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Ascorbic Acid Oxidation in Sucrose Aqueous Model Systems at Subzero Temperatures

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The reduction of Tempol by ascorbic acid in concentrated sucrose solutions was measured by electron paramagnetic resonance (EPR) at temperatures ranging from 16 to −16 °C. This method allowed the determination of the rate constants (*k*) of this fast reaction, by recording the Tempol reduction as a function of time. The two reactants were initially separated and had to migrate for the reaction to occur. The experimental findings were compared with predicted values according to the equation for diffusion-controlled reaction proposed by Atkins. The experimental reaction rate constants were observed to be lower than the calculated ones. However, the experimental values were found to be controlled by the temperature and viscosity changes of the reaction media, as expected for a diffusion-controlled reaction.

KEYWORDS: Vitamin C; Tempol; electron paramagnetic resonance; negative temperatures; reaction; cryoconcentration; diffusion

INTRODUCTION

Vitamin C or L-ascorbic acid is a nutriment essential for humans; its antioxidant role confers beneficial properties in the struggle against certain chronic diseases such as cataracts and cancer. Vitamin C action is also important in the preservation of foodstuffs, neutralizing the alteration caused by oxygen. Ascorbic acid is transformed into dehydroascorbic acid, thus preventing the oxidation of other biological molecules, such as lipids, which would be harmful due to the potential generation of peroxides in the human body or the appearance of rancidity in food. Ascorbic acid oxidizes when reacting with oxygen in air (*1*, *2*) or with oxidant substances (*3*, *4*) or, again, with certain enzymes (*5*). Ascorbic acid is an antioxidant present in abundance in fruits and green vegetables, but its sensitivity to oxygen makes difficult its preservation in foodstuffs. The reaction with oxygen occurs in two steps; dehydroascorbic acid is produced, which can then interact with other components of the food (reductones or other products of oxidation), giving a brown color as a result of nonenzymatic reaction.

One of the most effective means to preserve this nutriment is the freezing process, because it slows the kinetics of oxidoreduction reactions without inhibiting it completely. Freezing causes the transformation of part of the water into ice, thus inducing the concentration of the solutes in the remaining liquid phase. This phenomenon is called cryoconcentration. The evolution of the concentration in the liquid phase during the temperature change is related to its composition and is described by the curve of ice-melting temperature (T_m) of the phase diagram (*6*).

Oxidation of the ascorbic acid in frozen pieces of asparagus was studied by several authors (*5*). They highlighted that the ascorbic acid quantity remaining in this vegetable after bleaching is maintained during a few months of storage at low temperatures (−18 °C). The effects of freezing on the reaction kinetics may be different and complex because, depending on the temperature range or reaction considered, freezing may cause an acceleration of degradations or reduce the deterioration rate of the product. The ice crystal formation during freezing can induce the break of cellular membranes and the subsequent mixing of solutes, which were initially separated in different compartments. Moreover, the cryoconcentration causes the bringing together of the substances that are potentially reactive (*4*). Thus, by facilitating the meeting of the reactive molecules, the reaction kinetics might be accelerated. Takenaka and collaborators (*7*) have observed an increase at negative temperatures in the kinetics of oxidation of nitric acid by oxygen in air and oxygen hyperoxide in water. This acceleration due to the cryoconcentration was also observed by Hatley and collaborators (*4*) for the oxidation of the ascorbic acid in a buffer solution containing oxygen hyperoxide at −20 °C. These authors showed that the reaction kinetics could be 50 times higher in the medium containing ice than in the same medium without ice. On the contrary, reaction kinetics can be strongly reduced by low temperature for several reasons. On the one hand, the transformation of water into ice decreases its availability for

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reactions with water-soluble reactants or for the reactions in which water acts as a reactant. On the other hand, the temperature decrease, coupled with the cryoconcentration, causes a significant increase in the viscosity of the aqueous phase, inducing the reduction of the molecular mobility of the reactants in the system. In fact, the reduction in molecular mobility is often used for the preservation of foods. Indeed, the rate of many modifications of organoleptic and nutritional qualities in foods is sensitive to the viscosity of the medium in which small molecules are dissolved (*8*). The mobility of a molecule can be characterized by its rotational and translational diffusion coefficients. Both types of diffusional motions depend on temperature, the viscosity of the medium, and the size of the diffusing molecule, as well as on molecular interactions.

In this work, the oxidation of ascorbic acid by Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidine-oxyl, a stable nitroxide free radical) was studied in concentrated media composed of water and 50% sucrose in a temperature range from 16 to −16 °C. It is known that nitroxides may react with ascorbic acid. The reaction proceeds with the consumption of two molecules of nitroxide per mole of ascorbic acid, and the final products are 4-hydroxy-2,2,6,6-tetramethylhydroxylamine and dehydroascorbic acid. Dehydroascorbic acid being hydrated to 2,3-diketogulonic acid cannot be converted back into either ascorbic acid or dehydroascorbic acid under these conditions (*9*). After having reacted with the ascorbic acid, the Tempol molecule loses its paramagnetic character and becomes undetectable by the technique of electron paramagnetic resonance (EPR). The reduction of the EPR signal amplitude as a function of time allows the kinetics of oxidation of ascorbic acid to be followed. This method was already used with success for the study of the oxidation of ascorbic acid in suspensions of blood cells at ambient temperature (*3*). In our study, the specific effects of temperature and cryoconcentration on these kinetics were highlighted.

Many studies were carried out on the stability in low-moisture systems, dehydrated (*10*−*12*) or frozen media (*13*−*16*), to determine the effects of the physical state and molecular mobility on the reaction kinetics. Most of these experiments were performed in media containing the two reactants homogeneously distributed. Our experimental setup was designed to better approach the possible reaction process in real food products. Initially, reactants were physically separated; the reaction could thus not take place without the diffusion of the reactive entities one toward the other. Indeed, foods are constituted mainly of several compartments, with initially separated potential reactants. Moreover, all cellular systems from animals or vegetables can be presented as compartmentalized systems, which are often disrupted during the process, allowing the reactants' diffusion. Our results are discussed with reference to the theory proposed by Atkins (*17*) for diffusion-controlled reactions in homogeneous media.

MATERIALS AND METHODS

The two compartments with Tempol or ascorbic acid were prepared separately.

Tempol Solution. An aqueous solution with 57.5% sucrose and 1% agar was heated at 85 °C during 15 min under gentle stirring to obtain the complete dissolution of the gelling agent. During cooling and before gelling, 765 μL of concentrated Tempol (Sigma Aldrich, France) solution (2 g/L) was added to 5 g of the mix. The final concentrations of sucrose and Tempol were, respectively, 50.0% (g/100 g of solution) and 0.03%, that is, 1.83×10^{-3} mol·L^{-1}. The solution was introduced into a tube (internal diameter = 1.2 mm) and kept in a vertical position during gelification.

Ascorbic Acid Solution. Ascorbic acid crystals (15.3 mg; Merck, France) were added to 5 g of a 50% sucrose solution under stirring. The ascorbic acid concentration was 23.8×10^{-3} mol·L^{-1}, and a new solution was prepared before each experiment to avoid ascorbic acid oxidation by oxygen. The solution pH was 6.2 (±0.4), depending on the used distilled water. A volume of this solution, equal to that of the gel containing Tempol, was put into the tube at the surface of the gel containing Tempol.

Detection of Tempol by EPR. The EPR technique allows the detection of stable radicals. The EPR spectra were obtained with an EMX 300 (Bruker, France) equipped with the TE_{102} cavity and electromagnet using 100 kHz magnetic field modulation. The sample height in the capillary was ∼1 cm to allow the detection of the total Tempol quantity in the cavity of the EPR spectrometer. In resonance conditions, the energy absorbed is proportional to the quantity of radicals, and the decrease of the signal due to the Tempol reduction by ascorbic acid can be followed as a function of time. The recorded EPR spectrum corresponds to the derivative of the absorption curve as a function of the magnetic field (H). A double integration of the EPR signal gives a quantification of the Tempol quantity in the capillary as a function of time (Abs_s).

Kinetics of Tempol Reduction by Ascorbic Acid. The initial quantity of Tempol ($[T]_{t=0}$) was evaluated by EPR (Abs_0) before the addition of the ascorbic acid solution. The tube was put in a quartz holder tube (length = 25 cm, i.d. = 4 mm) in the spectrometer cavity. A Chrome-Alumel thermocouple was placed in the proximity of the capillary tube, allowing the temperature control. After the introduction of the solution with ascorbic acid, a first spectrum ($Abs_{s=1}$) was recorded as soon as the resonance conditions were established and the temperature set point was reached (±0.3 °C). To obtain a stable temperature in the sample and good resonance conditions, the time between the addition of ascorbic acid and the recording of the first spectrum was dependent on the temperature. The spectra were then recorded every 92 s, during a maximal period of 4 h, depending on the time when good resonance conditions were maintained. **Figure 1** shows the evolution of the shape of EPR absorption spectra as a function of time during the reduction of Tempol by ascorbic acid.

RESULTS

Determination of the Kinetics of the Tempol Reduction by Ascorbic Acid. The Tempol remaining in the sample was first expressed as the ratio of the absorbed energy after the addition of the ascorbic acid solution (Abs_s) to the absorbed energy before this addition (Abs_0). Indeed, **Figure 2** shows the percentage of Tempol remaining in the sample relative to the initial quantity ($100 \times Abs_s/Abs_0$) as a function of reaction time. The initial quantity of Tempol was the same for each sample (Abs_0 almost constant). Each experiment is presented with a different symbol in **Figure 2**. Indeed, according to the time at which the first spectrum ($Abs_{s=1}$) was recorded after the addition of the ascorbic acid solution, the remaining quantity of the probe varied (**Figure 2**). The decrease of the paramagnetic Tempol quantity was nonlinear as a function of time (**Figure 1**) for all temperatures studied. The reaction kinetics of Tempol reduction were measured from the slopes of the Tempol percentage remaining in the sample versus time (**Figure 2**); the rate of the reaction was considered to be constant for a decrease of <10% of the quantity of Tempol. The slopes determining the reaction rates (V_s) were calculated from linear regressions with significant regression coefficient (r^2) for $\alpha = 0.01$.

Apparent Reaction Order. Our experimental results of the concentrations of reactants as a function of time or of reaction rates were used to determine an apparent global reaction order.

According to the Van't Hoff method, the rate of reaction was supposed to follow the equation

$$V_{s=k}[\text{Tempol}]^m$$

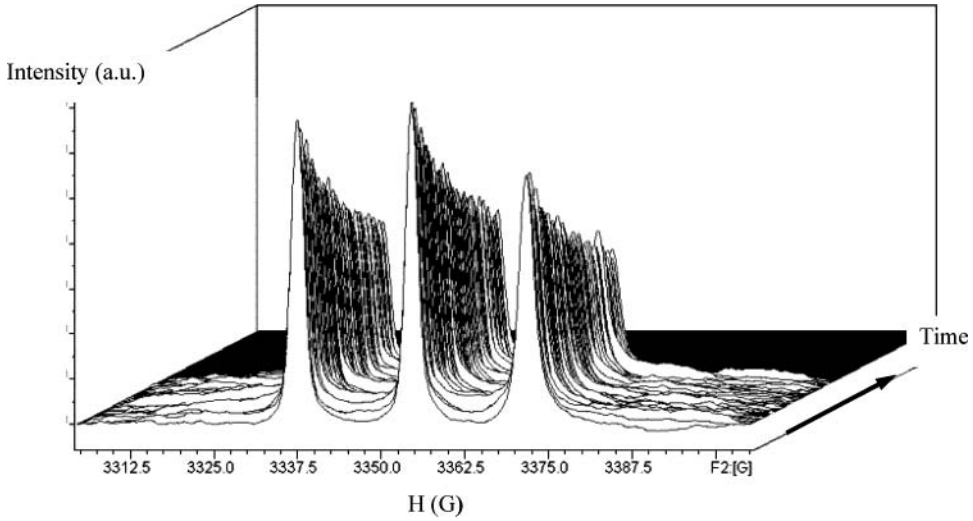

Figure 1. Evolution of EPR absorption curve of Tempol during its reduction by ascorbic acid in a 50% sucrose solution at 0 °C.

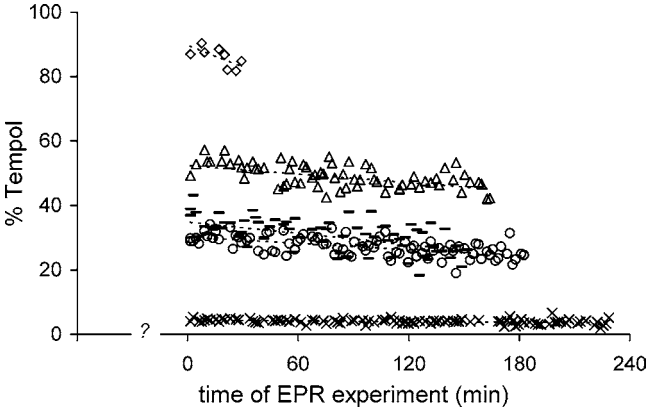

Figure 2. Evolution of the Tempol percentage remaining in the sample, during its reduction by ascorbic acid at −8 °C in a 50% sucrose solution. The different symbols correspond to experiments with different initial $Abs_{s=1}$; one spectrum is recorded each 92 s.

k is the reaction rate constant, [Tempol] is the concentration of Tempol, and *m* is the order of the reaction. Tempol concentrations as a function of time were obtained from the EPR signal intensity: $[\text{Tempol}] = Abs_s \times [\text{Tempol}]_{t=0}/Abs_0$. With our experimental data obtained at −8 °C, the logarithm of rates versus logarithm of Tempol concentrations plot gave a linear curve with a correlation coefficient $r^2 = 0.92$, and the *m* value determined with eq 1 was 1.04. Therefore, the partial order of reaction is 1, considering only Tempol as reactants. The same reaction was also studied by EPR by several authors (*3*, *18*). Craescu and collaborators (*3*) showed that the approximation to a pseudo-first-order reaction in an excess of ascorbic acid was correct, but in their experimental setup, the two reactants were initially mixed together. On the one hand, in our work, ascorbic acid was only slightly in excess (∼×10) in the medium compared to the Tempol quantity, and, on the other hand, the reactants were initially separated and their relative concentrations were dependent on the diffusion of these two molecules. According to Atkins (*17*), a bimolecular reaction is a second-order reaction because its rate is proportional to the rate at which the reactants meet. Therefore, on the other hand, the integrated rate law (eq 2) for a second-order reaction was tested with our experimental data in considering the two reactants, Tempol and ascorbic acid:

$$\frac{1}{([AA]_{t=0} - [\text{Tempol}]_{t=0})} \ln\frac{[\text{Tempol}]_{t=0}[AA]}{[AA]_{t=0}[\text{Tempol}]} = kt \quad (2)$$

[Tempol] and [AA] are, respectively, the Tempol and ascorbic acid concentrations (mol.L^{-1}) at time *t*. The plot of the logarithm of the ratio of ascorbic acid to Tempol concentrations as a function of time was analyzed with a linear regression for experiments carried out at −8 °C; the correlation coefficient was higher ($r^2 = 0.98$) than for a first-order reaction. In fact, the reaction scheme may be divided into two steps:

$$\text{Tempol} + \text{ascorbic acid} \xrightarrow{\text{diffusion}} (\text{Tempol} - \text{ascorbic acid}) \xrightarrow{\text{reaction}} \text{substates}$$

The first step corresponds to the meeting of Tempol and ascorbic acid molecules and the second step to their chemical transformation. Indeed, the chemical reaction may be a first-order reaction but was not expected to be the limiting factor of the reduction rate. Mainly, the diffusion of the two reactants in the highly concentrated sucrose solution and the cryoconcentration have to be taken into account as the limiting step of the reaction. The reactant molecules have similar sizes and should have similar diffusion coefficients. Therefore, a second-order process was considered to describe the reaction rate according to the first step of the reaction scheme and used for further analysis.

Influence of the Reactant Concentration. Table 1 shows the different reaction rates that were measured at a given temperature, depending on the remaining Tempol concentration in the sample. The reaction rate decreased with the decrease in the Tempol quantity. The concentration of ascorbic acid also decreases with time in the medium. As the reaction rate was assumed to be controlled by the reactant diffusion, an apparent reaction rate constant *k* ($L \cdot mol^{-1} \cdot s^{-1}$) was calculated from the reaction rates V_s ($mol \cdot L^{-1} \cdot s^{-1}$) and the concentrations of reactants:

$$k = V_s/([\text{Tempol}] \cdot [AA]) \quad (3)$$

The ascorbic acid concentrations were calculated according to the stoichiometry of the reaction, knowing the quantity of

Table 1. Experimental Reaction Kinetics (V_s) and Calculated Second-Order Reaction Constant (k) of Tempol Reduction by Ascorbic Acid at −8 °C as a Function of the Percentage of Remaining Tempol in the Sample, a 50% Sucrose Solution

% of Tempol for $Abs_{s=1}$	$10^8 \times V_s$ ($mol \cdot L^{-1} \cdot s^{-1}$) of Tempol reduction	reaction rate constant $10^3 \times k$ ($L \cdot mol^{-1} \cdot s^{-1}$)	mean reaction rate constant $10^3 \times k$ ($L \cdot mol^{-1} \cdot s^{-1}$)
87.6	6.89	2.04	
51.5	1.97	1.10	
38.6	2.62	1.80	1.55 (0.39)[a]
29.8	1.53	1.37	
4.02	0.23	1.52	

[a] Standard deviation is given in parentheses.

Table 2. Mean Reaction Rate Constant k of Tempol Reduction by Ascorbic Acid as a Function of Temperature in a 50% Sucrose Solution

T (°C)	reaction rate constant $10^3 \times k$ ($L \cdot mol^{-1} \cdot s^{-1}$)	no. of tests
16	9.79 (2.30)[a]	4
8	4.34 (1.18)	5
0	2.46 (1.06)	5
−8	1.55 (0.39)	5
−16	0.45 (0.09)	8

[a] Standard deviation is given in parentheses.

Tempol molecules that had reacted. Taking into account the evolution of reactant concentrations in the medium, the reaction rate constant corresponding to the average value was determined from the different measured reaction rates (V_s) at a given temperature and different ratios of $Abs_{s=1}/Abs_0$ (**Table 1**). The same evolution of the reaction kinetics as a function of Tempol quantity was observed whatever the temperature: the reaction rate, V_s, varied with the quantity of reactants in the sample, but the reaction constant k did not vary with reactant concentration and was dependent only upon temperature.

Temperature Effect on the Tempol Reduction Kinetics. The kinetics of the reaction were studied at 16, 8, 0, −8, and −16 °C. Statistical tests (variance analysis and Student−Newman−Keuls test with $\alpha = 0.05$) were done on k obtained for all experiments. The temperature induced a significant decrease of the reaction rate constant (**Table 2**).

DISCUSSION

Cryoconcentration Effect. The cryoconcentration of solutes allows the proximity of reactants, which may induce the acceleration of the reaction. Parker and Ring (*19*) have demonstrated that in a temperature range of 0 to −16 °C, the cryoconcentration of the reactants may induce an increase of the apparent reaction rate constant of a hydrolysis reaction, which is maximum between −2 and −3 °C, when the initial sucrose concentration is <20%. In this work, the initial sucrose concentration (50%) was higher and no maximum in the reaction rate was observed in the subzero temperature range, probably because the high viscosity of the medium was the most important factor. As compared to the sucrose percentage (50% g/g total solution), which contributes mainly to solution viscosity and ice-melting temperature decrease, the effect of the small quantities of Tempol (0.03%) and ascorbic acid (0.4%) was neglected.

Figure 3 shows an Arrhenius plot of the reaction constants. The cryoconcentration of the reactants in the liquid phase was taken into account for the calculation of k (eq 3) for the samples containing ice, in considering the sucrose state diagram (*6*). At −16 °C, the sucrose concentration of the liquid phase was 66.5%, due to the freezing of water. The reactant concentrations were supposed to follow the same cryoconcentration as sucrose, and concentrations of Tempol and ascorbic acid were recalculated as a function of temperature and ice formation. The viscosity of the reaction media was determined using (1) the sucrose state diagram (*6*) to determine the glass transition temperature (T_g) as a function of liquid-phase concentration and (2) the Williams−Landel−Ferry (WLF) expression (*20*)

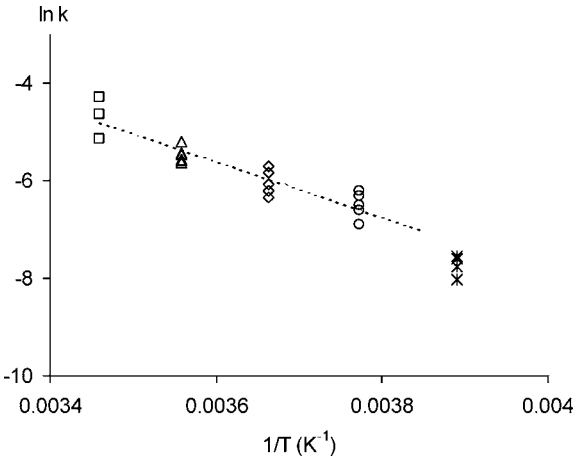

Figure 3. Arrhenius representation of the reaction rate constants k (16 °C, □; 8 °C, △; 0 °C, ◇; −8 °C, ○; −16 °C, +), calculated as a function of the concentrations in the whole sampe, and at −16 °C (×), calculated as a function of the concentrations in the nonfrozen phase. The dotted line is the linear regression taken into account for the apparent activation energy determination.

$$\log(\eta_T/\eta_{Tg}) = C_1(T - T_g)/[C_2 + (T - T_g)] \qquad (4)$$

where η_T and η_{Tg} are viscosities at T and T_g, respectively, and C_1 and C_2 are phenomenological coefficients. The values for η_{Tg}, C_1, and C_2 were determined from experimental viscosity data (*21*) and were, respectively, 1.6×10^{12} Pa·s, −19.8, and 51.6 K. At all temperatures down to −8 °C, there was no ice in samples; so the viscosity and thus the reaction rate constant decreased with the decrease in temperature according to a WLF evolution in the temperature range T_g, $T_g + 100$. However, in the small temperature range considered, an apparent Arrhenius behavior may be observed in the upper part of the WLF temperature domain. The apparent activation energy (E_a = 47 kJ/mol) of the evolution of k was determined from data obtained in the temperature range from 16 to −8 °C [linear part of the Arrhenius plot ($R^2 = 0.95$), i.e., for samples with no ice formation and thus no cryo-concentration (**Figure 4**)]. The data measured at −16 °C were under the influence of both cryoconcentration and decrease in the temperature difference $T - T_g$. Below −8 °C, in addition to the decrease in temperature, the increase in viscosity due to the cryoconcentration emphasized the decrease in the reaction rate. The higher is the activation energy value for viscosity, the higher is the sucrose concentration in the liquid phase (*22*). The diffusion of reactants became slower with the increase in viscosity and, as previously shown, the activation energy for diffusion of small molecules increases with the sucrose concentration (*21*). Our findings demonstrate that in relatively simple frozen systems such as our model, the influence of temperature change on the kinetics of a reaction may be evaluated from the changes in composition resulting from ice formation or melting.

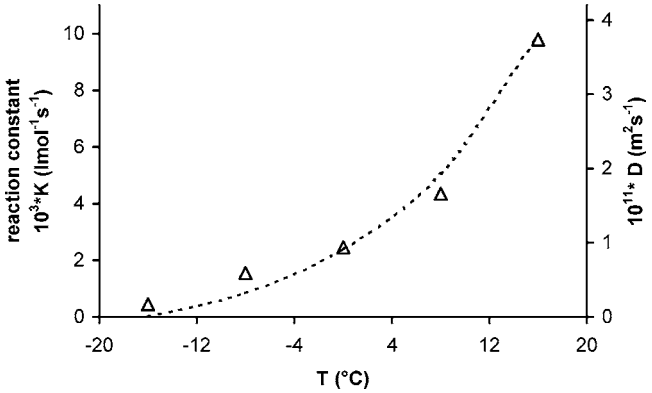

Figure 4. Reaction rate constants k (△) of Tempol (radius = 3.5 Å) reduction by ascorbic acid and calculated translational diffusion coefficients (dotted line) of a molecule with a radius of 3.5 Å as a function of temperature.

The rates of chemical reactions are likely to depend on the rates at which reactants and products can diffuse to and from regions of activity. The measured reaction rate depends on various factors, such as local properties of the surrounding media (viscosity), but most importantly in our experimental set up, it depends on the distance between reactants. Therefore, translational diffusion can play a significant role in influencing the reaction kinetics. The translational diffusion coefficient of small molecules (D_{trans}) was measured in cryoconcentrated sucrose solutions at temperature down to −15 °C (*21*, *23*). Using fluorescence recovery after photobleaching (FRAP), it was shown that fluorescein (r = 5 Å) diffused according to the Stokes−Einstein relationship (eq 5) when the temperature was not too close to the glass transition temperature ($T > 1.2T_g$) of the diffusion medium.

$$D_{trans} = k_B T/6\pi\eta r \quad (5)$$

k_B is the Boltzmann constant, T the temperature (K), r the hydrodynamic radius of the diffusing molecule, and η the viscosity (Pa·s). The translational diffusion coefficient of the Tempol molecule was calculated with eq 5 using r = 3.5 Å for the hydrodynamic radius of Tempol (*24*).

At each temperature, the reaction rate constant and Tempol translational diffusion coefficients (D_{trans}) were linearly correlated (**Figure 4**). Theoretical analysis of diffusion-controlled reaction kinetics was initiated in 1916 by Smoluchowski. Atkins (*17*) has proposed a development of the theory for bimolecular diffusion-controlled reactions, which is based on both Ficks' law and the Stokes−Einstein relationship and allows the determination of the reaction constant using the viscosity of the medium as a variable. The reaction model is

$$k = 4\pi d D_{trans} N \quad (6)$$

with d the minimal distance for the reaction to occur and N the Avogadro number. This equation was developed for a bimolecular reaction in which the reactant molecules have a similar size (r) and are initially mixed together. Combining eqs 5 and 6, Atkins (*17*) proposed the expression

$$k = 8RT/3\eta \quad (7)$$

assuming that d, the distance for collisions, is equal to the sum of reactant radii ($d = 2r$) and that D_{trans} is equal to the sum of the translational diffusion coefficients of each reactant (R is the ideal gas constant).

The experimentally measured reaction constants were lower than the theoretical prediction calculated from eq 5, by a factor >10 decades. On the basis of this calculation, the conclusions could be that the reaction was not diffusion controlled but was only influenced by temperature. However, it was noted that the E_a for small molecule translational diffusion coefficients and reaction rate constants were similar. Moreover, a linear correlation between k and D_{trans} was observed for all temperatures with a correlation coefficient of $r^2 = 0.987$, confirming that the kinetics of reaction may be simply derived from diffusion data. Therefore, the reaction rate constant may be predicted with the equation

$$k = A(4\pi d N) D_{trans} \quad (8)$$

with A a constant we introduced in the Atkins model to take into account our experimental setup, where the two reactants were not initially in the same solution but had to migrate to react. A similar expression was given by Shushin and Barzykin (*25*) to predict the reaction rate constant for anisotropic reactivity. They introduced a steric factor in eq 6 (instead of A) to take into account the distance-dependent anisotropic reactivity and molecular geometry. Their demonstration showed that even a relatively small thickness (compared to molecular dimension) of the reaction region could lead to a drastic acceleration of the reaction. In contrast to our work, the reaction region increasing with time could contribute to a slowing of the reaction.

Conclusions. The reduction of Tempol by ascorbic acid was studied in concentrated sucrose solutions at temperatures ranging from 16 to −16 °C by EPR. The technique was adapted to allow the measurement of the kinetics of this instantaneous reaction. To better mimic real food systems, the reactants were initially in different solutions before the two media were brought into contact. At the lowest temperatures, a cryoconcentration effect was taken into account, inducing no acceleration of the reaction or noticeable slowing of the reaction if rate constants were calculated by considering only the liquid phase. The reaction rate constants were found to be proportional to translational diffusion coefficients of the reactant molecules. The temperature was not the only parameter that controlled the reaction because through the translational diffusion coefficient, the influence of the viscosity was also taken into account, D_{trans} being inversely proportional to viscosity.

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