81. THE DETERMINATION OF VITAMIN C IN URINE

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THE estimation of vitamin C in urine by direct titration, even after treatment of the urine to remove certain interfering substances, presents a number of difficulties. Direct titration in acid solution with 2:6-dichlorophenolindophenol (hereinafter called indophenol) determines the amount of ascorbic acid present but does not measure dehydroascorbic acid or any non-reducing complex of ascorbic acid. Since dehydroascorbic acid is as active physiologically as ascorbic acid [Borsook *et al.* 1937], this point is of some importance in view of the ease with which ascorbic acid is oxidized by air and other reagents even in acid solution when traces of copper are present. A further complication is introduced by the fact that at hydrogen ion concentrations less than $pH\ 4.5$ dehydroascorbic acid undergoes a non-oxidative irreversible change [Ball, 1937] and cannot be reconverted into ascorbic acid by ordinary methods. The total amount of vitamin C, therefore, cannot easily be determined by direct titration with indophenol. Indophenol, in acid solution, oxidizes not only ascorbic acid but many other substances which may and do occur in urine. The method of direct titration cannot be considered specific and is of little use in the examination of urine from scorbutic subjects and cases of latent scurvy in which the total amount of vitamin C excreted may be very small compared with the total amount of indophenol-reducing material in the urine.

In this connexion we have examined the method of Scarborough & Stewart [1937] for the determination of vitamin C in urine and have introduced certain modifications; a new method has also been developed in which the reduction of dehydroascorbic acid and the hydrolysis of non-reducing ascorbic acid complexes are accomplished by means of a dilute solution of $SnCl_2$ in dilute HCl . In both methods the true vitamin C, which is all present finally as ascorbic acid, is determined by an enzymic procedure.

EXPERIMENTAL

Treatment of urine

The urine used in the experiments was a freshly passed or a 24-hr. specimen, according to the nature of the experiment, and, unless otherwise stated, was normal. It was immediately acidified with glacial acetic acid, 10 ml. being added to each 100 ml. urine. It was found that the loss of dehydroascorbic acid, which is of considerable magnitude in urine and which occurs to a lesser extent in acidified urine, could be prevented by addition to the collecting bottle of 50 ml. of the $SnCl_2$ solution described below; this procedure in no way interfered with the subsequent analysis.

Removal of interfering substances by 'clearing'

Two routine methods were used. The first was that of Scarborough & Stewart [1937] in which equal volumes of urine, 20 % mercuric acetate in 10 % acetic acid and 10 % acetic acid were mixed and centrifuged quickly (3 min.) and the supernatant liquid was saturated with H_2S until precipitation was complete. The black precipitate was filtered off and the filtrate freed from H_2S by bubbling through it a stream of wet CO_2 (10 min.).

The second clearing method was used after reduction by SnCl_2 when the urine filtrate was coloured and likely to contain sulphhydryl compounds etc. A measured and usually small volume of 20 % mercuric acetate in 10 % acetic acid was added to the urine filtrate and the whole quickly saturated with H_2S . The precipitated sulphides were filtered off and the filtrate aerated as before. When complete clearing was unnecessary the tin was precipitated alone as the sulphide and the filtrate aerated.

Reduction of dehydroascorbic acid

Reduction of dehydroascorbic acid was carried out according to the method of Scarborough & Stewart [1937] by saturation with H_2S for 24 hr. The solution was freed from H_2S by aeration with wet CO_2 (30 min.).

SnCl_2 was also used to reduce dehydroascorbic acid and the solution which was found to be most convenient in practice was $M/10 \text{ SnCl}_2$, $2\text{H}_2\text{O}$ in $M/4 \text{ HCl}$ solution. It was prepared by dissolving 22.25 g. SnCl_2 crystals (Analar) in 25 ml. conc. HCl with heat and diluting the solution to 1 l. This stock solution kept well on the addition of a small piece of granulated tin. 5 ml. were used to reduce a filtrate containing not more than 100 mg. dehydroascorbic acid.

The SnCl_2 solution made by B.D.H. Ltd. for arsenic tests is also a very useful stock solution: 0.20 ml. were sufficient to reduce a filtrate containing not more than 100 mg. dehydroascorbic acid. It is of particular value in the micro-method described below since excessive dilution is avoided.

Hydrolysis

The urine, or urine filtrate, after clearing, was refluxed for a measured time under an inert atmosphere and in acid solution. The acetic acid added to the urine made it sufficiently acid. Correction to the volume was applied in every case to allow for any slight loss of fluid while refluxing.

Enzyme oxidation

The acid filtrates obtained after hydrolysis and reduction were buffered with 25 % NaOH to $\text{pH } 6.0$ using bromocresol purple as an external indicator; the volume of NaOH was measured from a burette. The enzyme solution was prepared by autolysis of cucumber tissue and the juice was used without further treatment after being tested for activity. 1 ml. of this juice was added to approximately 20 ml. of the buffered filtrate and the mixture aerated in the blood urea apparatus manufactured by Quickfit and Quartz, Ltd.; the aeration was stopped after 30 min. and the enzyme action brought to an end by the addition of 2 ml. 50 % metaphosphoric acid. The solution was titrated after the protein precipitate had been removed by centrifuging.

Reductic acid was prepared according to the method of Reichstein *et al.* [1933]; it had $\text{m.p. } 210^\circ$ (uncorr.); quoted, 213° .

Reductone was prepared by the method of Euler & Martius [1933]. The material was not isolated but solutions were prepared freshly before use.

DISCUSSION AND RESULTS

The method of Scarborough & Stewart [1937] was investigated with regard to the hydrolysis of non-reducing complexes of ascorbic acid which they accomplished by refluxing the urine filtrate under an inert atmosphere for 24 hr.

Samples of urine were reduced with H_2S after clearing and weighed amounts of ascorbic acid added to aliquots of these to study the effect of hydrolysis at

Fig. 1.

Fig. 2.

higher concentrations of ascorbic acid than those in the urine filtrates. The samples were submitted to hydrolysis and small measured volumes were withdrawn at timed intervals for reduction, clearing and titration. As is evident from Fig. 1, a slight increase in the indophenol-reducing power after hydrolysis for 24 hr. need not be due to ascorbic acid being liberated from non-reducing complexes, since it is obvious that the acid is destroyed during the operation.

That this destruction is not a stoichiometric reaction with some other substance in the urine filtrate is shown by the following experiment. Urine samples were cleared and reduced as before and ascorbic acid was added. After hydrolysis for 4 hr., sufficient ascorbic acid was added to bring the concentration up to the original level and the hydrolysis was allowed to proceed; samples were withdrawn for reduction, clearing and titration and the results are expressed graphically in Fig. 2. The rapid rate of destruction during the second period of hydrolysis at a rate comparable with that during the first period of 4 hr. suggests a catalytic reaction rather than a stoichiometric reaction with some other substance in solution. The disappearance of ascorbic acid, presumably oxidative since it was measured by the decrease in the indophenol-reducing power, is rather difficult to explain in view of the anaerobic conditions prevailing, but it is quite real and non-reversible by reduction with H_2S . The same phenomenon was observed when mineral acid was used instead of acetic acid to acidify the urine, so that the hydrogen ion concentration was well within the range in which dehydroascorbic acid is stable if this substance were formed by some obscure oxidation process.

The graphs in Fig. 1 show that the main part of the hydrolysis which liberated indophenol-reducing material occurred within the first hour, so that the long 24-hr. period of hydrolysis previously included in the method of Scarborough & Stewart was not necessary. The total indophenol-reducing power was measured by prolonged treatment with H_2S which, as shown by Scarborough & Stewart, has a hydrolytic action as well as a reducing action.

It is also evident that with a suitable reducing agent for the rapid reduction of dehydroascorbic acid to ascorbic acid, the time required for a single estimation could be decreased considerably. Some inorganic reagents were investigated such as titanous and stannous chlorides but only SnCl_2 in very dilute HCl solution appeared to reduce solutions of dehydroascorbic acid. Since the tin salt could be removed either by clearing with mercuric acetate or by direct precipitation as the sulphide, the reagent was further investigated and the solution most suitable for the purpose was found to be $M/4$ HCl solution containing $M/10$ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$. The recoveries of ascorbic acid from solutions of dehydroascorbic acid by treatment with SnCl_2 solution in the cold for 10 min. are shown in Table 1.

Table 1. *The dehydroascorbic acid solutions were prepared by shaking up with charcoal standard solutions of ascorbic acid and removing the charcoal by filtration.*

Ascorbic acid, mg./5 ml. solution	Ascorbic acid, mg./5 ml. solution after oxidation	Recovery by SnCl_2 , mg.	Recovery %
0.050	0.000	0.050	100
0.086	0.001	0.087	101
0.102	0.000	0.100	98
0.490	0.005	0.491	101
0.671	0.001	0.670	100
0.880	0.002	0.878	99

Since the determination of ascorbic acid appeared to require both hydrolysis and reduction, operations which, under the acid conditions prevailing, would be difficult to separate, both procedures were combined in one operation so that the efficacy of SnCl_2 could be compared with that of H_2S . Preliminary experiments had shown that ascorbic acid was quantitatively recoverable after refluxing for 4 hr. with SnCl_2 solution under an inert atmosphere (Table 2), so an attempt was

made to hydrolyse and reduce the urine filtrates by refluxing them with SnCl_2 solution after clearing. The results of this experiment are shown in Fig. 3 along with the total indophenol-reducing power determined by the method using H_2S . The reducing power reached a maximum after hydrolysis for 60–90 min. on a boiling water bath, and this maximum was the same as that reached when

Table 2. *Recovery of ascorbic acid after refluxing with SnCl_2 solution on the hot water bath for 4 hr. under an inert atmosphere*

Ascorbic acid, mg.	Solvent, 50 ml.	Ascorbic acid recovered, mg.	Recovery %
12.3	N/100 HCl	11.9	97
10.5	"	10.2	97
8.23	5% HAc	8.22	100
21.0	"	21.6	103
5.50	1.5% HPO_3	5.50	100
4.27	"	4.27	100
21.0	10% HAc	20.5	98
12.5	"	12.5	100

Fig. 3.

using H_2S . The 90 min. interval was used for routine purposes and some comparative results for the two methods are shown in Table 3. It is interesting to note that the same total indophenol-reducing power may be reached in a variety of ways: refluxing on a water bath with SnCl_2 under N_2 for 90 min., treatment with SnCl_2 solution in the cold for 24 hr., and prolonged treatment with H_2S all give the same maximum indophenol-reducing power with a particular sample of urine. Treatment with SnCl_2 in the cold for 10 min. reduces only the dehydro-ascorbic acid in the urine. In Table 4 are collected the results of several analyses of urines by these different methods. It was found that simple hydrolysis followed by reduction rarely produced the same maximum as the other methods, but simultaneous reduction and hydrolysis proceeded satisfactorily.

Table 3. *Comparison of indophenol-reducing powers of various urines after reduction by SnCl_2 or H_2S*

No. of urine	SnCl_2 ascorbic acid mM/l.	H_2S ascorbic acid mM/l.
6	0.43	0.42
7	0.37	0.36
17	0.30	0.30
19	0.210	0.205
27	0.059	0.059
28	0.074	0.077
29	0.055	0.053

The last three urines were from a scorbutic subject.

Table 4. *Total indophenol-reducing power of 4 urines determined by different methods and amount of increase by simple reduction (10 min.)*

Figures represent mM/l. ascorbic acid

No. of urine	21	19	17	10
Cleared only	0.419	0.241	0.188	0.054
SnCl_2 10 min. (cold)	0.493	0.316	0.218	0.055
SnCl_2 90 min. (90°)	0.550	0.295	0.259	0.146
SnCl_2 24 hr. (cold)	0.548	0.298	0.257	0.145
H_2S method	0.551	0.296	0.257	0.146

The suggestion that ascorbic acid is present in urine partly in a non-reducing combined form is borne out by the results quoted above in which hydrolysis is necessary to produce the maximum reducing power of the urine filtrates, although treatment with SnCl_2 for 10 min. in the cold is sufficient to reduce dehydroascorbic acid in pure solution. Experiments with enzymic oxidation described below show that part of the increase in reducing power is due to ascorbic acid.

As a final and necessary test of the SnCl_2 method the true ascorbic acid was determined by enzymic oxidation and compared with the values obtained with the H_2S method. The results are shown in Table 5 and the agreement between the two methods is satisfactory.

Table 5. *The urines examined here are from a scorbutic subject before and after treatment with vitamin C. The figures represent the reducing power of the filtrates expressed as mM/l. ascorbic acid*

No. of urine	Total indophenol-reducing power		Reducing power after oxidation (non-vitamin material)		Difference, vitamin C	
	SnCl_2	H_2S	SnCl_2	H_2S	SnCl_2	H_2S
1 ₁	0.149	0.147	0.080	0.059	0.089	0.088
4 ₁	0.157	0.152	0.077	0.071	0.080	0.081
11 ₁	0.041	0.039	0.043	0.040	—	—
16 ₁	0.053	0.052	0.035	0.034	0.018	0.016
15 ₂	0.904	0.898	0.040	0.040	0.864	0.858
16 ₂	0.381	0.378	0.045	0.042	0.336	0.336
17 ₂	0.925	0.925	0.067	0.069	0.858	0.856

Specificity of the enzyme under the conditions of analysis

The specificity of the methods for the determination of ascorbic acid discussed above depend to a great extent upon the specificity of the cucumber oxidase used in the final operation. Zilva & Snow [1938] have shown that the

enzyme is not specific for ascorbic acid and that it oxidizes many other compounds of the same dienol type. Nevertheless, it seemed that the inclusion of the enzymic oxidation in the analytical procedure was desirable since, under the conditions of analysis, ascorbic acid is oxidized rapidly and completely, whereas other dienol compounds which have been examined are oxidized slowly and incompletely. There is as yet no evidence that these compounds are present in normal or scorbutic urines and, in addition, the enzymic oxidation excludes from the final result some indophenol-reducing material which is not oxidized by cucumber oxidase.

The experiments on the specificity of the enzyme were carried out in exactly the same way as the enzymic oxidations in the analytical procedure, using reductic acid and reductone solutions instead of urine filtrates. Control tubes containing known amounts of ascorbic acid were set up each time and it was found that the acid was oxidized completely in every case. The pH of the solutions was checked, before and after oxidation, in a Beckmann pH meter. The results are shown in Tables 6 and 7. The rates of oxidation of reductic acid and

Table 6. *The figures represent mM reductic acid in 20 ml. solution, correction being applied for the dilutions involved*

Reductic acid solution	Aerated at pH 6.0 + enzyme	Aerated at pH 6.0
0.270	0.160	0.179
0.164	0.083	0.083
0.083	0.025	0.024

The pH was checked before and after aeration in a Beckmann pH meter.

Table 7. *The figures represent mM reductone/20 ml. solution, correction being applied for the dilutions involved*

Reductone solution	Aerated at pH 6.0 + enzyme	Aerated at pH 6.0
0.375	0.148	0.182
0.624	0.431	0.432
0.740	0.446	0.456
0.787	0.313	0.388
0.948	0.406	0.415
1.301	0.662	0.762

The pH was checked before and after aeration in a Beckmann pH meter.

reductone were not significantly greater in presence of the enzyme than in its absence; under the conditions of analysis there was probably a fair amount of catalytic oxidation by copper as no special precautions were taken to exclude this metal. Thus, although it cannot be claimed that the procedure is completely specific, interference is limited to a few compounds of the same dienol type as ascorbic acid which are partly oxidized in the last operation and whose presence in normal or scorbutic urines is not yet conclusively proved.

Routine procedure for the determination of vitamin C in urine

Reagents. Glacial acetic acid and 10% acetic acid.

20% mercuric acetate in 10% acetic acid.

M/10 SnCl₂ in M/4 HCl (or B.D.H. SnCl₂ for arsenic tests).

H₂S gas (from Kipp apparatus), CO₂ and N₂.

Indophenol solution equivalent to 5 mg. ascorbic acid per 100 ml.

25% NaOH.

50% metaphosphoric acid, freshly prepared.

Autolysed cucumber juice.

Macro-method

The urine, acidified with glacial acetic acid to a concentration of 10 % acetic acid, need not be cleared unless it has a high specific gravity, is darkly coloured and is opaque and likely to contain considerable quantities of interfering substances as in the case of urines containing large amounts of sulphur.

To 50 ml. of urine or urine filtrate after clearing, add 10 ml. SnCl_2 solution or 0.50 ml. B.D.H. reagent and reflux under N_2 on a boiling water bath for 90 min. Cool quickly, add 20 ml. mercuric acetate solution, pass H_2S gas quickly through the solution until precipitation is complete and filter off the precipitate. Set aside 15–20 ml. for titration after removal of H_2S by aeration with CO_2 (10 min.) and buffer a 20 ml. portion with 25 % NaOH to pH 6.0, measuring the alkali from a burette. Add 1 ml. of autolysed cucumber juice and aerate in a blood urea apparatus for 30 min. Stop the aeration and add 2 ml. 50 % metaphosphoric acid solution, centrifuge and titrate an aliquot of the supernatant liquid. The difference between the results of the two titres, expressed as ascorbic acid and with due allowance for the dilutions involved, represents vitamin C.

Tables 8 and 9 contain results of analyses on urine and food samples carried out by this method.

Table 8. *Some analyses of scorbutic urines carried out by the SnCl_2 method*

Ascorbic acid is expressed as mg./100 ml. urine and the non-vitamin reducing material is also expressed as mg. ascorbic acid/100 ml. urine.

No. of urine	Day	Total indophenol-reducing power	Non-vitamin reducing material	Ascorbic acid	Daily excretion mg.
1	3	1.36	1.07	0.29	
1	5	1.14	0.86	0.28	
1	10	1.80	0.73	1.07	
1	12	7.50	0.70	6.80	
1	15	15.95	0.70	15.25	
1	16	6.70	0.79	5.91	
1	17	16.30	1.15	15.15	
2	6	1.23	0.62	0.61	5.96
2	7	0.53	0.61	—	—
2	8	2.50	0.73	1.77	16.6
2	9	2.27	1.28	0.99	7.1
2	14	1.04	0.72	0.32	5.7
2	16	0.87	0.63	0.24	4.2
2	17	0.83	0.59	0.24	4.0
2	19	0.71	0.62	0.09	1.3
2	20	0.86	0.61	0.25	2.9
3	11	0.75	0.65	0.10	1.4
3	14	1.01	0.68	0.33	4.7
3	18	0.50	0.53	—	—
3	19	0.78	0.68	0.10	1.4
3	20	2.17	0.61	1.56	27.0
3	21	6.00	0.76	5.24	86.4
3	22	5.31	0.68	4.63	51.4
3	23	1.86	0.72	1.14	11.4

Ur 1 and Ur 3 are urines from a scorbutic subject during treatment with vitamin C; Ur 2 is a urine from a scorbutic subject before treatment with vitamin C.

Table 9. *Analyses of dried vegetables*

The vegetables were extracted for a prolonged period with 5% metaphosphoric acid and the extract analysed by the SnCl_2 method. Owing, probably, to the drying process, the extraction was very easy to carry out, the solutions being coloured but free from excess of protein material.

Vegetable	Ascorbic acid mg./100 g.
Julienne	2.6
French beans	11.2
Turnips	10.8
Spinach	3.5
Cabbage	26.5
Onions	7.0

The values found in most cases are much the same as those quoted for cooked vegetables still retaining their tissue water but separated from the cooking water. The low value for spinach is interesting in view of the relatively high heavy-metal content of this vegetable.

Micro-method

The procedure in principle is exactly the same: smaller quantities are used and a micro-diffusion burette is used for the titration.

20 ml. acidified urine or urine filtrate are refluxed with 5 ml. SnCl_2 solution or 0.20 ml. B.D.H. reagent in a micro-reflux apparatus, with provision for a stream of N_2 to pass through the liquid, for 90 min. A 20 ml. portion of the mixture is cleared with 10 ml. mercuric acetate and the final filtrate is titrated in 1 or 2 ml. portions. Half quantities are used for the enzymic oxidation and a 2 ml. portion is taken for titration.

SUMMARY

The method of Scarborough & Stewart [1937] for the determination of vitamin C in urine has been investigated and certain modifications have been introduced.

A new method for the determination using SnCl_2 in dilute HCl solution for the reduction and hydrolysis of urine has been developed. In both methods, the true vitamin C was determined by an enzymic method.

The specificity of cucumber oxidase under the conditions of analysis has been investigated and the results uphold the utility of the enzymic procedure.

Ascorbic acid and other indophenol-reducing substances are liberated during the combined reduction and hydrolysis of urine.

Some analyses are reported. Scorbutic urines show very low concentrations of vitamin C and occasionally none is detectable.

Dried vegetables contain amounts of vitamin C of roughly the same order as the amounts in cooked vegetables retaining their tissue water but without the cooking water.

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REFERENCES

- Ball (1937). *J. biol. Chem.* **118**, 219.
Borsook, Davenport, Jeffreys & Warner (1937). *J. biol. Chem.* **117**, 237.
Euler & Martius (1933). *Ark. Kemi Min. Geol.* **11 B**, nos. 8 and 12.
Reichstein, Grüssner & Oppenauer (1933). *Helv. chim. Acta*, **16**, 988.
Scarborough & Stewart (1937). *Biochem. J.* **32**, 2232.
Silva & Snow (1938). *Biochem. J.* **33**, 1926.