanks are due to Prof. B. C. Guha for his advice and interest in this work. I am also indebted to the Students' Welfare Committee, Calcutta University, for a grant. Some expenses have been defrayed from a grant of the Indian Research Fund Association.

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Received May 10, 1940.