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Kinetic Investigation of the Reactions Connected to the System Ascorbate + O_2 by Amperometric Detection of H_2O_2 at a Modified Platinum Electrode

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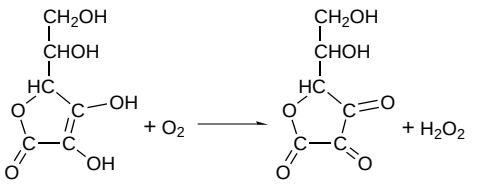
A Pt electrode modified by an electrochemically produced bilayer polymeric membrane [polypyrrole/poly(*o*-phenylenediamine)] entrapping the enzyme glucose oxidase proved able to detect (response time of few seconds) amperometrically (at +0.7 V vs Ag/AgCl) very low concentrations of hydrogen peroxide (micromolar range) in the presence of much higher amounts of ascorbate. The currents due to ascorbate, also electroactive at the given potential, were negligible under any conditions due to its almost complete rejection by the electrode-modifying membrane system. The very peculiar properties of the device setup were exploited to undertake a kinetic study of the reactions connected to the system ascorbate + O_2, following the concentration of H_2O_2 produced in the reaction mixture at 27 °C, pH = 7. The reaction between ascorbate and H_2O_2 was also considered; however, different kinetic models based on the two consecutive reactions proved unable to fit the data. An investigation on the single processes by the same experimental approach was then undertaken, leading to two explanations for the inadequacy of simple kinetic models. First, the presence of metal ion traces in the reaction mixture proved to be responsible for the nonlinear dependence of the rate of both reactions on the ascorbate concentration: a mechanism involving the role of ascorbate−metal complexes as the reactants was hypothesized to explain this result. Second, the influence of the reactivity of dehydroascorbic acid, the product of ascorbate oxidation, on the kinetics was ascertained.

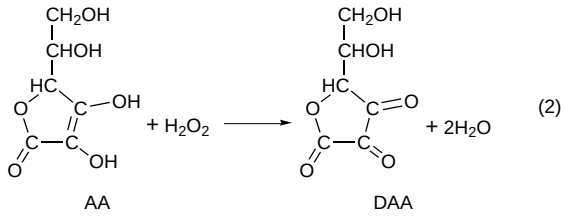
The oxidation of ascorbic acid (AA) by dioxygen (also known as autoxidation), leading to dehydroascorbic acid (DAA) and hydrogen peroxide according[1] to a reaction such as



(1)

has been the object of many kinetic investigations, due to its basic biochemical and practical significance. Most of the work done on this reaction has been recently reviewed.[1] The kinetic determinations relevant to AA autoxidation are usually carried out in the presence of known amounts of transition metal ions, e.g., Cu(II), Fe(III), Ru(III), etc., or their complexes, acting as catalysts. This procedure masks the small catalytic effects of metal ions impurities always present at variable and difficult-to-control concentration levels.

Furthermore, any kinetic study of reaction 1 should also take into account the consecutive oxidation process[2] between AA and H_2O_2:



(2)

In the course of the present study, an attempt was made to collect, during the same experiment, information on processes 1 and 2 by amperometric detection of the concentration of H_2O_2. A Pt electrode modified by an electrosynthesized bilayer polymeric membrane, i.e., polypyrrole (PPy) and poly(*o*-phenylenediamine) (PPD), entrapping the enzyme glucose oxidase (GOx), was employed. In fact, this device, Pt/PPyox/PPD(GOx), proved[3] able to detect very small concentrations of H_2O_2 (micromolar), drastically rejecting the electroactive AA (see also the Experimental Section).

The approach adopted made it possible to follow continuously the concentration of hydrogen peroxide, emphasizing the kinetic role of H_2O_2 consumption, usually ignored by previous investigations (see, e.g., ref 4) based on the periodic quantification of either AA or DAA.

In the present work, reactions 1 and 2 were studied at 27 °C and pH = 7 (phosphate buffer solutions), and different mathematical models were first adopted in order to fit the experimental [H_2O_2] vs time curves. Their failure led to a critical kinetic investigation on the single processes, which gave useful information on AA oxidation. In particular, the catalyzing effect due to

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(1) Davies, M. B. *Polyhedron* **1992**, *11*, 285−321 and references cited therein.
(2) Hand, D. B.; Greisen, E. C. *J. Am. Chem. Soc.* **1942**, *64*, 358−361.
(3) Losito, I.; Zambonin, C. G. *J. Electroanal. Chem.* **1996**, *410*, 181−187.
(4) Taqui Khan, M. M.; Martell, A. E. *J. Am. Chem. Soc.* **1967**, *89*, 4176−4185.

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the presence of metal ions, arising from the buffer (as traces) or added in known amounts (Cu^{2+}), was assessed, and the influence of DAA reactivity on the kinetics was examined.

These findings, besides permitting the rationalization of the inadequacy of relatively simple kinetic models, can be very helpful in drawing a reliable picture for the first steps of the quite complicated reacting system AA−O_2.

EXPERIMENTAL SECTION

Materials. GOx from *Aspergillus niger* (EC 1.1.3.4, 168 200 units/g) and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Hydrogen peroxide was obtained as a 36% (w/v) solution from Carlo Erba (Milano, Italy). *o*-Phenylenediamine (o-PD) was purchased from Aldrich (Steinheim, Germany) and purified just before use by vacuum sublimation at 90 °C. Pyrrole (Aldrich) was purified by vacuum distillation at 60 °C and stored under nitrogen at 0 °C.

The buffer solutions (pH = 7; I = 0.1) for the kinetic experiments were always prepared by using monobasic and dibasic sodium phosphates (ACS reagents) purchased from Aldrich.

Apparatus. Modified electrode preparation was carried out by a PAR 273 potentiostat−galvanostat (EG&G, Princeton Applied Research, Princeton, NJ) coupled to a conventional three-electrode system with a Pt wire as counter electrode and a Ag/AgCl/KCl (saturated) electrode as reference. The amperometric response during kinetic experiments was measured by a Model 400 EC Detector (EG&G) connected to a $Y-t$ strip chart recorder (LCI 100, Perkin-Elmer, Norwalk, CT).

The UV spectra were recorded in the range 210−400 nm using a Perkin-Elmer Lambda 2 UV/visible spectrometer.

Modified Electrode Preparation. The preparation of the modified electrode has been described elsewhere[3]. Briefly, a platinum disk (diameter, 3 mm), embedded in a Teflon cylinder, was used as the working electrode: its surface was polished with 0.3 μm alumina, washed, and pretreated by potential cycling between −0.21 and +1.19 V vs Ag/AgCl in 0.5 M H_2SO_4 until a steady state voltammogram was obtained. A PPy film was potentiostatically grown (at +0.7 V vs Ag/AgCl) from a 10 mM KCl solution containing pyrrole (0.4 M). The deposition charge employed was typically 300 mC/cm^2. The Pt/PPy-modified electrode was washed and transferred to another cell containing 5 mM *o*-PD (and 500 units/mL of GOx, when indicated) in a 0.1 M phosphate buffer (pH = 7.0) solution. A potential of +0.7 V vs Ag/AgCl was applied in order to potentiostatically deposit a PPD film; the process was stopped when no appreciable current flowed in the circuit. After preparation and thorough washing, the modified electrode was potentiostated at +0.7 V overnight in a 0.1 M phosphate buffer (pH = 7.0) in order to complete the PPy overoxidation process.

A calibration test was performed before each kinetic experiment in order to assess the sensitivity of the Pt/PPy_{ox}/PPD(GOx) electrodes to H_2O_2 in the range 2−100 μM, including the maximum H_2O_2 concentrations reached during the reactions. A response time ($t_{0.9}$) of about 5 s was estimated. The current values were controlled to be constant with time, which indicated that no decomposition of H_2O_2 was occurring under the present experimental conditions. The rejection of ascorbate from the electrode surface was also checked by measuring the amperometric response after addition of AA (final concentration, 1 mM) to phosphate buffer.

Modified electrodes showing relatively low ratios (<400) between the current responses obtained for H_2O_2 and AA respectively at the same concentration (1 mM) were not used for kinetic experiments. This choice was made to minimize the effect due to the AA electrooxidation products, which could alter the electrode surface,[3,5] thus influencing the sensitivity to H_2O_2 during a kinetic run (see also the Results and Discussion session).

Kinetic Measurements. A thermostated electrochemical cell was used as reaction vessel for all the kinetic experiments in this work. Particular care was taken to prevent any further introduction of oxygen (from air) in the reacting system after a run started. This was mandatory for the experiments involving oxygen, in which the concentration of dissolved O_2 was continuously reduced during the kinetic run.

Some of the experiments were performed by using, as oxygen initial concentration, the value corresponding to its partial pressure in the atmosphere (∼0.19 atm). The concentration was evaluated to be 0.23 mM, in good agreement with the data reported by Taqui Khan and Martell[4] for an aqueous solution with the same ionic strength as the buffer used in this work.

The cell was partially filled with phosphate buffer solution (I = 0.1, pH = 7), kept in a thermostatic bath at 27 °C, allowing O_2 to reach, under stirring, its equilibrium concentration. The three electrodes (working, counter, and reference) were then inserted, and O_2 saturated buffer was added to fill the reaction vessel. After the operating potential (0.7 V vs Ag/AgCl) was applied and the system allowed to achieve a steady value for the background current, the cell was protected from light and the kinetic experiment started by adding a known amount of AA to the solution (final concentration ranging from 0.1 to 1 mM). The AA stock solutions (50 mM) used for the injection were prepared just before each kinetic run and deaerated by flowing a UHP inert gas, in order to avoid any preliminary reaction with oxygen.

Other experiments were performed adopting lower oxygen concentrations. In these cases, a different preparation stage was necessary before starting the kinetic run. After the cell was filled, an inert gas was bubbled in the solution through the overflow pipe until the desired O_2 concentration was reached. During this stage, the O_2 concentration was followed (and finally calculated) by amperometric detection at an additional Pt electrode (operating potential, −0.4 V vs Ag/AgCl). The chosen AA aliquot was then injected and the kinetic study started.

In any case, particular care was used to eliminate small air bubbles inside the cell before each run.

Kinetic Models. Kinetic models based on two pseudo-first-order (1/1) or second-order (2/2) consecutive reactions were adopted, as a first approach, to study the oxidation of AA by oxygen and by H_2O_2 in the same system.

In particular, the 2/2 model was chosen on the basis of previous kinetic investigations[1,3,4,6] on processes 1 and 2, indicating a second-order dependence for their rate.

The mathematical treatment already available[7] for this model (with a ratio of 2 between the initial concentrations of the reactants) was extended to any ratio between the initial concentrations of the reactants. Briefly, as a final result, the following equation was obtained:

(5) Palmisano, F.; Zambonin, P. G. *Anal. Chem.* **1993**, *65*, 2690−2692.
(6) Taqui Khan, M. M.; Shukla, R. S. *J. Mol. Catal.* **1987**, *39*, 139−146.
(7) Pannetier, G.; Souchay, P. *Chemical Kinetics*; Elsevier: Amsterdam, 1967; pp 196−199.

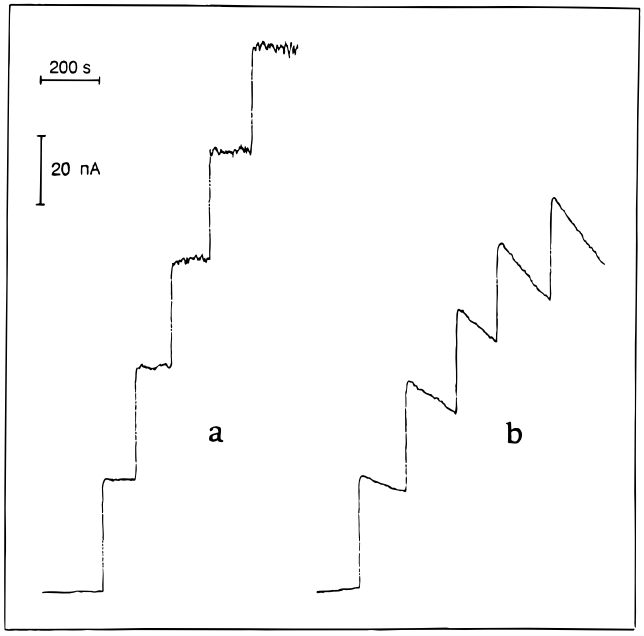


Figure 1. Calibration curves for hydrogen peroxide obtained with a Pt/PPY_{ox}/PPD(GOx) electrode in phosphate buffer (0.1 M, pH = 7) solution in the absence (a) and in the presence (b) of ascorbate (1 mM). Each step corresponds to a 2.5 μM increase in H_2O_2 concentration. Temperature, 27 °C; applied potential, 0.7 V vs Ag/AgCl (saturated KCl).

$$k_1[O_2]_0 t = \int_{\beta}^{1} \frac{1-r}{(1-2r)\beta^2 + (2-R)(r-1)\beta + \beta^{r+1}} \, d\beta \quad (3)$$

where k_1 is the second-order kinetic constant for reaction 1, r the ratio between the kinetic constants of reaction 2, k_2 and k_1, $\beta = [O_2]/[O_2]_0$, and $R = [AA]_0/[O_2]_0$.

A program was developed in Quick Basic (version 4.0) environment to perform the simulation of the concentration−time profiles and fit the experimental curves by means of the variable step-size *simplex* algorithm,[8] k_1 and k_2 being the dependent variables.

As far as the 1/1 model is concerned, commercial software was employed to perform the optimization of the constants values.

RESULTS AND DISCUSSION

A comparison between the calibration curves for H_2O_2 obtained by the Pt/PPyox/PPD(GOx) electrode both in the buffer solution as such and in the presence of ascorbate at the highest concentration level adopted in this work (1 mM) is shown in Figure 1.

The same increase of the current signal is observed for equal additions of hydrogen peroxide in both cases, which indicates that ascorbate does not influence the electrode sensitivity. However, the presence of AA results in a current decrease after each addition due to the occurrence of reaction 2; of course, its slope increases with H_2O_2 concentration.

Figure 2 shows the variation of $[H_2O_2]$ during a kinetic run with initial [AA] = 1 mM: the curve clearly suggests that hydrogen peroxide is a stable intermediate between consecutive processes, easily detectable by the electroanalytical method used, although its concentration was quite low under our experimental conditions (maximum recorded value was around 7 μM).

(8) Nelder, J. A.; Mead, R. *Comput. J.* **1965**, *7*, 308−313.

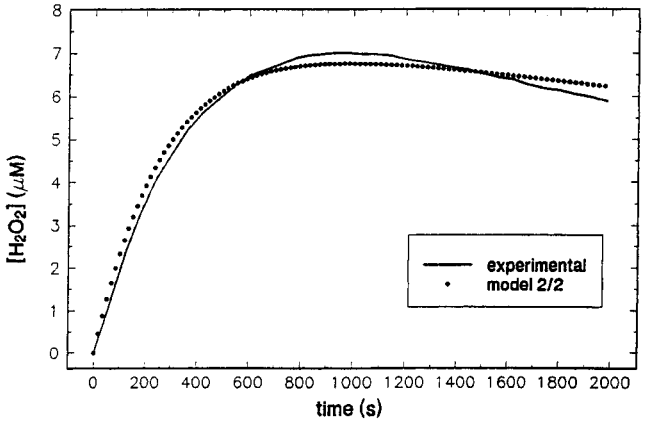


Figure 2. Kinetic analysis of the hydrogen peroxide concentration profile relevant to the reaction mixture AA (1 mM)−O_2 (0.23 mM) in phosphate buffer (0.1 M, pH = 7) solution at 27 °C. A model based on two consecutive second-order reactions was used. The H_2O_2 concentration profile was obtained from the current response corrected for the small contribution due to ascorbate.

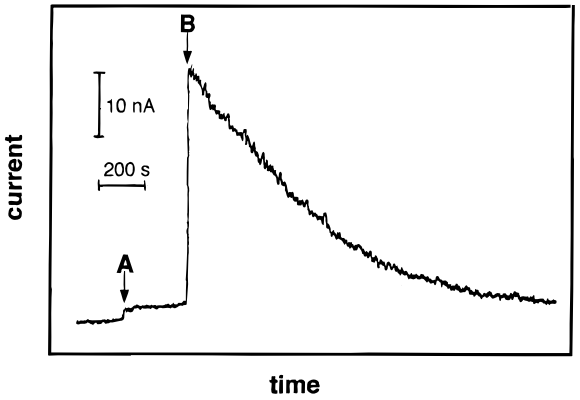


Figure 3. Current−time response obtained with a Pt/PPY_{ox}/PPD(GOx) electrode after addition of (A) ascorbate, final concentration 30 μM, and (B) H_2O_2, final concentration 2 μM, to a stirred phosphate buffer (0.1 M, pH = 7) solution containing Cu^{2+} (10 μM). Temperature, 27 °C; applied potential, 0.7 V vs Ag/AgCl (saturated KCl). The ratio between the sensitivities to H_2O_2 and AA at the given electrode configuration is around 500 (see text). The curve corresponds to the fourth experiment in Table 1 (vide infra).

The best curve calculated by means of the 2/2 model (dotted line) shows that the latter cannot fit the experimental data. The inadequacy of the model could be due to a difference between the real kinetic orders of the involved reactions (1 and 3) and those reported in the literature. Another possible explanation could be the occurrence of other reactions involving H_2O_2 and/or O_2 (e.g., with DAA, the first oxidation product of AA).

An investigation on the kinetic orders for the single reactions was then undertaken; the results are reported in the following paragraphs.

Investigation on the Kinetic Order: Reaction 2. A typical experimental curve for a reaction between AA and H_2O_2 is shown in Figure 3. In this case, oxygen is first removed from the phosphate buffer, and then AA and H_2O_2 are sequentially added. As was already reported, a first-order dependence of the reaction rate on $[H_2O_2]$ is found[3] under the same conditions used in the present work.

The dependence on the [AA] was then evaluated by performing kinetic runs in deareated solutions with different initial concentrations of AA. The pseudo-first-order kinetic constants were

Table 1. Kinetic Data Relevant to the Reaction between Ascorbate and H_2O_2, Performed in the Absence of Oxygen[a]

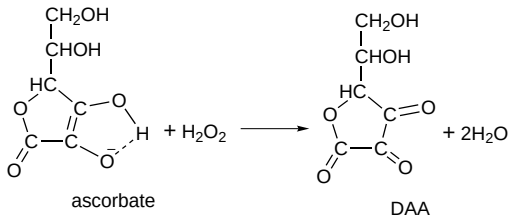
initial concentrations (mM)				
[AA]	[H_2O_2]	[Cu^{2+}][b]	$t_{1/2}$ (s)	k[c] (s^{-1})
0.030	0.002		1728	4.01×10^{-4}
0.100	0.002		852	8.14×10^{-4}
1.000	0.002		672	1.03×10^{-3}
0.030	0.002	0.010	396	1.75×10^{-3}
0.100	0.002	0.010	240	2.89×10^{-3}
1.000	0.002	0.010	198	3.50×10^{-3}

[a] Conditions: phosphate buffer solution (pH = 7, I = 0.1); temperature, 27 °C. [b] Final concentration due to Cu^{2+} added to the buffer solution. [c] Pseudo-first-order constants.

calculated from the half-life time ($t_{1/2}$) of H_2O_2, whose initial concentration was always 2 μM; the relevant data are summarized in Table 1.

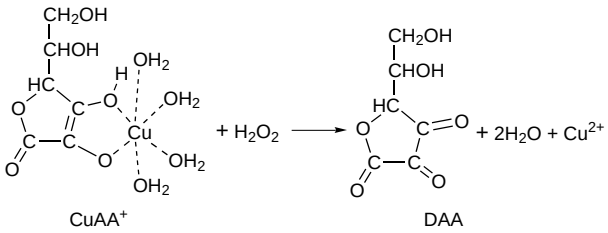
It is apparent that, although an increase of the kinetic constant with the initial AA concentration is observed, there is not a linear correlation between them. This finding can be explained by considering the simultaneous presence of two different mechanisms for the AA oxidation by H_2O_2, similar to those proposed[4] for its reaction with O_2.

The simplest model can be based on a first reaction path involving, as rate-determining step, a direct electron transfer between the ascorbate ion and H_2O_2 (eq 4), while a second one would involve H_2O_2 and one or more complexes between ascorbate and catalyzing metal ions, even present as traces in the reaction mixture, e.g., Cu^{2+}, Fe^{3+}, etc.



(4)

The case relevant to the mononuclear complex $CuAA^+$ is indicated in eq 5; it is worth noting that DAA formed by the reaction, though reported as the lactone, is probably present as 2,3-diketogulonate at the pH used for the kinetic study.



(5)

As recently pointed out,[9] the contribution due to metal ions should always be considered, even if they are in trace amounts, when the oxidation of AA is studied. In the present case, the concentrations of the main catalyzing ions (Cu^{2+} and Fe^{3+}) were measured, by means of ETAAS, in the buffer solution, prepared with analytical grade chemicals used for all the kinetic runs. The corresponding values (0.16 μM for Cu^{2+} and 0.15 μM for Fe^{3+}), though much smaller than those commonly used for studying catalyzed processes (often[4] larger than 10^{-5} M), do not exclude the influence of ion-involving mechanisms on the kinetics.

As a result, an expression for the overall reaction rate under "uncatalyzed" conditions could be the following:

$$v = -\frac{d[H_2O_2]}{dt} = k_{nc}[AA][H_2O_2] + \sum_M k_{c,MAA}[MAA][H_2O_2] + \sum_M k_{c,MAA_2}[MAA_2][H_2O_2] \quad (6)$$

k_{nc} being the kinetic constant for the uncatalyzed process, whereas $k_{c,MAA}$ and k_{c,MAA_2} are those relevant to the catalyzed ones. In eq 6, the effect on the kinetics due to the 1:1 (MAA) and 1:2 (MAA_2) complexes, which are those commonly formed by ascorbate with different metal ions,[10] has been taken into account.

According to the values of the stability constants for MAA and MAA_2 complexes reported in the literature,[10] it can be predicted that a large ascorbate excess may result in a complete displacement of the complexation equilibria toward the MAA_2 species, thus making the [MAA] values negligible and the [MAA_2] ones no more correlated to [AA].

As for the catalytic effect, the MAA_2 complexes are expected to be less efficient than the MAA ones, in analogy with the behavior[10] of the mixed chelates involving ascorbate and another ligand, like EDTA, in the AA oxidation by oxygen. The steric hindrance due to the second ligand, making the electron transfer between the metal ion and oxygen more difficult, has been hypothesized to explain the lower reactivity observed in this case.

It is likely that some of the metal ions initially present in the buffer solution used for the kinetic experiments of Table 1 are almost completely in the MAA_2 form, even when low AA concentrations are adopted, as in the first run. As a consequence, the contribution due to the MAA_2 species to the reaction rate (see eq 6) is not dependent on AA concentration, and a nonlinear increase of the kinetic constant is observed when [AA] goes from 0.03 to 0.1 mM.

A further increase of AA concentration could have the same effect also on the ions present at higher concentrations, thus reducing their catalytic effect, mainly due to the MAA complexes. This would balance the contribution to the reaction rate due to the nonmediated mechanism (corresponding to the first term in eq 6), which remarkably increases. As a result, only a slight variation of the kinetic constant is observed.

Kinetic runs were also performed by using the same concentrations of AA and H_2O_2 but with the addition of Cu^{2+} ions as catalysts, at a final concentration of 10 μM. The relevant data, reported in Table 1, confirm the small influence of the [AA] on the rate of reaction 2, though the latter is always greater than the one observed in uncatalyzed conditions. This was expected, since the concentration of Cu^{2+} complexes is higher in this case.

(9) Schaich, K. M. In *Methods in Enzimology*; Packer, L., Glazer, A. L., Eds.; Academic Press Inc.: New York, 1990; Vol. 186, pp 121−125.

(10) Martell, A. E. *Ascorbic Acid: Chemistry, Metabolism and Uses*; ACS Advances in Chemistry Series 200; American Chemical Society: Washington, DC, 1982; Chapter 7, pp 153−178.

Table 2. Kinetic Data Relevant to the Reaction between Ascorbate and O_2[a]

expt	initial concentrations (mM) [AA]	[O_2]	[Cu^{2+}][b]	v_0[c] (mM·s^{-1})
1	0.3	0.23	-	3.21×10^{-5}
2	0.3	0.11	-	1.45×10^{-5}
3	0.1	0.23	-	1.06×10^{-5}
4	0.3	0.23	0.005	5.98×10^{-4}
5	0.3	0.055	0.005	1.45×10^{-4}
6	0.1	0.23	0.005	3.86×10^{-4}
7	0.1	0.23	0.002	2.02×10^{-4}
8	0.3	0.23	0.002	4.22×10^{-4}

[a] Conditions: phosphate buffer solution (pH = 7, I = 0.1), temperature, 27 °C. [b] Final concentration due to Cu^{2+} added to the buffer solution. [c] Initial rates, $v_0 = d[H_2O_2]/dt \approx -d[O_2]/dt$.

The results suggest that an equation like $v = [AA]^n[H_2O_2]$ cannot be generally applied, even considering an apparent order $n < 1$, to describe the kinetics of the reaction between AA and H_2O_2 in uncatalyzed conditions. As a consequence, the 2/2 model is not suitable for studying the overall process due to reactions 1 and 2.

On the other hand, even when the 1/1 model was applied, considering the ascorbate concentration as a constant throughout the reaction, it was not possible to obtain a good fit of the experimental curve.

In any case, the experimental [H_2O_2] is lower than the predicted one for long reaction times; this finding could indicate the occurrence of other H_2O_2-consuming processes along with reactions 1 and 2 (vide infra).

Investigation on the Kinetic Order: Reaction 1. Kinetic information on process 1 was also obtained through H_2O_2 monitoring, using the slope of the [H_2O_2] vs t curves for $t \rightarrow 0$ as a reliable estimate for the initial rate v_0 of reaction 1.

Results relevant to kinetic runs performed in the presence of oxygen are reported in Table 2.

Data relevant to experiments 1−3 suggest that the reaction is unimolecular both toward AA and O_2 in uncatalyzed conditions. In fact, the initial rate is directly proportional to the different concentrations of one of the reactants when the other is constant.

A different behavior is observed when Cu^{2+} is added as a catalyst, at final concentration 5 μM: while v_0 is still proportional to $[O_2]_0$ (experiments 4 and 5), the linear correlation between v_0 and $[AA]_0$ is no longer observed (experiments 4−6). This finding appears to be in agreement with the observations made on the data obtained for reaction 2 under comparable conditions: a prevailing mechanism based on parallel processes, like the one summarised in eq 6, can be invoked for reaction 1 in the presence of small concentrations of catalysts.

An expression for v_0 in the case of reaction 1 could be then

$$v = -\left(\frac{d[O_2]}{dt}\right)_{t=0} = k_{nc}[AA]_0[O_2]_0 + \sum_M k_{c,MAA}[MAA]_0[O_2]_0 + \sum_M k_{c,MAA_2}[MAA_2]_0[O_2]_0 \quad (7)$$

where the symbols have the same meaning as in eq 6.

Data relevant to experiments 7 and 8 in Table 2 further support this hypothesis: in this case, a concentration of Cu^{2+} lower (2 μM) than those in experiments 4−6 was adopted. As a result, though the correlation between v_0 and $[AA]_0$ is still not linear, the influence of $[AA]_0$ on v_0 is higher (an apparent order 0.67 for AA can be estimated in this case, compared with 0.40 obtained when 5 μM Cu^{2+} was added). This is in agreement with eq 7, since the contribution due to the complexes is lowered on going from 5 to 2 μM Cu^{2+}.

It is worth noting that the reaction between AA and O_2 shows the "expected" second-order kinetics (first order in respect to each reagent) in uncatalyzed conditions. Thus, the contribution due to the last two terms in eq 7 seems to be negligible in this case, whereas the corresponding ones for the process AA + H_2O_2 have already an influence on the kinetics (vide ante).

The method based on the initial rate has also enabled us to evaluate directly the influence of metal ion traces on the AA reactivity when virtually identical systems are considered.

In effect, kinetic experiments performed by using buffer solutions prepared with salts (monobasic and dibasic sodium phosphate) from different sources showed variations, even remarkable, in the v_0 value. This can be attributed only to differences in the trace amounts of metal ions in the buffer solutions, since the same concentrations of AA (1 mM) and O_2 (0.23 mM) were always used to make the comparison.

Kinetic Runs with Low Oxygen or Ascorbate Concentrations: Effects of DAA Reactivity. As cited before, the dehydroascorbic acid (DAA) produced by reactions 1 and 2 could be responsible for the decrease of [H_2O_2] after long reaction times not explained by kinetic models based only on two consecutive processes. The possibility of a reaction between H_2O_2 and DAA has been reported in a recent work[11] based on a GC/MS study, suggesting that DAA, supposed[12] to be rapidly hydrolyzed to 2,3-diketogulonate in aqueous solution at pH = 7, can be oxidized by H_2O_2 to 2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid.

On the other hand, the possibility of a reaction between oxygen and DAA has been also previously hypothesized,[13] though no kinetic data are available on this process.

A careful analysis of the [H_2O_2] vs t profiles for experiments performed under particular conditions provided an indirect confirmation of the DAA reactivity. As an example, the response obtained during experiment 5 in Table 2 is shown in Figure 4. In this case, the global process is limited by the oxygen concentration, and the almost complete disappearance of H_2O_2 is observed within a relatively short time (about 20−25 min).

Contrary to the case of the uncatalyzed reaction between AA (1 mM) and O_2 (0.23 mM), shown in Figure 2, a very good fit was obtained by using the 1/1 model, whose application was possible because AA was in excess in comparison with O_2 and H_2O_2. The fitting results suggest that the possible influence of the reaction between H_2O_2 and DAA is quite small, at least under these conditions, also due to the fact that H_2O_2 has been already consumed when DAA reaches appreciable concentrations.

A very different behavior is observed in experiments 4 and 6−8 of Table 2, in which the initial concentration of AA is

(11) Deutsch, J. C.; Santosh-Kuwar, C. R.; Kolhouse, J. F. *Anal. Chem.* **1994**, *66*, 345−350.
(12) Tolbert, B. M.; Ward, J. B. *Ascorbic Acid: Chemistry, Metabolism and Uses*; Advances in Chemistry Xeries 200; American Chemical Society: Washington, DC, 1982; Chapter 5, pp 101−123.
(13) Dunn, J. A.; Ahmed, M. U.; Murtiashaw, M. H.; Richardson, J. M.; Walla, M. D.; Thorpe, S. R.; Baynes, J. W. *Biochemistry* **1990**, *29*, 10964−10970.

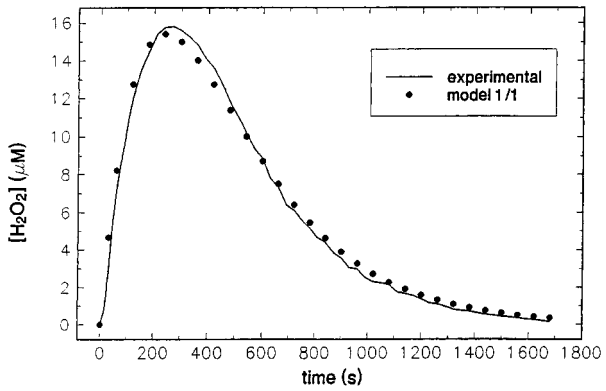


Figure 4. Kinetic analysis of the concentration profile for hydrogen peroxide relevant to the reaction mixture AA (0.3 mM) + O_2 (0.05 mM) in phosphate buffer (0.1 M, pH = 7) solution containing 5 μM Cu^{2+} (temperature, 27 °C). The calculated concentrations were obtained by using a model based on two consecutive pseudo-first-order reactions. The reported concentrations of H_2O_2 were calculated on the basis of the current response corrected for the contribution due to ascorbate.

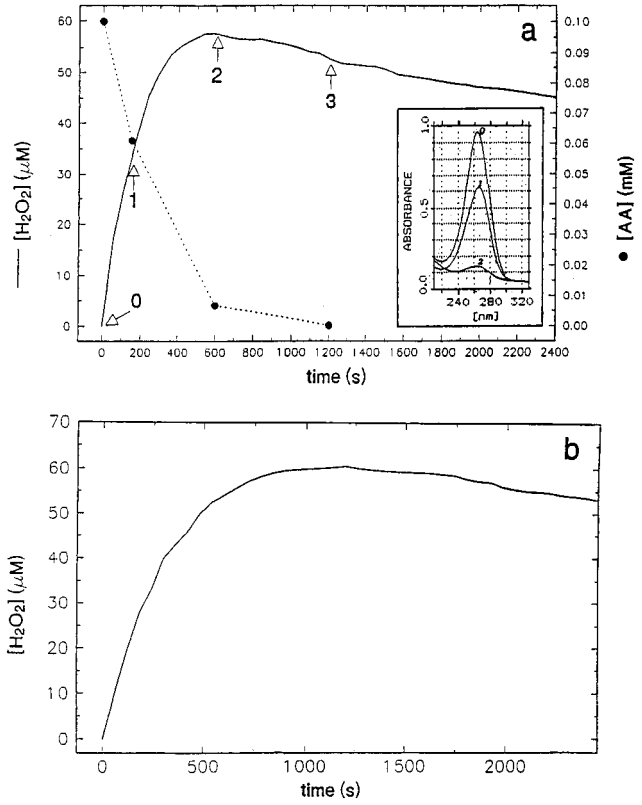


Figure 5. Concentration profiles for hydrogen peroxide relevant to the reaction mixtures AA (0.1 mM) + O_2 (0.23 mM) in phosphate buffer (0.1 M, pH = 7) solution containing (a) Cu^{2+} (5 μM) and (b) Cu^{2+} (2 μM). In (a), the concentration of ascorbate in the reaction mixture, determined by UV spectroscopy at different times (see the inset for the relevant spectra, with the exception of point 3, for which no appreciable absorption at 260 nm is detectable), is also reported. H_2O_2 concentrations were obtained from the corresponding current values corrected for the contribution due to ascorbate.

stoichiometrically lower than that of oxygen, the "correct" value being equal to $2[O_2]_0$, according to eqs 1 and 2. As an example, the $[H_2O_2]$ vs t profiles obtained in two cases (with different Cu^{2+} concentrations) are reported in Figure 5.

According to the reaction scheme based on processes 1 and 2, a stabilization of the H_2O_2 concentration should be reached after the total consumption of AA. The concentration of the latter was then monitored by UV spectroscopy (absorption at $\lambda \approx 260$ nm) on small aliquots of the reaction mixture with $Cu^{2+} = 5$ μM at different times. The corresponding values are also reported in Figure 5a; in the inset, the detail of the UV spectra (with the exception of point 3, for which no absorption at 260 nm is detectable) is shown. It is apparent that a decrease of $[H_2O_2]$ occurs even after ascorbate has been completely consumed. An experiment performed by adding 50 μM H_2O_2 to a phosphate buffer solution containing 5 μM Cu^{2+} showed that the presence of Cu^{2+} ions has a negligible effect on H_2O_2 decomposition. These findings seem to confirm the already observed[11] reactivity between H_2O_2 and DAA. At the same time, reaction with oxygen cannot be excluded.

It is also interesting to point out that the slopes of the $[H_2O_2]$ vs t curves after AA disappearance are not significantly influenced by Cu^{2+} concentration, thus suggesting that metal ions have a slight catalytic effect in this case. This finding is consistent with the weak affinity[10] of DAA for metal ions, which minimizes the possibility of an ion-mediated mechanism for the DAA−H_2O_2 process.

The simultaneous presence of ascorbic and dehydroascorbic acids in the reaction mixture makes the system too complex to draw direct kinetic information on the oxidation processes involving DAA. However, the peculiar experimental device setup for the present study seems also suitable for investigating systems in which only DAA (or other oxidation products of AA) is present as an oxidizable species. This investigation could contribute to clarify, from a more general point of view, the network of reactions involving ascorbate oxidation.

CONCLUSIONS

In the present work, a novel approach has been adopted to investigate, from a kinetic point of view, the processes connected to ascorbate oxidation by oxygen.

In particular, hydrogen peroxide, present as a stable intermediate in the reaction mixture, was monitored amperometrically by a specially modified electrode able to drastically prevent the simultaneous electrooxidation of ascorbate.

This peculiar experimental technique allowed us to obtain an interesting picture of the first steps of the AA−O_2 reacting system, likely the most complete presented up to now by a single experimental approach (comparison of homogeneous data), showing that other processes probably involving the byproduct of AA, dehydroascorbic acid, have to be considered, together with the already known reactions AA + O_2 and AA + H_2O_2. Moreover, it emphasized the role played by catalyzing metal ions, even if present in very low amounts.

In most cases, these effects could be quantitatively determined, though, as a matter of fact, they prevented the successful application of kinetic models based on simple consecutive reactions.

Because of its complexity, the problem of ascorbate "autoxidation" is far from being solved; however, the experimental device presented in the paper offers new, original possibilities for further studies on this system.

Furthermore, the experimental approach adopted seems to be a very promising method to get useful information also on similar reaction systems involving hydrogen peroxide.

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