CCXXXI. THE CHEMICAL IDENTIFICATION OF ASCORBIC ACID IN URINE

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DURING the past 3 years several methods have been proposed, and widely used, for the estimation of ascorbic acid in urine. They all depend on the reducing properties of ascorbic acid and are all non-specific. By careful limitation of conditions it is possible, with these methods, to exclude interference by a number of substances known to be present in urine and to reduce the reagents employed. There is, however, no proof that any of the methods is really estimating ascorbic acid, and the lack of unanimity among the various protagonists suggests that none of the methods, even with carefully defined conditions, is really specific. Indeed there is no complete proof that ascorbic acid is normally present in urine, though indirect evidence obtained by analysis of urine from normal and scorbutic subjects suggests that it is. However, attempts at biological identification have failed, though the failures may reasonably be attributed to toxicity on the part of the urine [Ahmad, 1936; Wieters, 1935]. More serious was the failure of Hinsberg & Ammon [1936] to isolate ascorbic acid from urine. They attempted to obtain it in the form of the 2:4-dinitrophenylhydrazine derivative of dehydroascorbic acid, and concluded, from their failure with normal urine and their success with added ascorbic acid, that urine could not contain, at most, more than 0.3 mg. ascorbic acid per 100 ml., i.e. less than a third of the amount indicated by titration with dichlorophenolindophenol.

Some time ago two of us [Scarborough & Stewart, 1937] found that the indophenol-reducing substance in normal urine was partly, though not completely, oxidized aerobically in the presence of the cauliflower hexoxidase described by Szent Györgyi [1928] and by Hopkins & Morgan [1936]. This rather strengthened the view that ascorbic acid is excreted normally, but did not amount to proof, since the enzyme has not been shown to be completely specific. It was therefore decided to attempt the actual isolation of a derivative of ascorbic acid from normal urine, obtained from persons receiving an ordinary mixed diet without ascorbic acid supplements. A preliminary note on this attempt, which was successful, has appeared [Stewart *et al.* 1937]; while this note was in the press, a similar success was announced by Meuwissen & Noyons [1937] who used a very similar method. As, however, the identification of the isolated substance by the present authors seems to be more rigorous than that of Meuwissen & Noyons, and since another point of interest arose during the work, it seems desirable to give a fuller account of the work than is possible in the columns of *Nature*.

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EXPERIMENTAL

Isolation of 2:4-dinitrophenylhydrazine derivative of dehydroascorbic acid

Freshly passed normal urine was mixed with sufficient A.R. oxalic acid to produce a concentration of 4% of the acid. 12 l. of the acidified urine were evaporated at atmospheric pressure in carbon dioxide to 2 l., a procedure which was shown by control experiments not to destroy ascorbic acid. The dark brown concentrate was kept overnight and filtered from the precipitated urea oxalate etc. The filtrate was cleared by two successive treatments in the cold with 25 g. of norite. This treatment removed no ascorbic acid, but oxidized it to dehydroascorbic acid.

At this point it would have been possible to proceed with the removal of excess oxalic acid and the preparation of the 2:4-dinitrophenylhydrazine derivatives. As, however, it was desired to know the amount of indophenol-reducing substances present, the straw-coloured liquid was allowed to stand in an atmosphere of hydrogen sulphide for 48 hr. to reduce the dehydroascorbic acid. Hydrogen sulphide was then removed by a current of carbon dioxide. The excess of oxalic acid was precipitated by treatment first with a thin paste containing 60 g. of calcium hydroxide and then, when the solution was nearly neutral, with solid calcium carbonate. The filtrate was then re-treated with hydrogen sulphide for 48 hr. to complete the reduction of dehydroascorbic acid. At this point indophenol titration, using the mercuric acetate treatment [Emmerie & van Eekelen, 1934], showed the presence of 5.7 mg. of reducing substances per 100 ml. of the liquid, which meant that little or no loss had occurred. In another experiment the initial indophenol-reducing power of the urine was measured, and an actual increase was found to occur during the concentration. This phenomenon is to be discussed elsewhere.

Hydrochloric acid was added to the urine concentrate to make a 2.5*N* solution and *N* iodine solution was run in until a faint blue was given with starch, and any ascorbic acid had been completely oxidized to dehydroascorbic acid. The 2:4-dinitrophenylhydrazine was then added (16 g. in 500 ml. of hot 2.5*N* HCl) and the mixture was incubated at 40° for 4 days. After cooling for 24 hr. the reddish brown precipitate was filtered off, boiled with two successive lots of 2.5*N* HCl to remove unchanged dinitrophenylhydrazine and then washed with warm water until the washings were no longer acid. The residue, dried *in vacuo* at room temperature, weighed 4.0 g. This was extracted in the cold with 2 l. of a mixture of equal volumes of absolute alcohol and dry acetone, and the insoluble residue was filtered off (it failed to dissolve in more fresh solvent). The filtrate was passed through a column of aluminium oxide (Merck, *Standardisiert nach Brockmann*), 14 cm. long and 3 cm. diameter, previously saturated with the mixed solvent (five such columns were used). The top of the column became brick-red and this narrow zone overlapped a broad dark purple zone, below which there were no further definite zones of adsorption, although the solution remained coloured. The chromatogram was developed with acetone, until the liquid passing through was colourless; the two zones were fairly well differentiated, but did not separate completely. The whole of the coloured aluminium oxide was extracted with hot glacial acetic acid, the eluate was concentrated *in vacuo* and kept overnight. The purple precipitate was filtered off, washed with water, dried and weighed (1.4 g.); a second crop (0.35 g.) was obtained by further concentration of the filtrate. Only a small amount of these precipitates dissolved in alcohol-acetone. The acetic acid mother-liquor was poured into 2 vol.

of water, and the immediate reddish precipitate (0.85 g.) was filtered off. On standing for 3 days a further precipitate (0.42 g.) was obtained. These two precipitates dissolved readily, but not completely, in the acetone-alcohol mixture.

The acetone-alcohol extracts from all four precipitates were combined and readsorbed on aluminium oxide. Again two zones were formed, a narrow red one at the top of the column, and a broad purple zone below and contiguous with it. These two zones, after development with acetone, were separately extracted with glacial acetic acid. The two eluates were treated separately with 2 vol. of water, and the flocculent reddish brown precipitates were filtered off after 3 days. The two precipitates were (separately) dried, dissolved in acetone-alcohol mixture, and passed through columns of aluminium oxide. In the case of the fraction derived from the upper, red, zone of the previous chromatograms, there were again two zones, but the lower, purple one was very small. The eluate from the purple zone now gave a very narrow upper red zone. The two purple zones from this set of chromatograms (the third) were combined and eluted with glacial acetic acid. As before, water was added to this eluate and the precipitate (which appeared only on standing) was dried, weighed (0.118 g.), dissolved in alcohol-acetone and once more adsorbed on aluminium oxide. The very narrow reddish zone at the top of the column was discarded, and the broad purple zone below it was extracted with glacial acetic acid. Water precipitation of this eluate yielded 65 mg. of precipitate.

This material was crystallized from a small volume of hot glacial acetic acid. It was not completely soluble, a small amount of an aluminium salt remaining undissolved. The substance deposited from the hot acetic acid on cooling was crystalline. A drop of the solution, on spontaneous evaporation, left rosettes of very small needles (Pl. VI, fig. 1*a*) identical with those similarly obtained from the 2:4-dinitrophenylhydrazine derivative of dehydroascorbic acid (Pl. VI, fig. 1*b*). Heated in a capillary tube it melted at 269–271° (decomposing sharply at 271°). The "synthetic" substance, prepared from pure ascorbic acid, melted at 270–272° (decomposing at 272°) and there was no depression when the two were mixed (270–272°).

After recrystallization of the urine derivative from acetone-alcohol, the m.p. rose to 279° (decomp.). The "synthetic" substance, similarly recrystallized, melted at 280° (decomp.), as did a mixture of the two. Herbert *et al.* [1933] describe two 2:4-dinitrophenylhydrazine derivatives of dehydroascorbic acid, prepared by the interaction of the components in 2.5*N* HCl at 70°. One, obtained when the reaction mixture was momentarily made alkaline before addition of the hydrazine, melted at 268°; the other, without use of alkali, melted at 280° after recrystallization from alcohol-acetone. We have used both methods, but found no difference in the ultimate melting points of the purified products.

The crystalline forms of the two substances were identical, fine needles often in sheaves or clumps. By slow evaporation of a solution in nitrobenzene, both appeared in long fine red needles, sometimes forming sheaves or clumps (Pl. VI, fig. 2*a* and *b*).

The yield of the purified substance was 20 mg.

The substance from urine contained 20.52% N by micro-Dumas; that prepared from pure ascorbic acid contained 20.56%. The calculated figure for $C_6H_6O_4[N.NH.C_6H_3(NO_2)_2]_2$ is 21.00% N.

With conc. H_2SO_4 , both substances gave a deep red coloration, changed to salmon-pink by dilution with water and to a reddish purple following a transient blue on making alkaline with NaOH. The colour faded to yellow in 5–10 min.

Fig. 1 *a*. $\times 150$.

Fig. 1 *b*. $\times 150$.

Fig. 2 *a*. From urine. $\times 100$.

Fig. 2 *b*. Synthetic. $\times 100$.

The purple colour with alkali was given without previous treatment with H_2SO_4 .

Examined through a hand spectroscope, both substances in dilute acetone solution showed a broad diffuse absorption band in the green extending from below the *E* line to above the *b* line. Several other red dinitrophenylhydrazine derivatives (e.g. those of furfuraldehyde and salicylaldehyde) showed absorption in the visible spectrum but nearer the blue (above *b*).

In further support of the identity of the "synthetic" hydrazone and that from urine, the distribution between two immiscible solvents was determined. In each case exactly 1 mg. of the substance was dissolved in 5 ml. of nitrobenzene, and the solution repeatedly shaken with 5 ml. of aqueous acetic acid (75 % by weight of acetic acid). After standing overnight in the dark, the corresponding layers were compared by means of a Pulfrich photometer using a 0.5 cm. cell and an S 53 filter. The corresponding layers contained identical concentrations, as is shown by the photometer readings (no attempt was made by use of standard solutions to convert these figures into actual concentrations).

	<i>E</i> Substance from urine	<i>E</i> Substance from pure ascorbic acid
Water-acetic acid layer	0.259	0.263
Nitrobenzene layer	1.45	1.45

Isolation of an unidentified 2:4-dinitrophenylhydrazone

Acetic acid elution of the final chromatogram in the isolation of the ascorbic acid derivative gave a solution which, on addition of water, gave a precipitate (weighing 65 mg.) from which the ascorbic acid derivative was isolated. This precipitate appeared after 3 hr. When the mother-liquor was kept 3 days longer, a further precipitate, much more definitely red, had appeared. This weighed 32 mg. Crystallized from glacial acetic acid it formed dark red micro-crystals which melted sharply at 221°. Recrystallization from acetone failed to raise the m.p. About 10 mg. of purified material were obtained. (Found: N, 20.01 % by micro-Dumas.) Although obtained from the same chromatogram zone as the ascorbic acid derivative (and on reabsorption forming a similarly placed purple zone) it seemed not to be merely an impure specimen. Thus: it depresses the m.p. of the pure ascorbic acid derivative; it gives a yellow colour with conc. H_2SO_4 , changing to a pure violet with NaOH; it absorbs in the green and blue of the visible spectrum, the band extending from the *E* line to above the *F*; and the crystalline form is different.

It was tempting to suspect that it might be the 2:4-dinitrophenylhydrazine derivative of reductic acid, which, according to Meuwissen & Noyons [1937], melts at about 225°. This substance, however, on the basis of the formula for reductic acid suggested by Reichstein & Oppenhauer [1933], should contain 23.7 % N. A closer agreement with the figures is given by furfuraldehyde 2:4-dinitrophenylhydrazone which is dark red, melts at 222° and contains 20.29 % N [Simon, 1932]. This substance could conceivably be formed from pentoses during the preliminary concentration of the urine, or even [Roe, 1936] from ascorbic acid itself. This possibility, however, was quickly disproved by the fact that a mixture of the two substances melted at 200°. Pyruvic acid 2:4-dinitrophenylhydrazone (m.p. 218°, 20.9 % N) is yellow; the hydrazone of methylglyoxal, though red, contains 25.9 % N and melts at 298°; that of glucose (20.8 % N) is reported by Torres & Brosa [1933] to melt with decomposition at 238°. According

to Torres & Brosa, and to Brady [1931], α -hydroxyaldehydes do not readily react with 2:4-dinitrophenylhydrazine under the conditions we employed, and in any case Meuwissen & Noyons [1937] have found that the hydrazones of lactose, galactose, glucose, glycuronic acid, dihydroxyacetone and glyceraldehyde were not adsorbed in the same way as that of ascorbic acid.

The substance must therefore remain unidentified until a repetition of the preparation has yielded enough for further investigation. The nitrogen content suggests that it is either a monohydrazone of a 3-carbon compound (among which the glucoreductone of von Euler & Martius [1933] remains a possibility) or a dihydrazone of a 6-carbon compound. Among the latter a polymeride of glucoreductone must be considered, and in view of the M.P. of the substance and its behaviour on adsorption, reductic acid cannot be entirely excluded in spite of the low nitrogen content. It is possible, too, that it may be another derivative of dehydroascorbic acid itself.

SUMMARY

From 12 l. of urine, 20 mg. of the pure 2:4-dinitrophenylhydrazine derivative of dehydroascorbic acid were isolated after repeated adsorption on aluminium oxide and crystallization from acetic acid and from acetone or acetone-alcohol.

The substance was identified by crystalline form, M.P. and mixed M.P., colour reaction with sulphuric acid and with sodium hydroxide, absorption in the visible spectrum and distribution between two immiscible solvents.

A second hydrazone, similarly adsorbed, has been isolated, but not yet identified.

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