39. *The Isolation and Some Properties of Dehydro-*l*-ascorbic Acid.*

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A crystalline solid possessing the characteristics of anhydrous dehydro-*l*-ascorbic acid has been isolated, the preparation together with that of two derivatives being described. A study of its properties has confirmed the view that in aqueous solution dehydro-*l*-ascorbic acid exists as a hydrate. In addition to those of 2 : 3-diketo-*l*-gulonic acid, a second series of salts has been obtained from dehydroascorbic acid. A possible structure of these salts is discussed.

The chemistry of dehydro-*l*-ascorbic acid—the primary reversible oxidation product of vitamin C—has hitherto been mainly investigated by utilising solutions of the freshly oxidised vitamin. In the course of early work on the constitution of *l*-ascorbic acid (Herbert *et al.*, *J.*, 1933, 1270), formulation of the dehydro-compound as the γ-lactone of 2 : 3-diketo-*l*-gulonic acid was established, absorption spectra data suggesting that hydration of the keto-groups in aqueous solution was probable. The labile nature of the substance has, however, prevented its isolation hitherto in a pure state, although Hirst and Woodward (*vide* Crook and Morgan, *Biochem. J.*, 1944, **38**, 10) have prepared it as a solid from which, by treatment with hydrogen sulphide, *l*-ascorbic acid could be regenerated in 65—75% yield.

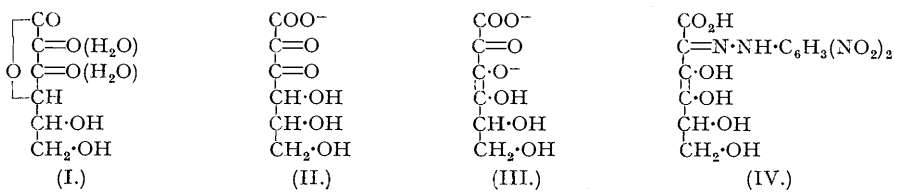
During the course of renewed investigation on this problem it has been found that when the solid product obtained by the method of Hirst and Woodward (*loc. cit.*) is intensively dried and dissolved in ethanol at room temperature, the solution on standing deposits a crystalline colourless compound which has the characteristics of anhydrous *dehydroascorbic acid*, although its existence as a polymer is not excluded. The absence of colour in an $\alpha\alpha$-diketone is surprising, but that the same property is found with 3 : 4-diketo-2 : 5-dicarbethoxytetrahydrofuran (Johnson and Johns, *Amer. Chem. J.*, 1906, **36**, 290), the structure of which in many respects resembles that of dehydroascorbic acid, is worthy of note.

Owing to its insolubility in neutral solvents measurement of many physical constants has been impossible. It was found necessary to warm to 60° to achieve immediate solution of anhydrous dehydroascorbic acid in water. On evaporation of the resultant solution a viscous syrup was obtained from which the anhydrous material could only be recovered, in low yield, by application of the drying procedure already mentioned.

The hydration hypothesis is thus confirmed, the syrupy product representing a hydrate form in which dehydroascorbic acid normally exists. In contrast to the anhydrous form, the hydrate is readily soluble in many organic solvents such as alcohols.

Acetylation of anhydrous dehydroascorbic acid with acetic anhydride and sulphuric acid yielded a *diacetyl* derivative. This compound, which was also colourless, was not dissolved by water even on boiling. The behaviour of aqueous solutions prepared from anhydrous dehydroascorbic acid was similar to that of solutions of freshly oxidised *l*-ascorbic acid; *e.g.*, hydrogen sulphide effected reduction to *l*-ascorbic acid which was isolated in good yield (crude, 90%). In water alone, the opening of the lactone ring to give free 2 : 3-diketo-*l*-gulonic acid was found to be extremely rapid in its initial stages. Immediately after preparation the solutions were invariably slightly acid, having pH 3·0—3·5. In optical rotation measurements and in titrimetric assays of *l*-ascorbic acid regenerated by hydrogen sulphide it was necessary to use as solvent a buffer solution of pH 3·5 in order to obtain maximum values. The characteristic osazone of 2 : 3-diketogulonic acid, which is described in the literature (Herbert *et al.*, *loc. cit.*; Drum, Scarborough, and Stewart, *Biochem. J.*, 1937, **31**, 1874; Penney and Zilva, *ibid.*, 1943, **37**, 39, 403) and is also obtainable from *l*-ascorbic acid and oxidised ascorbic acid, was produced by condensation with 2 : 4-dinitrophenylhydrazine in aqueous alcohol when starting with crystalline anhydrous dehydroascorbic acid.

The increase in acidity and accompanying mutarotation in oxidised *l*-ascorbic acid solutions has been studied by many workers (Herbert *et al.*, *loc. cit.*; Penney and Zilva, *loc. cit.*; Borsook, Davenport, Jeffreys, and Warner, *J. Biol. Chem.*, 1937, **117**, 237; Ghosh and Rakshit, *Biochem. Z.*, 1938, **299**, 394; Rosenfeld, *J. Biol. Chem.*, 1943, **150**, 281), the production of free 2 : 3-diketogulonic acid being later demonstrated by the isolation of the corresponding calcium and barium salts by Penney and Zilva (*Biochem. J.*, 1945, **39**, 1). We have similarly prepared the barium salt from solutions of crystalline dehydroascorbic acid after partial neutralisation with sodium hydroxide. It has been found, however, that in aqueous solution anhydrous dehydroascorbic acid titrates with alkali as a dibasic acid though its rearrangement to give the necessary H^+ ions is slow and the titration has to be carried out potentiometrically and in an indirect manner. The source of the second H^+ ion has been elucidated by the preparation of a further series of salts of the formula $C_6H_6O_7M$ (M = Na_2, Ca, Ba). These are orange-red in colour, the *disodium* salt giving an orange-coloured aqueous solution while the *calcium* and *barium* salts were almost insoluble in water. All were soluble in excess of mineral or acetic acid giving yellow solutions which possessed considerable reducing activity, taking up two equivalents of oxidising agent on treatment with bromine or iodine and slowly reducing 2 : 6-dichlorophenolindophenol to a like extent. This reducing activity was unaffected by treatment with hydrogen sulphide. The structure of the salts would be explained by the theory of Ghosh (*loc. cit.*); this author also observed that dehydroascorbic acid reacts with more than one equivalent of alkali and suggested that the ions might be derived severally from enolisation of the second keto-grouping and from opening of the lactone ring. It is now apparent, of course, that if this be the explanation, opening of the lactone ring must preceed enolisation (formulæ I, II, III).



(I.) (II.) (III.) (IV.)

The development of yellow colour in oxidised *l*-ascorbic acid solutions on keeping is well known, and Rosenfeld (*loc. cit.*) has found a correlation between the colour intensity and the reducing activity of such solutions.

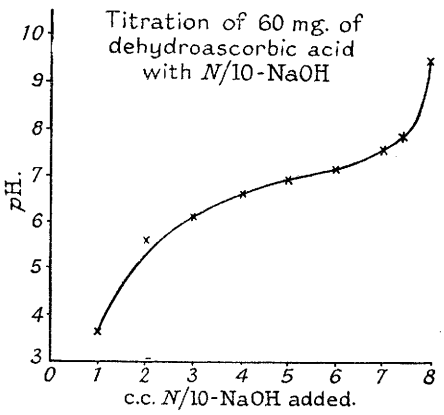
Further evidence for the enol–acid structure is the fact that only one molecule of 2 : 4-dinitrophenylhydrazine will condense with the free acid (III) obtained in solution by treatment of the salts with sulphuric acid in ethanol; the resulting dark red dinitrophenylhydrazone has the formula $C_{12}H_{12}O_{10}N_4$, which is in accord with the postulated structure (IV).

It is, however, difficult to understand how a compound having such a structure could give only oxalic and threonic acids on decomposition as already established with dehydroascorbic acid (Hirst and Reynolds, *Nature*, 1932, **129**, 576; **130**, 888; Herbert *et al.*, *loc. cit.*).

Experimental.

*Anhydrous Dehydro-*l*-ascorbic Acid.*—Pure *l*-ascorbic acid (8·8 g.) was shaken with resublimed iodine (12·4 g.; 1·96 mols.) in methanol (75 c.c.; water content, 0·5%). Excess of precipitated lead carbonate (30—35 g.) was gradually added until completion of the reaction, *i.e.*, until a colourless solution was obtained after settling of the lead salts. The solution was filtered and the residues were washed with methanol (50 c.c.). Traces of lead remaining in solution were precipitated by a few bubbles of hydrogen sulphide, excess of this reagent being immediately removed by a current of air. Failure to remove excess of hydrogen sulphide with the minimum of delay results in the presence of small amounts of sulphur in the final product. The dehydroascorbic acid–methanol solution was filtered from lead sulphide through kieselguhr and subsequently evaporated at *ca.* 30—40° (bath temp.)/20 mm. to a syrup. The bath temperature was then gradually raised (during 20 minutes) to 100° and maintained thereat for a further hour before cooling to room temperature, the dehydroascorbic acid being kept under vacuum. After cooling, the resulting crisp glass was shaken with absolute alcohol (30 c.c.) and the solution kept at 0° for 2 days. The microcrystalline *dehydroascorbic acid* was collected, washed twice with absolute alcohol (25 c.c.) followed by cold distilled water (10 c.c.) and finally with a further 50 c.c. of absolute alcohol, before being dried in a vacuum. Yield, 2·0 g. (Found: C, 41·2, 40·9; H, 3·5, 3·8. $C_6H_6O_6$ requires C, 41·4; H, 3·5%).

Freshly prepared anhydrous dehydroascorbic acid was white, and had m. p. 225° (decomp.); after 6 months at ordinary temperature it had become yellow-brown and then had m. p. 204—205° (decomp.). Purification by crystallisation was not found possible owing to its insolubility in all available neutral organic solvents. It dissolved readily in basic solvents such as pyridine but could not be recovered. Complete and immediate solution in water was only obtained on warming to 60°; no solid product separated on cooling.



Properties of Dehydroascorbic Acid in Aqueous Solution.—(a) *Regeneration of ascorbic acid by hydrogen sulphide.* Anhydrous dehydroascorbic acid (2 g.) was dissolved in water (100 c.c.) at 60°; the solution was rapidly cooled to 0° and a current of hydrogen sulphide passed for 90 minutes, the temperature meanwhile being allowed to rise to 14°. The solution was kept in a closed vessel for 2 days before the excess of hydrogen sulphide was removed in a current of carbon dioxide. The precipitated sulphur was filtered off and washed with a further 100 c.c. of water, and the combined filtrates were evaporated at 30° (bath temp.)/10 mm. in a current of carbon dioxide. The slightly yellow residue was triturated with acetone (30 c.c.), filtered off, and dried. Yield, 1·8 g. (90%); m. p. 173—176° (decomp.); content of *l*-ascorbic acid by iodine titration, 91%. By recrystallisation from methanol–ether–petrol the purity was raised to 99%; m. p. alone and mixed with authentic *l*-ascorbic acid, 188—189° (decomp.).

Anhydrous dehydroascorbic acid (100 mg.) was dissolved with warming in 15 c.c. of a phthalate–hydrochloric acid buffer of pH 3·5. A further 15 c.c. of cold buffer were immediately added and the solution was saturated with hydrogen sulphide during ¾ hour before being kept corked for 12 hours. Excess of hydrogen sulphide was removed by a current of carbon dioxide (—ve test to lead acetate paper), one drop of glacial acetic acid added, and the solution titrated with N/10-iodine (titre, 11·0 c.c.; calc., 11·4 c.c.).

This experiment was repeated, the buffer solution being replaced by distilled water (titre, 10·5 c.c.).

(b) *Specific rotation.* A 1% dehydroascorbic acid solution prepared by dissolving the anhydrous material in a phthalate–hydrochloric acid buffer of pH 3·5 at 60° and immediately cooling to 20°, had $[\alpha]_D^{20°}$ +56° (initial); +53·5° (after 2 hours); +19·0° (3 days); −2·0° (5 days); −6·0° (6 days). The solution had become red-yellow when readings were discontinued. It was found necessary to cool the freshly dissolved dehydroascorbic acid as rapidly as possible in an ice-bath, otherwise a slightly lower initial specific rotation was obtained.

A 1% solution similarly prepared in distilled water had $[\alpha]_D^{20°}$ +50° (initial); +44° (after 2 hours); +23° (2 days); +16° (3 days); 0° (6 days).

(c) *Acidity of dehydroascorbic acid and titration with alkali.* A 1% aqueous solution obtained on warming followed by cooling to 20° had pH 3·37 (initial); 2·08 (after 6 days). Anhydrous dehydroascorbic acid (100 mg.) likewise dissolved in distilled water (10 c.c.) required 0·4 c.c. of N/20-sodium hydroxide for titration to methyl-red. Anhydrous dehydroascorbic acid (0·600 g.) was dissolved in hot distilled water and the solution made up to 100 c.c. Aliquots (10 c.c.) were removed and quantities of N/10-sodium hydroxide (from 1 to 8 c.c.) added to each before these were warmed, under nitrogen, to 70—80° for 20 minutes. Each solution was then cooled, made up to 25 c.c. with water, and the pH measured electrometrically.

No rapid increase in alkalinity was found until just over 2 equivs. of alkali were present. The slight discrepancy is probably due to the production of acid breakdown-products in the more alkaline solutions as has been observed by other investigators. Another experiment was carried out in which the pH was measured immediately. Downward drifts of pH at values over 6 were encountered; these were slow and sustained, and the foregoing procedure was therefore adopted. Attempts to measure the intensity of yellow–red colour produced in the more alkaline solutions failed owing to fading.

(d) *Recovery of anhydrous dehydroascorbic acid.* Anhydrous dehydroascorbic acid (1 g.) was dissolved with gentle warming in distilled water (30 c.c.) and the resultant solution immediately evaporated

below 30°, first at 10 mm. and finally at 0·1 mm. A very viscous, pale yellow syrup was obtained which did not crystallise on being kept for 1 week at 0°. 1·089 G. of syrup dried to constant weight at 90—100° in a vacuum lost 0·160 g. (14·7%). The resultant reddish glass was powdered, shaken with 20 c.c. of absolute alcohol, and kept at 0° for 3 days. 60 Mg. of anhydrous dehydroascorbic acid [m. p. 221° (decomp.)] were recovered.

Derivatives of Anhydrous Dehydroascorbic Acid.—2 : 4-*Dinitrophenylosazone.* The acid (0·5 g.) was dissolved with gentle warming in water (4 c.c.), ethanol (15 c.c.) added, and the solution treated with 2 : 4-dinitrophenylhydrazine (1·0 g. + 2 c.c. of concentrated sulphuric acid + 20 c.c. of ethanol). The osazone was collected next day and well washed with ethanol before being dried; m. p. 282° (decomp.) alone and when mixed with an authentic osazone prepared from *l*-ascorbic acid (Found : N, 21·0%). *Acetylation.* The acid (0·5 g.) was gently warmed with acetic anhydride (15 c.c.) containing 2 drops of concentrated sulphuric acid until solution was complete. The solution was poured into cold distilled water (50 c.c.) and stirred for 5 minutes to precipitate the white *acetyl* derivative which was washed by decantation with cold water until free from acid and finally boiled with 50 c.c. of water, filtered off, and dried. Yield, 0·45 g.; m. p. 246—247° (decomp.; darkens and shrinks *ca.* 230°). It can be recrystallised from aqueous pyridine, m. p. unchanged (Found : C, 46·4, 46·2; H, 3·8, 3·7. $C_{10}H_{10}O_8$ requires C, 46·5; H, 3·9%).

Preparation of Salts from Dehydroascorbic Acid.—*Barium diketogulonate.* To a solution of anhydrous dehydroascorbic acid (0·87 g.) in water (6 c.c.) N-sodium hydroxide (5 c.c.) was added gradually. The yellow solution was adjusted to pH 3 with N-hydriodic acid, when it became colourless; barium iodide (0·8 g.) was then added. The barium diketogulonate which separated after the addition of ethanol (100 c.c.) was filtered off, washed with ethanol and ether, and dried in a vacuum (Found : Ba, 21·0. Calc. : Ba, 20·7%). The salt gradually turned yellow in a vacuum desiccator at room temperature.

Salts from dibasic dehydroascorbic acid. A solution of anhydrous dehydroascorbic acid (1·74 g.) in warm distilled water (5 c.c.) and methanol (25 c.c.) was gradually stirred into 1·6 g. (4 equivs.) of sodium hydroxide in methanol (100 c.c.). The *disodium* salt which appeared as an orange precipitate was collected and washed with 3 × 50 c.c. of methanol, being well stirred in the filter each time. This hygroscopic salt was immediately dried in a vacuum desiccator then triturated with more methanol (25 c.c.), collected again, and redried to constant weight in a vacuum, first at ordinary temperature and finally at 60°. Yield, 1·5 g. (Found : Na, 19·7. $C_6H_6O_7Na_2$ requires Na, 19·5%).

This disodium salt (1·0 g.) was dissolved in cold distilled water (10 c.c.) and a solution of 0·42 g. (0·8 equiv.) of calcium chloride in water (10 c.c.) stirred in. The *calcium* salt was precipitated as a gelatinous solid which was filtered off with some difficulty. Washing was carried out by stirring in the filter with 50 c.c. of water in 2 parts. The salt was dried in a vacuum, first at 20° and finally at 100° (Found : C, 31·7; H, 2·8; Ca, 17·5. $C_6H_6O_7Ca$ requires C, 31·3; H, 2·6; Ca, 17·4%). The *barium* salt was prepared in a similar way using 1·0 g. of disodium salt and 0·7 g. of barium chloride (Found : Ba, 42·7. $C_6H_6O_7Ba$ requires Ba, 41·8%).

The calcium and barium salts dissolved in excess of calcium or barium chloride solution to give dark red solutions, and it was for this reason that less than the calculated quantities of these reagents were used in their preparation.

The disodium salt (0·8 g.) was stirred in ethanol (20 c.c.) containing 4—5 drops of concentrated sulphuric acid until reaction was complete. The residual sodium sulphate was filtered off, and excess of alcoholic 2 : 4-dinitrophenylhydrazine (1·0 g. + 2 c.c. of concentrated sulphuric acid + 20 c.c. of ethanol) added. After 4 days at ordinary temperature, the dark red derivative was collected, washed with ethanol, and dried in a vacuum. Yield, 0·25 g.; m. p. 208° (decomp.) (Found : C, 39.5; H, 3·1; N, 15·0. $C_{12}H_{12}O_{10}N_4$ requires C, 38·7; H, 3·2; N, 15·1%).

Reducing action of the disodium and the calcium salt. To a freshly prepared solution of the calcium salt (0·125 g.) in hydrochloric acid (25 c.c. of N/5), bromate–bromide solution (20 c.c. of N/10) was added. After ½ hour, excess of potassium iodide solution was added and the solution titrated with N/10-thiosulphate. (N/10-Bromine uptake, 10·5 c.c. Calc. for 2 equivs. : 10·8 c.c.)

Disodium salt (0·104 g.) was dissolved in the calculated quantity (8·8 c.c.) of N/10-hydrochloric acid for exact neutralisation. N/10-Iodine (20 c.c.) was added and the solution kept for 15 minutes before titration with N/10-thiosulphate. (N/10-Iodine uptake, 9·1 c.c. Calc. : 8·8 c.c.)

5 C.c. of a phosphate–citrate buffer of pH 3·5 containing 1 mg. of disodium salt were titrated with standard 2 : 6-dichlorophenolindophenol (1 c.c. = 0·30 mg. of ascorbic acid). No sharp visual end point was obtained, dye decolourisation becoming progressively slower. After 20 minutes the dye uptake was *ca.* 2 equivs. (2·3 c.c.), further reduction being negligible. Saturation of the disodium salt solution with hydrogen sulphide for 2 hours followed by removal of excess by means of carbon dioxide had no effect on the titration values subsequently obtained.

In these titrimetric oxidations freshly prepared salts were used, their reducing power having been observed to deteriorate on keeping.

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