Accelerating Effect of Ascorbic Acid on N-Nitrosamine Formation and Nitrosation by Oxyhyponitrite

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ABSTRACT

The reaction of nitrite ion with ascorbic acid and its effect on the rate of nitrosation of secondary amines have been investigated by differential pulse polarography in aqueous acidic solution. Ascorbic acid shows nonuniform behavior: it accelerates the nitrosation of N-methylaniline between pH 1.00 and 1.95, allows the nitrosation of diphenylamine and iminodiacetanilide, but inhibits the nitrosation of secondary amines, such as dimethylamine, diethylamine, proline, hydroxyproline, N-methylaminopropionitrile, and sarcosine. The nitrosating agent generated by the reaction between ascorbic acid and nitrite ion appears to be oxyhyponitrite ion (N₂O₃⁻).

INTRODUCTION

The importance of N-nitrosamines as carcinogens (16) and the ready availability of the necessary precursors, nitrite and amines, in the environment and in food products have prompted many investigators to study the kinetics of nitrosamine formation (11-13, 15, 17, 20, 24, 25, 29). Of particular interest has been the search for inhibitors to block the formation of N-nitrosamines (14, 21). Ascorbic acid and ascorbates have been among the most popular compounds studied probably because of their presumed safety in various food and drug products.

The effect of ascorbic acid on the formation of N-nitrosocompounds in vitro and in vivo has been examined (2, 10, 18, 19), and the kinetics of the reaction between ascorbic acid and nitrous acid have been studied (9). Ascorbic acid effectively inhibits the formation of many nitrosamines, including dimethylnitrosamine, methylnitrosourea, nitrosomorpholine, and mononitrosoipiperazine, but it is not completely effective in blocking the formation of NMNA (18).

Because of the current interest in vitamin C and its suggested role in cancer prevention and nitrosamine formation (3), we are reporting preliminary observations on the interaction of nitrite ion with secondary amines in the presence and absence of ascorbic acid.

Kinetic studies on nitrosamine formation performed to date usually use spectroscopic or chromatographic techniques requiring special sampling techniques that do not allow measurement to be made readily during the initial and intermediate stages of reaction. DPP as used in this study avoids this difficulty and allows reactions to be studied in situ, yielding interesting results. The use of DPP as an analytical method for N-nitrosamines and as a technique to study some of the chemistry of M-nitrosamines has been previously reported by us (6-8, 31, 32).

MATERIALS AND METHODS

Instrumentation. The instrumentation, cells, and conditions used for DPP and spectral studies have been previously described (6, 7). The pH was measured with a Leeds & Northrup expanded-scale pH meter. Constant temperature studies were made by using an Exacal 300 temperature controller.

Reagents. Diphenylamine and iminodiacetanilide were recrystallized twice from methanol:water and benzene, respectively. NMA was purified by vacuum distillation. The N-nitrosamines were prepared in the usual way (30). The sodium salt of oxyhyponitrous acid was prepared by the method of Naik et al. (23). These workers obtained the salt as Na₂N₂O₃; our results show it to be the monohydrate Na₂N₂O₃·H₂O. The hydrate can be converted into the anhydrous form by heating in a vacuum at 120°.

Na₂N₂O₃·H₂O
Calculated: N 20.01, Na 32.84
Found: N 20.04, Na 33.34

The water used was triple distilled, and the nitric oxide was Matheson CP. All inorganic reagents were reagent grade and were used without further purification.

Procedures for Rate Studies. A stock solution of 5.0 mM NMA was prepared in an appropriate mixture of NaClO₃ and HClO₄ so that the solution was 0.10 M in ClO₄⁻ after pH adjustment. A portion (20.0 ml) of this solution was placed in the polarographic cell and deaerated with N₂. A stock solution of NaNO₂ was prepared in the appropriate mixture of NaClO₃ and HClO₄, and a small aliquot was delivered to the cell, so that the solution was 0.10 mM in NO₂⁻. The solution was then stirred magnetically for 10 to 15 sec. Nitrite was the limiting reagent in all cases. When ascorbic acid was used, it was prepared in the corresponding NaClO₃·HClO₄ mixture, and an aliquot was delivered to the cell prior to nitrite addition to make the solution 0.80 mM in ascorbic acid. All solutions were freshly made before each run. The pH's studied were 1.00, 1.20, 1.50, 1.70, and 1.95. The reduction peak potential of the NMNA versus the saturated calomel electrode was found to be -0.61 V at pH 1.95. Three different solutions were studied at each pH: Solution 1, a control in which only NMA and NO₂⁻ were present; Solution 2, a solution containing NMA, NO₂⁻, and ascorbic acid, with the polarographic cell "open" to the environment; and Solution 3, a solution containing NMA, NO₂⁻, and ascorbic acid, with the polarographic cell "closed" to any reduction occurring at the surface of the electrode.
atmosphere; and Solution 3, Solution 2 with the polarographic cell “closed” with Parafilm to retard the loss of any gaseous products from the cell. The peak current (i') due to the NMNA was monitored as a function of time. “Time zero” was taken as the moment of addition of nitrite.

RESULTS AND DISCUSSION

Chart 1 shows the results of the polarographic study of the nitrite ion-ascorbic acid system. Curve A is that of the background solution free of NO$^{-}_{2}$ and ascorbic acid at pH 1.0. Upon the addition of the nitrite stock solution to the background solution such that nitrite concentration was 0.10 mM, Curve B, characteristic of nitrite, was obtained (8). When more than an equivalent amount of ascorbic acid was then added, Curve C with $E_{p} = -0.87$ V appeared along with the complete disappearance of Curve B due to the NO$^{-}_{2}$. On addition of excess diphenylamine to this solution, Curve D resulted with the disappearance of Curve C. Curve D is identical with the polarogram of diphenylnitrosamine (8). This was verified by the addition of authentic diphenylnitrosamine to the solution that yielded Curve D; Curve D increased in height with no change in peak potential or bandwidth.

The species giving rise to Curve C has interesting properties. If nitrogen is bubbled through the solution yielding Curve C for only a few moments, the curve disappears. If, instead, the solution is blanketed with nitrogen and allowed to remain open to the atmosphere, Curve C gradually diminishes with time. If NMA or iminodiacetonitrile is added in place of diphenylamine, the peak due to the corresponding N-nitrosamine appears and Curve C disappears. If, however, dimethylamine, diethylamine, sarcosine, proline, hydroxyproline, N-methylaminopropionitrile, or N-methylaminopropionitrile is added, no nitrosamine peak appears. Solutions containing the species giving rise to Curve C were sealed under N2 with the latter amines for 3 to 4 days and then scanned for the presence of the N-nitrosamines. No nitrosamines were found, and the species yielding Curve C was still present.

The species giving rise to Curve C was also generated from nitrite ion by reagents other than ascorbic acid, such as cysteine, pyrogallol, and iodide ion. (The iodide solution yielded free iodine.) The reaction of iodide ion with nitrous acid is a standard laboratory preparation for nitric oxide. NO, however, does not yield a curve with a peak at $-0.87$ V when passed through an aqueous solution at pH 1.0 to 2.0. It produces a curve similar to that of nitrite ion, i.e., Curve B. This is probably due to reaction of NO with trace amounts of oxygen, remaining in solution, generating NO$_2$ which produces nitrite and nitrate ions in acidic water. It has also been shown that NO is not an effective nitrosating agent (4). On the basis of these observations, the possibility that NO was the species giving rise to Curve C was ruled out.

It has been suggested that dinitrogen trioxide (N$_2$O$_3$) is involved in the reaction between ascorbic acid and nitrite ion (9). This species, however, is the anhydride of nitrous acid (22) and is unlikely to exist at concentrations large enough to give rise to a peak such as that seen in Chart 1. Its formation does, however, agree with the observed stoichiometry (9), which is 2:1, nitrite:ascorbic acid.

Accordingly, oxyhydroxynitrous acid (H$_2$N$_2$O$_3$) was studied as the possible species giving rise to Curve C. When the sodium salt of oxyhydroxynitrous acid was added to the solution yielding Curve C, an increase in peak height with no shift in peak potential was produced. Aqueous solutions containing only Na$_2$N$_2$O$_3$ at pH 1.0 also yielded a curve with a peak at $-0.87$ V. The peak disappeared rapidly when nitrogen was bubbled through the solution.

When NMA, diphenylamine, or iminodiacetonitrile was added to aqueous solutions of oxyhydroxynitrite at pH 1.0, the corresponding nitroso compound was rapidly formed as evidenced by the appearance of the respective nitrosamine peak and the disappearance of Curve C. Other amines, such as sarcosine, proline, hydroxyproline, N-methylaminopropionitrile, N-methylaminopropionitrile, and diethylamine, were not nitrosated under identical conditions. Oxyhydroxynitrous acid is not a well-characterized chemical species, and its structure is not known. However, aqueous solutions of the compound are unstable and decompose to yield nitric oxide and water (23). It is quite possible that oxidation-reduction reactions between nitrite ion and a reducing agent, such as ascorbic acid, at low pH, proceed via oxyhydroxynitrite as an intermediate.
Dehydroascorbic acid had been previously suggested (9) as one of the products of the ascorbic acid-nitrite ion reaction. Its presence in the solution in this study was shown by the appearance of a small kinetic peak at about -0.4 V characteristic of dehydroascorbic acid (27). In addition, after thorough degassing to remove all $\text{NO}_2^-$, the ascorbic acid could be regenerated by bubbling $\text{H}_2\text{S}$ through the solution. $\text{H}_2\text{S}$ is known to reduce dehydroascorbic acid to ascorbic acid (1).

If the reaction proceeds as suggested by Equation A, one would expect to see NO$_2$ appear over the solution when the gaseous product comes in contact with air. The concentrations of reactants used in the polarographic study are, however, too small to permit the observation of NO$_2$. However, if the concentrations are increased to 0.5 M ascorbic acid and 1.0 M nitrite ion, a brown gas (presumably NO$_2$) is seen over the solution.

Since the species generated by the reaction between ascorbic acid and nitrite ion can nitrosate certain secondary amines, it was important to study the rate of nitrosamine formation. Among the 3 amines examined, NMA was chosen for the rate study since its nitroso compound is an established carcinogen. Results are shown in Chart 2. All data points were obtained in quadruplicate; reproducibility was ±3%. Although not shown here, the results at pH 1.20 and 1.70 fit the trend shown in Chart 2. In Chart 2, the yield was calculated from the ratio of $I_p$, measured at any given time, to the $I_p$ of the control reaction when no further nitrosation was occurring.

Chart 2 shows that ascorbic acid accelerates the rate of formation of NMNA even though at certain pH levels the final yield of nitrosamine is reduced. The effect differs slightly depending on whether the cell is open or closed; the closed cell gives the higher yield. This result is consistent with the hypothesis that the nitrosating species is volatile or decomposes yielding a volatile product. The effect is also very dependent on pH, as seen by comparing the results in Chart 2. These show that if one used an analytical technique that examined the solution after only 5 or 10 min, at pH 1.0, one might conclude that partial inhibition had occurred without any evidence of acceleration.

The reason for the increase in the reaction rate is not immediately clear from these preliminary studies. As shown in Equation A and Chart 1, ascorbic acid converts all nitrite into oxyhyponitrite which can nitrosate certain secondary amines. The increase in rate may simply be due to the increase in the concentration of the nitrosating species, or be due to a change in the reaction mechanism, or a combination of both of these effects. Aqueous solutions of nitrous acid contain many species, only a few of which can act as nitrosating agents.

The reason only certain amines are nitrosated by the oxyhyponitrite may be related to the base strength of the amines. The 3 amines that are nitrosated are relatively weak bases ($pK_a \approx 0.9$ to 4.8), whereas those that are not are relatively strong ($pK_a \approx 10$ to 11). These strong bases should be largely in the form of their salts at pH 1 to 2, thus reducing their nucleophilicity substantially and slowing nitrosation by electrophilic nitrosating species.

Rapid formation of nitrosamines in aqueous systems is of concern not only because some of them are known carcinogens but also due to their ability to transnitrosate under appropriate conditions (5, 26, 28). The addition of ascorbic acid to a commercial product containing a variety of unknown amines might slow down the formation of some nitrosamines, but in
contrast, it might accelerate the formation of others which, although less potent carcinogens, might generate more potent ones by transnitrosation.

REFERENCES

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