

Initial Reaction Intermediates in the Oxidation of Ascorbic Acid by Nitrous Acid

JAY B. FOX, JR., ROSEMARY N. FIDDLER and AARON E. WASSERMAN*

Eastern Regional Research Center¹, Philadelphia, Pennsylvania 19118

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ABSTRACT

Nitrite and ascorbate react to form nitric oxide at pH 5.5. In the initial stages of the reaction, seven intermediates can be identified spectrally and chromatographically; these include two colorless nitroso derivatives which contain 30-60% of the initial nitrite, two nitroso reductant derivatives absorbing at 345 and 412 nm, diketogulonic acid and two further decomposition products. None of the intermediates was paramagnetic; except for diketogulonic acid, all decomposed rapidly during or after isolation. Based on the order of appearance of the ultraviolet and visible absorption bands in the reaction mixtures, the observed characteristics of the intermediates, and the lack of organic radicals, a sequence is proposed for the initial steps in the oxidation of ascorbic acid by nitrous acid.

Studies of the kinetics of oxidation of ascorbic acid by nitrous acid (6-11.17) have led to a postulated mechanism in which the first step is formation of N_2O_3 from the bimolecular reaction of nitrous acid. N_2O_3 then nitrosates ascorbic acid to form the semistable intermediate, nitrosoascorbic acid, which in turn, decomposes by forming either NO and the ascorbyl radical or a dimer from which the original reactants are regenerated (17). None of the intermediates was isolated in these studies. There is, however, direct evidence that semistable intermediates are formed, for measurement of the optical absorbance of reacting solutions show formation of maxima at 290, 340, and 415 nm (16). Absorption bands at 290 and 340 nm have also been observed during oxidation of ascorbic acid by other oxidizing systems (3,20,25), but there is some question as to the identity of the compounds producing them. Bielski et al. (3) claimed that compounds absorbing at ca. 290 and 340 nm were the acid-base forms of the ascorbyl radical produced during the oxidation. This claim seemed unusual because it has always been held that the absorbance at ca. 290 nm was due to diketogulonic acid

(DKGA), the hydrolysis product of dehydroascorbic acid (DHA) (20). DKGA and DHA are generally accepted to be two electron oxidation products of ascorbic acid, hence neither would show an EPR signal. Furthermore, Laroff et al. (25) proved that the observed radical is fully ionized ($pK = -0.17$) at higher pH values, which would preclude any equilibrium between compounds absorbing at 290 and 340 nm, since the ionized radical would have only one form at the pH values at which the other studies were made. This still does not preclude either the 290- or 340-nm absorbing material from being a radical compound. In view of the importance of radical reactions in oxidative processes, it is of interest to know whether or not the oxidation of ascorbate by nitrite produces the ascorbyl radical. In free radical chain reactions, nitric oxide acts as a chain inhibitor (12). In view of these questions, we isolated the reaction intermediates and characterized them to further elucidate the oxidation mechanism.

EXPERIMENTAL

Reagent grade or highest purity chemicals from a variety of sources were used. Nitrite and ascorbate, 4 mM each, were reacted with each other at pH 5.5, 70 C. The buffer was $\Gamma/2 = 0.3$ sodium acetate. At gradually lengthening time intervals beginning every 5 min, the reacting solutions or appropriate dilutions thereof were scanned for absorbance in the ultraviolet and visible regions. The spectra so obtained were separated into their component Gaussian peaks by a program written by C. R. Eddy and V. Metzger of the Center for use in an IBM 1130 computer. The intensities of the resolved peaks were plotted as a function of time.

Bio-Rad² anion exchange resin AG1-X2, 200-400 mesh, was used for separation of the reaction products. Five milliliters of the reaction mixture were placed on a column with a resin bed 2.5 cm diameter \times 20 cm long (100 ml) and eluted with the reaction buffer. Eluant from the column was continuously monitored at 288 nm by means of a flow cell in a recording spectrophotometer. The eluant was then collected in fractions and, after the ultraviolet and visible absorptions were recorded, each fraction was analyzed for its nitrite and carbonyl content.

The nitrite concentration of each fraction was determined with sulfanilamide and 1-naphthylamine,³ which produce a pigment absorbing at 525 nm (2). Ascorbic acid and its derivatives were measured in the fractions by reaction with dinitrophenylhydrazine, which forms an osazone with diketogulonic acid (19,28). The absorbance of the brown-red osazone was measured at 520 nm.

The electron spin resonance measurements (e.s.r.) were made by Dr. Raymond McKay of Drexel University, using a Varian E-12 spectrometer operating at 9 gigahertz with 100 kilohertz modulation. To obtain as high concentrations as possible for the e.s.r. measurements, after fractions had separated on the column, the resin was extruded

¹Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118.

²Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

³1-Naphthylamine is listed as a carcinogen under Title 29, Chapter XVII, Code of Federal Regulations, Occupational Safety and Health Administration. Necessary precautions were taken for using a less than 1% solution.

and sliced into various fractions which were then extracted with 1 N NH_4Cl .

RESULTS

The reaction of 4 mM ascorbate and nitrite at 70 C

A scan of the absorbance of a reaction mixture after 25 min and the resolved absorbance bands at 288, 345 and 415 nm are shown in Fig. 1. The baseline for the spectrum is proportional to $1/\lambda^4$. This dependence is typical of light scattering curves due to the presence of large molecules in the system (23). The curve was not present initially, but developed during the reaction, indicating formation of polymers from oxidation of ascorbic acid. Absorbance values of the absorption bands resolved from the spectra recorded at various time

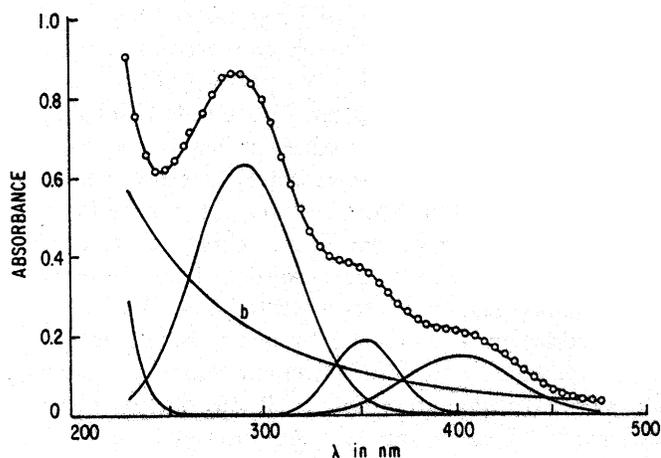


Figure 1. Resolution of the optical absorption spectrum (+) of a mixture of ascorbate and nitrite into its components absorption maxima. 4 mM ascorbate and nitrite, pH 5.5 $t = 70$ C. Baseline curve (b) proportional to $1/\lambda^4$. Solid line through data points (+) is reconstituted from baseline and computed Gaussian maxima.

intervals were plotted as a function of time (Fig. 2). The absorption maximum at 265 nm (ascorbic acid) decreased logarithmically; that is, the initial oxidation step was first order with respect to ascorbic acid. The absorption maximum at 345 nm did not originate at zero, but had a value of 0.040 AU at zero time. Nitrite (4.0 mM) has an absorption of 0.024 AU at 345 nm, but the absorption value immediately obtained when nitrite and ascorbate were mixed was always higher than 0.024. The 288-nm band did not appear in the reaction until the 345-nm absorption had reached 75% of its maximal value (Fig. 2). Since the relative proportions of the absorbances of the two bands varied with time, the compounds represented by the 288- and 345-nm absorption bands were not in equilibrium with each other. After about 4-5 h, all the absorption bands disappeared, leaving only a very large terminal absorption ($\lambda_{\text{max}} < 200$ nm) and light scatter baseline. The rate of disappearance of the 288, 345, and 415 nm maxima were about equal.

Chromatography

Chromatography of the reacting mixture was done

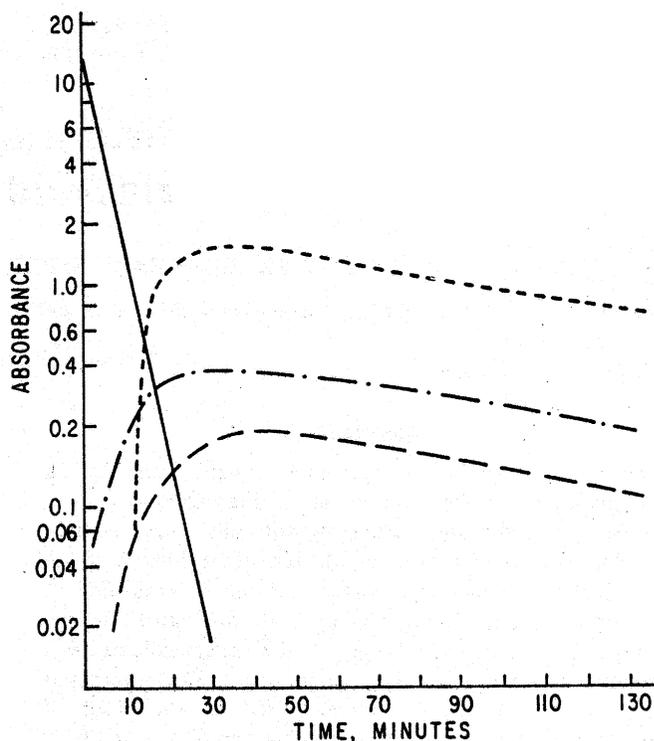


Figure 2. Individual absorption maxima from spectral scans of solutions of nitrite and ascorbate at various times, as a function of time. —, $\lambda = 265$ nm (ascorbate). ----, $\lambda = 288$ nm (diketogulonic acid) — · — ·, $\lambda = 345$ nm. — — —, $\lambda = 415$ nm (3-nitro-ascorbic acid?).

after the 265-nm absorption band had disappeared from the spectrum of the solution. The optical absorption of the eluate at 288 nm is shown in Fig. 3a. The arrows labeled "DHA" and "DKGA" indicate where dehydro-ascorbic acid and diketogulonic acid eluted in accord with the results of Hegenauer and Saltman (19). When the individual fractions were optically scanned for absorption, only those fractions corresponding to the 288-nm bands in the eluate had any absorption, with a single maximum at 288 nm. The results of the analysis for nitrite and carbonyl content are shown in Fig. 3b. The third chromatographic band appears to be diketogulonic acid, since it had the same chromatographic affinity and optical absorption as DKGA and was a carbonyl compound which contained no nitrite. The first two bands (Fig. 3b) contained both nitrite and carbonyl groups. Neither band represents free nitrite, since the latter was bound to the column and was eluted only by strong alkali. The bands therefore represent nitrosated forms of ascorbic acid and/or one of its derivatives.

The fractions of the first chromatographic band gave weak dinitrophenylhydrozine responses immediately upon elution, but when allowed to stand for several hours produced much more of the osazone. Absorbance bands at 265 and 288 nm also developed, indicating conversion to ascorbic and diketogulonic acids. Since the oxidation process is not reversible, formation of ascorbic acid can occur only if electron transfer has not taken place, which means the compound was a nitrosated ascorbic acid derivative. The first band had a low affinity for the

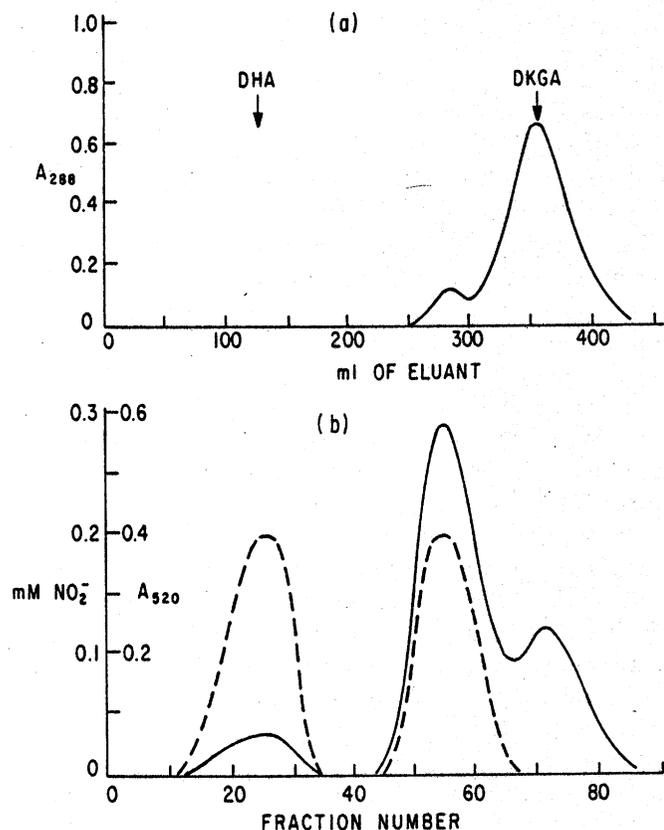


Figure 3. a. Absorbance at 288 nm of the eluate from the chromatography at 20 C of 5 ml of a solution of 4 mM nitrite and ascorbate which had been reacted at pH 5.5, $t = 70$ C, for 30 min. Dowex AG1-X2, 200-400 mesh, volume 100 ml (2.5 cm dia \times 20 cm). b. Nitrite content and osazone formation in fractions collected of above eluate. —, Absorption at 520 nm of osazone formed from dinitrophenyl hydrazine and carbonyl in eluate. ---- Concentration of nitrite in fractions as measured by diazo dye using Griess reagents.

column and eluted with the solvent front. The same lack of affinity in DHA has been attributed to the loss of molecular charge due to oxidation of the ionizing C_3 hydroxyl (15.24) to a carbonyl group (19). Since DHA is in the lactone form, the carboxyl group is not ionized and the molecule is electrically neutral. Nitrosation of the C_3 hydroxyl of ascorbic acid would also eliminate the charge, and we conclude, therefore, that the first chromatographic band is the postulated first step reaction intermediate, 3-nitrosoascorbic acid (6-8.17), compound II in Fig. 4.

The second chromatographic band (Fig. 3b) also contained nitrite, gave a positive carbonyl (DNPH) reaction and had a weak, variable 288-nm absorption. It was less stable than the first chromatographic band, giving a strong carbonyl response immediately upon elution, and showing a rapid increase in the 288-nm absorption on standing. Finally, the compound had almost as strong an affinity for the resin as did DKGA, which marks it as having a free carboxyl group. From the foregoing evidence we deduce that the compound was nitrosodiketogulonic acid. Such a compound was to be expected, since nitrite continued to oxidize the observed

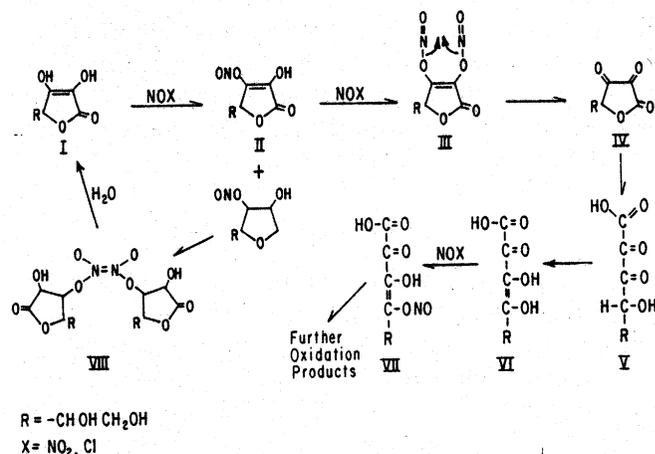


Figure 4. Reaction sequence for the formation of the initial intermediates in the oxidation of ascorbic acid by nitrous acid.

reaction intermediates (Fig. 2), the first step in such oxidations being the nitrosation of a hydroxyl group.

The amount of nitrite in the first two chromatographic bands (Fig. 3b) was about 60% of the initial nitrite. In replicate experiments the amount of nitrite in the two bands was variable, but ranged from 30 to 60% of that added. The values are probably low, since the carbonyl compounds produced by decomposition to yield nitrite are reducing compounds, i.e., ascorbate, which can interfere in the colorimetric analysis (1).

In addition to the bands shown in Fig. 3a and 3b, two other chromatographic bands with absorbance maxima at 288 nm eluted at fractions 96 and 145. There was also a brown material that bound to the resin at the top of the column and could not be removed with 1 N NaOH or NH_4OH . This was probably the polymerized material produced in the reaction, since none of the eluant fractions showed the light scattering curve of the initial reaction solution.

Electron spin resonance

The appropriate parameters in the Varian E12 were established for measuring the e.s.r. signal of the ascorbyl radical produced in a solution of ascorbic acid by air oxidation at pH 5.5. Solutions of the nitrite/ascorbate reaction mixture, as well as solutions of the individual fractions, were then successively placed in the e.s.r. cell. No ascorbyl signal was observed in any of the solutions or chromatographic fractions. The instrument was then set for broad scanning, but no other signal was observed. The reaction of ascorbate with nitrite, therefore, proceeds without formation of the ascorbyl radical, or apparently any other radical intermediate. We conclude that the reaction is a two-electron transfer, which in turn requires formation of a dinitroso intermediate, since the nitrite to NO reduction is a one electron step.

Stability

The intermediates were highly polar and unstable. To apply other identifying techniques, it was necessary to transfer the intermediates to other solvents, isolate, or concentrate them. None of the observed intermediates was extractable from the aqueous phase. Solvent

evaporation or sublimation techniques were tried, but the residues were all yellowish compounds absorbing at 288 nm. The compounds decomposed with evolution of the brown gas, NO₂, formed from oxidation of NO, in turn formed by electron transfer during decomposition of the nitrosoascorbic acid derivatives.

DISCUSSION

The two compounds represented by the 288- and 345-nm bands are not the acid-base forms of each other in this system, since they are not in equilibrium with each other. From its observed characteristics, the compound absorbing at 288 nm is identified as diketogulonic acid (19,20). The compound absorbing at 340-350 nm in this system cannot be identified specifically, since bands in this region are produced not only during ascorbate oxidation, but also are characteristic of nitrous acid and nitrite oxidation systems (16,27). The band therefore may represent more than one compound in the oxidation of ascorbic by nitrous acid, but it is not, nevertheless, a radical compound.

The only nitrous acid oxidation compounds for which absorption bands in the 415-425-nm region have been observed are the ortho-nitrosophenols (5,29). The molecular structure of these compounds consists of a nitroso and a hydroxyl group on vicinal π bonded carbon atoms of a benzene ring. Whether or not such a structure produced in ascorbic acid would show absorption bands in the same region is speculative. It is possible to form the vicinal nitroso-hydroxyl group structure, either by direct N-nitrosation of the C₃ carbon (31,33) or by a rearrangement, similar to that taking place during nitrosophenol formation (29,32). The π bond electrons of ascorbic acid are highly conjugated, as are the π electrons of phenol ($\lambda_{\text{max}} = 265$ and 271 nm, respectively), but the π bond energy levels of the nitroso derivatives may not necessarily be the same. The 415-nm band is therefore only suggestive of the formation of a C₃ (or C₂) nitrosocarbon derivative.

Both the amount of nitrite bound to and the stability characteristics of the two nitrosocarbonyl intermediates are significant with respect to the role of ascorbate in reducing nitrosamine formation. High levels of ascorbate have reduced the levels of dimethylnitrosamine in frankfurters with high levels of nitrite (13), and the levels of dimethylnitrosamine and nitroso pyrrolidine in bacon (14). Similar results have been found for isoascorbic acid (18). However, even at 550 ppm of ascorbate, the levels of nitrosamines were only reduced, not eliminated. Such a result is consistent with the binding of nitrite in a readily dissociable structure. While less nitrite is available for reaction, thus slowing nitrosamine formation, the nitroso-carbonyl compounds represent a reserve of nitrite available for long-term formation of the more stable nitrosamines. If such a reserve were to protect nitrite from oxidative processes converting the nitrite to unreactive forms, the net effect could well be to increase, not decrease, nitrosamine formation. Such a condition

may explain the anomalous, but reproducible, increased formation of dimethylnitrosamine in bacon at high ascorbate levels (14).

The reaction sequence

A reaction sequence proposed for the initial steps in the oxidation of ascorbic acid by nitrous acid is shown in Fig. 4. The initial nitrosation, I \rightarrow II, occurs on the C₃ hydroxyl to form 3-nitrosoascorbic acid. From kinetics of formation of nitric oxide from ascorbic and nitrous acids, this intermediate is a semi-stable compound which decomposes by a bimolecular backward reaction to re-form the initial reactants, II \rightarrow VIII \rightarrow I (17). Nitroso monomers readily form dimers (4), which would explain the bimolecular character of the backward reaction. We have accordingly shown the intermediate as the nitroso dimer, VIII.

Formation of an internal vicinal nitroso dimer from nitrosoascorbic acid would account for the absence of an ascorbyl radical during the reaction. We propose that the electron transfer reaction begins with the further nitrosation of the nitrosoascorbic acid intermediate to form the 2,3-dinitrosoascorbic acid, II \rightarrow III. A two-electron transfer then takes place to yield dehydroascorbic acid, IV, and N₂O₂. The electron transfer would be facilitated by formation of the dimer, a stable form of NO (26).

The reaction sequence from IV to VI was proposed earlier (21) to explain the continued oxidation of the reaction products of ascorbic acid, such as double bond migrations being very common in browning reaction oxidations (22,30). Opening of the lactone ring produces diketogulonic acid (V), and the double bond is then free to migrate to form further ene-diol structures; VII is the nitrosodiketogulonic acid, second band in Fig. 3b, which leads to further oxidation. The two weak 288-nm bands which eluted at fractions 96 and 145, as well as the brown material that adsorbed to the top of the column, are probably products of this further reaction.

SUMMARY

Oxidation of ascorbic acid by nitrous acid was studied by spectral analysis of reacting solutions, chromatography of the intermediates and electron spin resonance measurements of the reacting system and isolated fractions. Presence of semistable nitrosated ascorbic acid derivatives has been confirmed, including nitrosoascorbic acid and nitrosodiketogulonic acid. The reaction proceeds without formation of any detectable ascorbyl radical, by a mechanism involving 2,3-dinitrosoascorbic acid. There was no evidence of any unusual intermediates that by reaction with other compounds might lead to nitrogen-oxygen derivatives other than nitric oxide. Nitrite is bound in the form of nitroso-derivatives in significant quantities during the reaction, which can explain its role in reducing nitrosamine formation.

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