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Possible explanation and implications of the reaction of ascorbic acid with some disulphide reagents

Ascorbic acid has been postulated as a reducing agent for disulphide groups (Lewin, 1976). Evidence for this role comes from experiments in which ascorbic acid was required for the photochemical reduction of Ellman's thiol estimating reagent, Nbs_2, in the presence of chromatophores and low potential dyes (Newton, 1962). Ascorbic acid inhibits the thiol/disulphide enzyme barley β-amylase by reducing endogenous cupric ion to cuprous ion which reacts with enzyme thiol groups (Rowe & Weill, 1959). While investigating aspects of this inhibition we noted that Nbs_2 was reduced when it was incubated with the enzyme and ascorbic acid. This reaction interfered with our experiments so we endeavoured to establish the chemical basis of the effect of the effect by using simple thiol compounds.

Reactions were performed at room temperature. A reaction mixture containing 140 μmol of phosphate buffer, pH7.0, 0.4 μmol of $CuSO_4$ and 2 μmol of ascorbic acid per 3ml was used in most experiments, but the copper and ascorbic acid concentrations were sometimes altered as indicated. A disulphide compound (either 1 μmol of Nbs_2, 0.4 μmol of cystine or 2 μmol of oxidized glutathione) was added to the reaction mixture.

Addition of copper ion to a reaction mixture containing Nbs_2 caused an immediate increase in absorbance and the reaction proceeded to equilibrium (Fig. 1). Copper concentrations as low as 0.08 μmol/3ml still catalysed reduction but gave a lower final absorbance (0.38). When the amount of Nbs_2 reduced was plotted against the concentration of ascorbic acid, the graph was sigmoidal at lower concentrations (up to 2.5 μmol/3ml), which was suggestive of binding phenomena. After correcting for other components in the reaction mixture the ratio of Nbs^- formed (412nm) to Nbs_2 reacting (323nm) indicated a 2:1 stoichiometry. The concentration of Nbs^- was calibrated using solutions in which Nbs_2 was fully reduced by an excess of cysteine.

Abbreviation used: Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid).

The reducing effect of ascorbic acid on other disulphides was assessed by incorporating cystine or oxidized glutathione into the reaction mixture and incubating for 2h. No reduction of the cystine or glutathione was detected by standard methods for detecting thiol groups (Stadtman, 1957). However, at the end of the incubation period addition of Nbs_2 and 5 μmol of EDTA resulted in reduction of the Nbs_2. The 2h incubation period was sufficient to oxidize all the ascorbic acid (measured at 265nm) in the absence of biological disulphides, but 18% of original ascorbic acid remained in the presence of cystine.

In another experiment using the oxygen electrode to measure oxygen uptake by solutions of ascorbic acid, 2.1 μmol of oxygen were consumed during the oxidation of 1.19 μmol of ascorbic acid in the absence of disulphides, whereas Nbs_2 and cystine reduced oxygen consumption to 1.14 μmol and 0.73 μmol respectively.

Ascorbic acid oxidation proceeds via the formation of complexes with copper and oxygen (Bauernfeind & Pinkert, 1970). From the above results we suggest that a derivative of ascorbic acid forms complexes with copper and disulphide

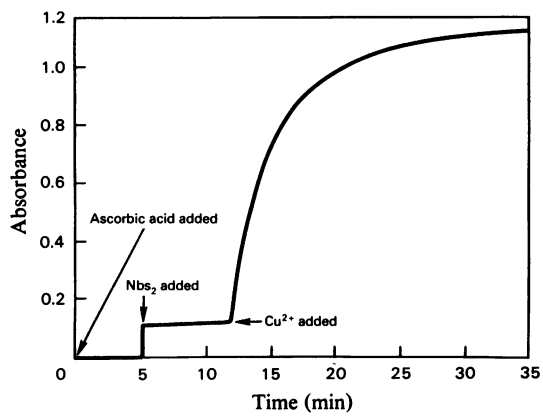

Fig. 1. *Effect of copper ion on the reduction of Nbs_2 by ascorbic acid measured at 412nm*

Cuvette contained, per 2.8ml, 140 μmol of phosphate buffer, pH7.0, and 2 μmol of ascorbic acid. Nbs_2 (0.1ml of 10mM) and $CuSO_4$ (0.1ml of 4mM) were added as indicated. In other experiments oxidation of ascorbic acid was initiated by a similar addition of $CuSO_4$.

groups. The complexes with cystine and glutathione are probably relatively stable, whereas Nbs_2 is more readily reduced because of the electron-withdrawing effect of its aromatic groups. The actual reductant is unknown, but could be the monodehydroascorbate anion or the ascorbate free radical.

As ascorbic acid has medical and technological uses, the Nbs_2 reduction phenomenon is being further investigated as a sensitive method for following either metal or enzyme-catalysed ascorbic acid oxidation. The striking effect of copper ion on Nbs_2 reduction in the presence of ascorbic acid must be considered when assaying for free thiol groups in preparations rich in the vitamin. Conversely, assay results for ascorbic acid in food or tissue samples containing disulphides may be affected if copper is present in significant concentrations.

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