CCLXXIV. OBSERVATIONS ON THE EXCRETION OF ASCORBIC ACID IN URINE

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(Received 15 October 1937)

It is well known that 2:6-dichlorophenolindophenol in acid solution oxidizes not only ascorbic acid but many other substances which may or do occur in normal urine. Hence the direct titration of urine with the indophenol does not constitute a specific method of estimating ascorbic acid. Certain interfering substances, such as glutathione, react slowly, and, though they make the end-point of the titration rather vague, do not seriously affect the result [Bessey & King, 1933; Harris *et al.* 1933]. Others, however, such as thiosulphates, react as rapidly as ascorbic acid itself; they ought therefore to be removed as an essential preliminary to any titration with indophenol, and a suitable method of precipitation by mercuric acetate has been suggested by Emmerie [1934], Emmerie & van Eekelen [1934], and van Eekelen [1934].

In attempting to estimate ascorbic acid in urine, using the preliminary treatment with mercuric acetate, we encountered certain peculiarities of behaviour which led us to investigate the matter further.

EXPERIMENTS AND RESULTS

All urine used was freshly passed, immediately acidified with acetic acid (10 ml. of glacial acetic acid per 100 ml. of urine) and submitted to the experimental procedure. The preliminary treatment which we used was a modification of that recommended by Emmerie & van Eekelen [1934]. To the urine, acidified with acetic acid, an equal volume of water was added, and this was followed by the same volume of a 20% solution of mercuric acetate. The mixture was then immediately centrifuged, the precipitate was discarded, and the excess of mercury removed from the solution by hydrogen sulphide which was passed in within 5 min. of the mercuric acetate being added. After removal of mercuric sulphide by filtration, dissolved hydrogen sulphide was removed by a current of carbon dioxide. Emmerie & van Eekelen [1934] recommend diluting the urine with six times its volume of water and adding a half volume of mercuric acetate. We wished, however, to avoid excessive dilution, which makes the end-point in the indophenol titration difficult to observe, and therefore chose the conditions which, with minimum dilution, gave a colourless final solution and allowed the maximum removal of indophenol-reducing substance. The substance (or substances) so removed cannot be ascorbic acid, since the treatment did not precipitate ascorbic acid from pure solution, and, moreover, additional ascorbic acid added to urine could be quantitatively accounted for by indophenol titration after the mercuric acetate treatment. These points are of some importance, since we used more mercuric acetate than did Emmerie & van Eekelen and, as Emmerie found (and we have confirmed), an excessive amount of the reagent causes the re-solution of the precipitated interfering substances.

Since the preliminary treatment with mercuric acetate does not affect the ascorbic acid content of the urine, and since it removes at least some of the

¹ In receipt of a part-time grant from the Medical Research Council.

interfering substances including (as van Eekelen suggests [1934]) and as we have confirmed) thiosulphate, besides giving a clear colourless solution for titration, we have adopted it in all the experiments described in this paper. For the actual titration we have added the ascorbic acid solution from a burette to a measured volume of the standard indophenol solution acidified with acetic acid, so obtaining a clearer end-point than is yielded by the reverse procedure.

The removal of excess mercuric acetate by hydrogen sulphide is, of course, complete in a few minutes. Emmerie & van Eekelen [1934], however, after saturating the solution with hydrogen sulphide, allowed it to stand overnight before completing the procedure. In order to see whether this was necessary, we filtered off the precipitated mercuric sulphide as soon as possible (10–15 min.) and kept the filtrate saturated with hydrogen sulphide, removing samples at intervals for titration (the hydrogen sulphide, of course, being swept out of each sample by carbon dioxide). In dealing with urine in this way, we found that the amount of indophenol-reducing substance (I.R.P.) increased to a maximum which was reached only after 72 hr. treatment with hydrogen sulphide (Table I). Incidentally, it may be remarked that we have found lemon

Table I

Figures represent I.R.P., mg. per 100 ml., calculated as ascorbic acid

Hours of reduction	Urine	Lemon juice
0	1.4	64.7
24	4.6	73.8
48	5.5	—
72	7.5	76.1
96	7.5	77.0
120	7.5	76.9

Table II

mg. I.R.P. per 100 ml.

Hours of reduction	Solution of dehydroascorbic acid	Dehydroascorbic acid added to urine
0	0	0
10 min.	3.3	0.91
24 hr.	8.0	2.7
48 „	10.0	2.7
72 „	9.0	2.7
96 „	9.5	2.8

juice to behave similarly (Table I). A solution of dehydroascorbic acid prepared from ascorbic acid either by simple aeration or by the aerobic action of norite, showed maximum indophenol-reducing power after treatment with hydrogen sulphide for 24 hr., and equally rapid reduction was obtained when the dehydroascorbic acid was added to urine (Table II). This difference in rate of reduction by hydrogen sulphide between dehydroascorbic acid and the potential indophenol-reducing substances of urine could not be ascribed to differences in *pH*. We therefore considered it possible that even after treatment with mercuric acetate, urine still contained (possibly associated with dehydroascorbic acid) some interfering substance capable of slow reduction to an indophenol-reducing compound. An alternative, or possibly supplementary, explanation was that the acetic acid present was slowly hydrolysing some compound containing ascorbic acid. This explanation would demand the combination of the ascorbic acid in some way which would destroy its reducing properties, e.g. an ester

linkage involving one hydroxyl of the reductone group, the possibility of which has been suggested by the work of Reichstein *et al.* [1934].

Van Eekelen [1934] had, actually, found the indophenol-reducing power of urine to be increased by boiling with 5% sulphuric acid for 15 min. in an atmosphere of carbon dioxide. He considered that this might be due to hydrolysis of a non-reducing ascorbic acid derivative, or to the production of reductic acid from glycuronic acid [Reichstein & Oppenauer, 1933].

In our own experiments, involving the prolonged passage of a stream of hydrogen sulphide through the solution, there was the additional possibility of the formation of traces of thiosulphate. This, however, can be discounted, since we found that the extra indophenol-reducing power produced by the hydrogen sulphide treatment was not removed by addition of mercuric acetate and rapid precipitation of the excess mercury by hydrogen sulphide passed for only 10 min. (a procedure which will be referred to again as the "rapid clearing process"). Added thiosulphate was removed by such treatment. Equally, the extra reducing power could not be due to reductic acid formed from glycuronic acid, for we found that glycuronic acid, which does not itself reduce the indophenol reagent, is equally inert after treatment with hydrogen sulphide for 72 hr., boiling with 10% acetic acid for 24 hr., or both.

To test the hypothesis that the action of the hydrogen sulphide in the acidified urine was really a hydrolysis, we boiled urine containing 10% of acetic acid, for 24 hr. in an atmosphere of carbon dioxide, removing samples at intervals, and submitting them to the rapid clearing process before titration. There was a gradual increase in indophenol-reducing power, maximum in 18 hr. (Table III). This result confirms that of van Eekelen, and in view of the control

Table III

mg. I.R.P. per 100 ml. calculated as ascorbic acid

Hours of hydrolysis	Urine A	Urine B
0	1.3	1.4
1.5	1.8	2.7
7	3.3	3.8
12	4.9	4.7
18	6.0	5.5
22	5.9	5.6
24	6.0	5.6

Table IV

mg. I.R.P. per 100 ml. calculated as ascorbic acid

Hours of reduction	After hydrolysis	
	Urine A	Urine B
0	3.5	6.0
24	4.7	6.8
48	5.5	7.9
72	7.5	8.8
96	8.0	10.0
120	8.0	10.1

experiments just mentioned, it seems that it is most probably due to hydrolysis of some non-reducing substance. If, however, the urine, after hydrolysis has proceeded until the indophenol-reducing power has risen to a constant level, is again treated with hydrogen sulphide, a further increase in reducing power occurs, and this reaches a maximum constant level only after 96 hr. (Table IV).

It is evident, therefore, that of the total potential indophenol-reducing substances of urine (apart from those precipitated by mercuric acetate), part is present as such, part in a combined hydrolysable non-reducing form, and part in a reversibly oxidized form identical with or analogous to dehydroascorbic acid. It is noteworthy that the behaviour of urine when boiled with acid is similar to that of vegetables as reported by (for example) Guha & Pal [1936]. These observers interpreted their results as indicating the presence of ascorbic acid in a combined form, though their interpretation has since been questioned [e.g. Mack, 1936]. An alternative explanation has been advanced—that boiling inactivates ascorbic acid dehydrogenase which, being active in the unboiled material, decreases the apparent amount of ascorbic acid. The results with urine, however, cannot be due to enzyme inactivation, and suggest that possibly the interpretation of Guha & Pal may be partly correct. In the case of vegetables, there is room for both explanations, as Levy [1936] has pointed out.

The finding that urine contained a hydrolysable substance from which acid liberated an indophenol-reducing substance was capable of explaining the very slow increase in reducing power when acidified urine was treated with hydrogen sulphide. It was not necessarily the whole explanation, however, and the suspicion that some substance other than ascorbic acid or its derivatives was present was strengthened by a study of the rate of reoxidation by air of the reducing substances present in urine after full treatment with acetic acid (boiling) and hydrogen sulphide. Whereas ascorbic acid in 10% acetic acid, or in urine to which 10% of acetic acid had been added, was oxidized very slowly on aeration, the indophenol-reducing substances of urine itself, whether untreated or boiled with acetic acid and then treated with hydrogen sulphide, disappeared relatively quickly on aeration (Table V). This certainly suggested the presence of some

Table V

mg. I.R.P. per 100 ml. calculated as ascorbic acid

Hours of aeration	Ascorbic acid	Ascorbic acid added to urine	Urine	
			Before hydrolysis	After hydrolysis
0	10.0	2.4	4.6	6.0
4	9.9	2.35	1.2	2.3

substance other than ascorbic acid, and indeed, at the time the observations were made it seemed possible that none of the indophenol-reducing power of normal urine (at any rate without previous administration of large doses of vitamin C) was really due to ascorbic acid. Wieters [1935] and Ahmad [1936] had failed, by feeding experiments, to detect ascorbic acid in urine, while Hinsberg & Ammon [1936] had failed to detect it by chemical means. Since then, however, ascorbic acid has been detected in normal urine by chemical means [Meuwissen & Noyons, 1937; Drumm *et al.* 1937]. The methods which led to the isolation of ascorbic acid derivatives were far from quantitative, and it was necessary to rely on other means to determine whether the ascorbic acid was actually accompanied by other indophenol-reducing substances as the experiments already described had suggested.

Evidence on this point was obtained by observing the effect, on the indophenol-reducing substances of urine, of the ascorbic acid dehydrogenase discovered in cauliflower by Szent-Györgyi [1928] and further studied by Hopkins & Morgan [1936]. For the first series of experiments, urine was submitted to the rapid clearing process, was then boiled with 10% acetic acid for 24 hr. and

treated with hydrogen sulphide for a further 96 hr., after which it was again rapidly cleared and finally freed from hydrogen sulphide by a stream of carbon dioxide. The pH was adjusted to 6.0 by addition of sodium hydroxide. Enzyme solution, prepared as described by Hopkins & Morgan [1936], was then added to the treated urine (4 ml. enzyme to 20 ml. urine solution), and the mixture was aerated. For titration with indophenol, samples of 10 ml. were withdrawn, added to 2 ml. of freshly prepared 50% metaphosphoric acid and centrifuged. Trichloroacetic acid, for acidifying the mixture and precipitating the enzyme, was avoided since it itself decolorizes indophenol.

We used fresh enzyme solutions, prepared by expressing the juice of cauliflower and diluting it with (usually) four times its own volume of water. The amount of dilution was varied in accordance with the results of preliminary experiments to test the activity of the enzyme. The dilution was always the maximum consistent with complete oxidation of ascorbic acid within 20 min. using the volumes specified above. The adjustment of the pH of the ascorbic acid solution to 6.0 must be made before the enzyme is added, since, when dealing with urine containing acetic acid, the low pH causes precipitation and consequent inactivation of the enzyme.

The results of such an experiment are shown in Fig. 1. There is a rapid disappearance of indophenol-reducing substance, which, after half an hour, has fallen to half its original level (in this particular case, the magnitude of this rapid fall varies in different urines). Control experiments showed that ascorbic acid was completely oxidized by the enzyme preparation in this time. Thereafter, there is a slow, steady, disappearance of indophenol-reducing power which we suggest is due to the non-enzymic oxidation of a substance other than ascorbic acid. That the reducing substance still present is, in fact, not ascorbic acid is shown by the rapid disappearance of added ascorbic acid (Fig. 1). It is further demonstrated by the results of similar experiments in which the enzyme action

Fig. 1.

Fig. 2.

Fig. 1. Aerobic oxidation of indophenol-reducing substances of urine by cauliflower hexoxidase. pH 6.0. Ordinate: mg. indophenol-reducing substance per 100 ml. urine.

Fig. 2. After the initial rapid oxidation in the presence of hexoxidase, the subsequent slow oxidation of substances other than ascorbic acid continues and is not affected by addition of KCN, though the enzyme, as shown by its inability to oxidize added ascorbic acid, is completely inactivated. pH 6.0. Ordinate: mg. indophenol-reducing substance per 100 ml. urine.

was stopped after the initial rapid fall in reducing power, by addition of $M/1000$ potassium cyanide. As Fig. 2 shows, the addition of cyanide does not alter the rate of oxidation of the residual indophenol-reducing substance. Moreover, the rate is still unaltered after addition of ascorbic acid (which the enzyme, now inactivated, does not attack).

The use of the enzyme preparation from cauliflower not only confirms the presence in urine, after acid hydrolysis and prolonged treatment with hydrogen sulphide, of an indophenol-reducing substance other than ascorbic acid; in so far as it may be regarded as specific for ascorbic acid, it permits an estimate of the actual amount of that substance. The figures obtained must still, however, be regarded as maximum, for proper proof of the specificity of the enzyme is lacking. We have determined the indophenol-reducing power before and after aeration with the enzyme using urine (*a*) without any preliminary treatment; (*b*) submitted to the rapid clearing process; (*c*) treated with mercuric acetate followed by hydrogen sulphide for 72 hr.; (*d*) boiled with 10% acetic acid in an atmosphere of carbon dioxide for 24 hr. and then rapidly cleared; (*e*) treated as in (*d*) and subsequently as in (*c*). The results with untreated urine must be regarded as approximate only, as the end-point of the titration is by no means sharp. In these experiments the difference between the two titrations represents the true ascorbic acid (with the proviso mentioned above); the second titration represents the non-ascorbic acid reducing substance (or substances). Results are shown in Table VI. Rapid treatment with mercuric acetate is without effect

Table VI

mg. I.R.P. per 100 ml. calculated as ascorbic acid

Method	Urine A		Urine B		Urine C	
	Ascorbic acid	Other reducing substance	Ascorbic acid	Other reducing substance	Ascorbic acid	Other reducing substance
Immediate direct titration	1.1	0.3	0.29	0.92	0.28	0.56
"Rapid clearing" method	0.26	0.25	0.12	0.66	0.26	0.99
Full reduction	2.8	1.8	2.44	0.73	1.33	1.07
Hydrolysis plus reduction	3.9	2.1	2.74	1.58	1.53	0.88

on the amount of ascorbic acid which, however, is increased, though not always to the same extent, both by the hydrolysis and by the reduction with hydrogen sulphide. We consider this to be strong evidence that ascorbic acid is present in freshly passed normal urine as such, as a non-reducing ester-like derivative, and further, as dehydroascorbic acid which also occurs in part as a hydrolysable derivative. It is probable that dehydroascorbic acid is present, for urine acidified with acetic acid and allowed to stand at room temperature in an inert atmosphere does not show the increase in reducing power which follows prolonged saturation with hydrogen sulphide; the hydrogen sulphide is therefore probably acting as a reducing agent. The concentrations of these substances vary independently in different samples of urine.

The amount of non-ascorbic acid reducing substance is also increased by hydrolysis and by reduction with hydrogen sulphide. This suggests that it, too, exists in an "oxidized" and a combined form. Titration of untreated urine fails to afford evidence of its existence in a "reduced" form, since other interfering substances, removable by mercuric acetate, are still present. Since, however, its oxidized form appears to be reduced very slowly by hydrogen sulphide in acid solution, and since some non-ascorbic acid reducing substance is present after the rapid clearing process, it is probable that the "reduced" form is also present in urine, and that it is not an artefact. These facts suggest a substance similar to ascorbic acid, a suggestion which is supported by the facts that it, also, is slowly oxidized by aeration even in acid solution (10% acetic acid) and is rapidly oxidized, though not absorbed, in the presence of norite. One may recall that

Drumm *et al.* [1937] isolated from normal urine an undoubted 2:4-dinitrophenyl-hydrazone, melting at 221°, of similar composition to the hydrazone of ascorbic acid which it closely resembled in solubility and in adsorption properties.

We have shown that the unknown substance is neither glycuronic acid nor a derivative formed by the experimental procedure. It may conceivably be reductic acid or a similar substance. One possibility we considered was that of an isomeride of ascorbic acid. Chemical reduction of dehydroascorbic acid might conceivably give rise to both *cis* and *trans* isomerides, of which only one would, presumably, be oxidized by the enzyme, though both might be oxidized (possibly at different rates) by molecular oxygen. This possibility however, is inadmissible since the "oxidized" form of the substance in urine is reduced by hydrogen sulphide more slowly than is dehydro-ascorbic acid, and furthermore, a solution of ascorbic acid, oxidized by aeration alone or with charcoal and then re-reduced by hydrogen sulphide, is completely and rapidly oxidized in presence of the enzyme.

As a point of practical importance, it is evident that the existence of this non-ascorbic acid reducing substance must be taken into account in any method intended to estimate accurately the amount of vitamin C in urine, as must also the existence of dehydroascorbic acid and the non-reducing hydrolysable derivative. Such a method, though obviously a laborious one and still open to suspicion as to the specificity of the enzyme, could be founded on titration before and after aeration in presence of Szent-Györgyi's "hexoxidase" after hydrolysis and prolonged reduction.

The technique, as we have used it, is as follows: All urine is acidified to 10% with acetic acid. We use either a fresh specimen (as in all the above experiments) or a 24 hr. specimen preserved by 10% acetic acid in a brown stoppered bottle. Take 20 ml. urine and add 20 ml. water and 20 ml. 20% mercuric acetate. Centrifuge for 2-3 min. immediately. Pour off supernatant fluid and pass H_2S at once for 15 min. Filter and remove H_2S by CO_2 —about 30 min. Place filtrate in 150 ml. flask and reflux under CO_2 for 24 hr. Make volume up to 75 ml. and pass in H_2S for 96 hr. Take 10 ml. and to this add 10 ml. water and 10 ml. 20% mercuric acetate. Centrifuge and remove Hg as before. Titrate sample for total I.R.P. Take 20 ml. into an aeration tube. Adjust pH to 6.0 by addition of 20% NaOH. Add 4 ml. of enzyme preparation suitably diluted and tested for activity. Remove 10 ml. immediately into 2 ml. freshly prepared 50% HPO_3 (Spec. A). Allow aeration to proceed for 30 min. and again remove 10 ml. into

Fig. 3. Effect of aeration, in presence of reduced glutathione and cauliflower hexoxidase on the indophenol-reducing substance of urine. The total indophenol-reducing substance increases. The increase is not due to GSH, which is removed by mercuric acetate before titration. Aeration was stopped after 5 hr., and the mixture kept for 16 hr. in a CO_2 atmosphere. The glutathione had then been completely oxidized; the enzyme was still active, but only part of the indophenol-reducing substance disappeared rapidly when aeration was resumed. pH 6.2. Ordinate: mg. indophenol-reducing substance per 100 ml.

2 ml. HPO_3 (Spec. B). Centrifuge both specimens for 10 min. and titrate. The indophenol-reducing power of B is due to substances other than ascorbic acid. The difference between this value and that of A represents the total ascorbic acid.

One further observation, of which, however, we can offer no satisfactory explanation, remains to be mentioned. Hopkins & Morgan [1936] reported that, in presence of reduced glutathione, ascorbic acid was not oxidized by the hexoxidase of cauliflower. The glutathione was oxidized, and it was only when the nitroprusside reaction disappeared that the ascorbic acid was oxidized in the normal way. In testing urine in this way, we found, as we expected, that the indophenol-reducing power was not diminished by aeration in presence of hexoxidase and reduced glutathione; to our surprise, however, we found that it was actually increased, and remained so for several hours during which the nitroprusside reaction remained positive (Fig. 3). Later, with the nitroprusside reaction negative, the indophenol-reducing power remained high, although the enzyme was still active as was shown by the rapid disappearance of added ascorbic acid.

SUMMARY

It is confirmed that mercuric acetate, in suitable concentration, removes from urine certain substances including thiosulphate, which interfere with the indophenol titration method for the estimation of ascorbic acid. The use of "hexoxidase" from cauliflower shows that reducing substances other than ascorbic acid are still present.

Prolonged treatment with H_2S increases the indophenol-reducing power of urine, and it is confirmed that acid hydrolysis has a similar effect. Hydrolysis followed by reduction produces the maximum increase. Enzyme experiments show that both these increases are due in part to production of extra ascorbic acid (if the enzyme is specific) which is probably present in urine partly in the "reduced" form, partly as dehydro-ascorbic acid, and partly as non-reducing, hydrolysable derivatives of these substances.

Associated with ascorbic acid is another indophenol-reducing substance, closely resembling it in properties, and also existing in non-reducing "oxidized" and "combined" forms.

A method of estimating ascorbic acid in urine is suggested.

Our thanks are due to the Moray Research Fund of this University for a grant which partly covered the expenses of this work.

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