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The Oxidation of Ascorbic Acid by *o*-Dinitrobenzene, and the Detection of Dehydroascorbic Acid

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During a survey of the natural sources of ascorbic acid, three reactions have been observed that may be of analytical value: (1) the rapid reduction of *o*-dinitrobenzene by ascorbic acid to a substance giving a violet pigment in alkaline solution, (2) the formation of a green pigment when dehydroascorbic acid is boiled alone in aqueous solution at pH 3.5–4.5, and (3) the spontaneous formation of a red pigment when dehydroascorbic acid is kept in alkaline solutions out of contact with air.

(1) Reaction of ascorbic acid with *o*-dinitrobenzene

(a) *Experimental.* When 2–5 ml. of a dilute (0.02%) aqueous solution of ascorbic acid are treated in the cold with 5 drops of a saturated aqueous solution of *o*-dinitrobenzene, and then made alkaline by addition of 5 drops 20% NaOH, a violet colour rapidly develops, and is stable for several hours. Under these conditions the colour is not given by dehydroascorbic acid, cysteine, glutathione, uric acid or creatinine. Fructose gives a slow positive reaction in the cold, but only after 10–15 min., when the solution contains at least 0.2% of this sugar. The other reducing sugars may require 2 hr. or longer. Proteins, simple alcohols, aldehydes, ketones, and the commoner hydroxy-, keto-, and amino-acids do not give the test. The test loses its selectivity if the reaction mixture is heated, and a positive result is then given by all the reducing sugars and by uric acid. Thus, by controlling temperature and concentration of alkali, it becomes possible to detect ascorbic acid in presence

of other compounds of biological importance. On acidification, the violet pigment changes to yellow, and can be extracted by chloroform or by peroxide-free ether. Addition of alkali removes the pigment from the organic solvent, and regenerates the violet colour. By this means, small amounts of ascorbic acid can be detected in solutions the colour of which obscures the test.

(b) *History of the reagent.* Lipschitz [1920] reported that living tissues, such as muscle and yeast, contain a catalytic system capable of reducing '*m*-dinitrobenzene' to a yellow pigment, at pH 7.4, under anaerobic conditions. Addition of alkali converted the yellow into violet. The test was developed colorimetrically, and it was later found to depend on the presence of *o*-dinitrobenzene as a contaminant in the *m*-isomer [Lipschitz & Osterroth, 1924]. Apparently unaware of this, Ekkert [1934] claimed that reducing sugars gave a violet colour when heated in alkaline solution with *m*-dinitrobenzene, but v. Szecseny-Nagy [1935] showed that this reaction was due also to contamination by the *o*-isomer, and that pure *m*-dinitrobenzene gave no colour. This was confirmed by Truhaut [1937].

(c) *Mechanism of the test.* From an inspection of the formulae of the reactants, the test seems to depend on the presence of the enediol system, —C(OH)=C(OH)— , which occurs in ascorbic acid, though, of course, the reagent is not specific for this system, since powerful inorganic reducing agents such as hydrosulphite give a similar response.

Aldoses and ketoses react only when conditions bring about enolization. Kept in alkaline solution at room temperature for some hours they give immediate violet on addition of the reagent, which

thus may be used to demonstrate the relative rate of transformation. Polyhydroxy compounds incapable of enolization, such as glycerol, mannitol, sorbitol, mucic and tartaric acids, do not react. Pyrogallol, when dissolved in oxygen-free water, and protected from atmospheric oxidation by a layer of toluene, gives an immediate reaction, as also does 1:3-dihydroxyacetone, and glucoreductone.

The pigment formed by the reduction of *o*-dinitrobenzene has been investigated by Bamberger & Hübner [1903], and by Meisenheimer [1903]. Using hydroxylamine as the reducing agent, the end-product was identified as *o*-nitro-nitrosobenzene, and isolated as the Na salt. Working under less drastic conditions, Patzig & Patzig [1906] were able to recover *o*-dinitrobenzene from the pigment after acidification and atmospheric oxidation.

The reaction with ascorbic acid has been found to proceed on similar lines. When equimolecular proportions of ascorbic acid and *o*-dinitrobenzene interact in *N*/10 NaOH, the primary product is the violet-coloured ion of the *o*-nitroxylic acid, which on acidification is converted into the yellow form of the free acid, soluble in ether.

After recrystallization from ethanol, the acid separates out in wisps of very fine yellow needles which turn violet on exposure to NH_3 . Melting-point determinations were unsatisfactory, as the acid readily dehydrates to *o*-nitro-nitrosobenzene, which is recognizable by the fact that it gives no violet colour on addition of alkalis.

The low solubility of *o*-dinitrobenzene in water (about 0.03% at room temperature) restricts the yield of the pigment, while if the ascorbic acid be in excess at the start of the reaction the reduction proceeds beyond the nitroxylic stage.

(d) *Preparation of the pigment.* 100 mg. ascorbic acid (Roche or B.D.H.) were dissolved in 50 ml. Cu-free distilled water in which were suspended 100 ml. finely powdered *o*-dinitrobenzene. 1 ml. 20% NaOH was added drop by drop, the mixture being shaken continuously. A deep violet colour appeared immediately, and became more intense as the dinitrobenzene slowly dissolved. After 12–24 hr. at room temperature, all the ascorbic acid had been oxidized, as was shown by withdrawing samples, discharging the violet colour by acetic acid, and then titrating with standard 2:6-dichlorophenolindophenol. The mixture was then filtered free from undissolved dinitrobenzene, and treated with CO_2 until the colour became orange-yellow. It was then concentrated *in vacuo*, acidity being maintained by passing in CO_2 whenever the violet colour reappeared. At the end of 2–3 days, yellow crystals of *o*-nitro-nitrosobenzene (m.p. 87–90°) began to separate out in small clusters on the surface. The yields were only about 10% of the theoretical, the bulk of the original *o*-dinitrobenzene having undergone further reduction to a mixture of at least three different products, which separated out in a yellow crystalline crop when the solution was concentrated to a syrup.

In terms of current formulation, the primary reaction is:

(2) Pigment formation from dehydroascorbic acid alone

(a) *At pH 4.* 5 ml. of a dilute (0.005–0.5%) aqueous solution of dehydroascorbic acid, adjusted to pH 3.5–4.5 by means of an acetate-acetic acid buffer, and gently boiled for a couple of minutes, develops a stable grass-green colour. The colour is not given by the reduced form of ascorbic acid, unless boiling is prolonged sufficiently to allow of atmospheric oxidation. Other reducing agents, such as cysteine, glutathione, the monosaccharides, uric acid and creatinine, do not give the test. Colour formation is not dependent on the presence of acetate ions, but can be observed in mixtures suitably buffered by phosphate or $(\text{NH}_4)_2\text{SO}_4$ and H_2SO_4 . The green colour is stable for several hours, but may turn brown if the solution is too concentrated, the acidity insufficient, or the boiling greatly prolonged. The solution may be diluted with water for colorimetric comparison. Multivalent ions, especially Al^{+++} , Ca^{++} , Zn^{++} , and Pb^{++} , intensify the colour and change it to yellow, but accelerate the subsequent bleaching by atmospheric oxidation.

The reaction can be demonstrated by buffering 3 ml. of a 0.1% aqueous solution of ascorbic acid by addition of about 1 g. of solid Na acetate and 1 ml. of glacial acetic acid. The mixture is then carefully oxidized by addition of 1% iodine drop by drop, any excess of iodine being bleached by addition of a particle of thiourea or a drop of ascorbic acid solution, as it tends to destroy the pigment. After boiling the mixture for 1 min., the green colour appears and reaches a maximum within 5 min. The pigment can be extracted directly by amyl alcohol, or by ethanol or acetone after saturation of the mixture with $(\text{NH}_4)_2\text{SO}_4$. Addition of alkali converts the green into a red pigment rapidly bleached by exposure to air. Formation of the green pigment can also be observed when dehydroascorbic acid is kept in contact with concentrated HCl at room temperature, or warmed gently, but the test is best obtained by boiling at pH 4.

The reaction is not obviously affected by proteins, amino-acids, sugars, or the urinary solutes; and as none of the common biological compounds examined gives a similar response, the test can be used to show the presence of dehydroascorbic acid in natural sources, such as the white inner rind of citrus fruits. It can be applied to coloured solutions after acidification by acetic acid and agitation with 'norite' charcoal, which also oxidizes any ascorbic acid present. Although the intensity of the green pigment is not great, small amounts may be detected by extraction into an amyl alcohol layer.

(b) *At pH > 9.* When 5 ml. of a 0.1 % aqueous solution of dehydroascorbic acid, preferably in freshly boiled water, are made strongly alkaline by addition of about 1 ml. 20 % NaOH, and protected from atmospheric oxidation by a layer of toluene, a bright yellow-green colour develops within 30 min. at room temperature, and in the course of some hours slowly changes into a deep carmine red. The red pigment is rapidly bleached by exposure to air during the stage of its formation, but is stable for upwards of a week if protected from oxidation, unless the mixture be too strongly alkaline, when it changes to brown and tends to precipitate. The colour reaction is not given by reduced ascorbic acid, reducing sugars, simple ketones or other enolizable compounds. Colour formation may be hastened by gentle warming, but the pigment is then less stable. While the green precursor of the

$=C=C(OH)-CO-R$. Hence, it was first assumed that the pH 4 green pigment was merely the true diketone form of the oxidized vitamin. This is disproved by the fact that the pigment does not yield an osazone with phenylhydrazine, and cannot be reconverted into recoverable ascorbic acid by reduction. During the process of boiling, CO_2 is evolved, which suggests that the $-CO-COOH$ end of the chain is being decarboxylated, as would happen if a fural derivative were being formed. That a furfural is obtained by the action of acids on dehydroascorbic acid can be demonstrated by warming some of the solution with an equal volume of concentrated HCl and a few mg. of pyrogallol, when a deep purple colour develops. Reduced ascorbic acid gives this reaction only on prolonged heating and aeration. The pH 4 green pigment does not give the pyrogallol reaction, nor can furfural be obtained by distillation of the mixture, which indicates that furfural formation precedes the formation of the green pigment, and enters into its construction.

Furfural can also be detected in the alkaline colour reaction by addition of acetone, which combines with it to form difurfurylaceton, stable to atmospheric oxidation and yielding a bright carmine on acidification. This can be demonstrated only in the early stages of the test.

red pigment closely resembles the green pigment obtained from dehydroascorbic acid at pH 4, it is not identical, since it is bleached by acidification, whereas acidification of the red pigment changes it into a green indistinguishable from the pH 4 pigment. The red pigment in neutral solution yields a series of lakes, especially with Al and Zn, and also can be separated by adsorption on $MgCO_3$ suspensions.

(3) Mechanism of the tests

Pending actual isolation of the pigments, which has not yet been accomplished, the following suggestions are advanced. Since freshly prepared dehydroascorbic acid is colourless, it presumably does not contain the chromogenic 2:3-diketone system, $=CH-CO-CO-R$, but exists as the enol form,

The fact that ascorbic acid is quantitatively converted into furfural by boiling with HCl is well known [Cox, Hirst & Reynolds, 1932; Roe, 1934], while the development of a yellow colour in alkaline solutions of the vitamin has been noted [Herbert, Hirst, Percival, Reynolds & Smith, 1933], but neither the acid green pigment nor the red alkali pigment appears to have attracted attention, although the latter may be concerned in the reaction described by Kruger [1906], in which milk made strongly alkaline and kept at room temperature for several days often develops a red colour.

SUMMARY

1. A colour reaction between ascorbic acid and *o*-dinitrobenzene is described.

2. Solutions of dehydroascorbic acid spontaneously form pigments under certain conditions.

3. The specificity of these colour reactions is described, and their mechanism discussed.

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Studies in Detoxication

13. THE BIOSYNTHESIS OF AMINOPHENYL- AND SULPHONAMIDOAMINOPHENYL-GLUCURONIDES IN THE RABBIT AND THEIR ACTION ON HAEMOGLOBIN *IN VITRO*

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Sieberg [1914] fed 'cupferron' (ammonium salt of nitroso- β -phenylhydroxylamine) to dogs and isolated from their urine a crystalline compound, m.p. 179–180°, which he claimed to be the *lactam* form (I) of *p*-aminophenylglucuronide. He isolated the same compound from urine after the administration of β -phenylhydroxylamine. When this substance was neutralized with Na_2CO_3 and the solution kept for a day, it was able to convert a solution of haemoglobin to methaemoglobin. It should be noted that the β -glycosidic link present in this compound is stable to dilute Na_2CO_3 , so that in this case formation of methaemoglobin could not be ascribed to hydrolysis of the glucuronide to *p*-aminophenol, which is a methaemoglobin-former *in vitro* and *in vivo* [Bernheim, Bernheim & Michel, 1937]. From this it would appear that the 'detoxicated' form of *p*-aminophenol could still exert a toxic effect on blood. Therefore, it was important to confirm this observation of Sieberg and to extend it to the study of the properties of the aminophenyl- and sulphonamidoaminophenylglucuronides, none of which has been prepared or described hitherto.

Aminophenylglucuronides

Aminophenylglucuronides are produced in the animal body in response to the administration of the aminophenols themselves, or of certain aromatic amino compounds which are oxidized or converted in some way to aminophenols. When aromatic amino compounds are oxidized *in vivo* to aminophenols, *o*- or *p*-phenols only are produced and in no case studied has *m*-aminophenol been detected.

o-Aminophenol is produced when dimethylaniline or its oxide [Horn, 1936] or acetanilide [Jaffe & Hilbert, 1888] is fed to dogs, or when salvarsan is injected into humans [Sieberg, 1916]. It is excreted in conjugation both with sulphuric and glucuronic acids, and in the rabbit some 26–29% (dose, 0.25 g./kg.) is excreted as an ethereal sulphate [Williams, 1938]. In the present work *o*-aminophenylglucuronide has been isolated from the urine of rabbits fed with *o*-aminophenol. The yield of the crystalline glucuronide was equivalent to 25.5% of the phenol fed and since no free aminophenol was excreted, the remainder, if excreted, must be in