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Studies on the strand-breaking activity of the ascorbate/copper(II) system in poly(adenylic acid)

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The role of the ascorbate/Cu(II) system on the formation of strand breaks in poly(adenylic acid) (polyA), was studied by conductivity measurements in aqueous solution at pH 7.2 and 3.5 under aerobic conditions. Solutions of poly(adenylic acid) were incubated with freshly prepared ascorbate/Cu(II) solutions at various temperatures and the resulting conductivity increases were followed by measuring the conductivities of the solution at different time intervals. The apparent activation energies for the strand breakage were found from the conductivity measurements to be 59.8 and 22.4 kJ mol^{-1} for pH 7.2 and 3.5, respectively.

(Keywords: poly(adenylic acid); ascorbic acid; strand breaking)

INTRODUCTION

Ascorbic acid, vitamin C, is extensively used as an antioxidant in the food industry. It can act as a reducing agent, a free-radical scavenger and a source of free radicals which could damage macromolecules within a cell. Despite their low reactivities, ascorbate ion radicals have been suggested as posing a considerable threat to biomolecules because they can react more selectively with the target at critical cell locations and may also diffuse to some distances away from the site of their generation[1].

The DNA cleavage by ascorbate in the presence of Cu(II) is well documented[2–6]. It has been suggested that the enediol group of the ascorbate has an essential role to play in the damage to nucleic acids, and that Cu(II) increases this action[2,3]. The precise mechanism of DNA damage may be multifactorial. These biologically important compounds function as polyelectrolytes and when dissociated will bring about changes in the ionic strength or conductivity of the solution. It is well known that polyelectrolytes are not fully dissociated[7], and Schindewolf[8] has found that the degree of dissociation increases with a decreasing chain length. The introduction of chain breaks in a polyelectrolyte, therefore, increases the concentration of free counterions and hence the conductivity of the solution increases[9,10]. Measurement of the conductivity change was shown to be a reliable tool in the investigation of strand breakage in single-stranded polynucleotides and DNA[11–13]. This method has the advantages of being applicable at low polymer concentrations and can be used with polyelectrolytes of comparatively low molecular weight. Only a few cm^3 of solution are needed for the measurements. In addition, the rate of formation of strand breaks can be conveniently determined with this method[13]. In this present work, which is part of a research study on the strand breaking of DNA and polyA by radiation or other types of radical initiator, we have studied the strand-break activity of the ascorbate/Cu(II) system on polyA by measuring the conductivity built up after incubating aqueous polyA solutions with ascorbate/Cu(II). The potassium salt of poly(adenylic acid), (polyA), was used as a model compound instead of DNA, since it is a simpler compound than DNA and only contains one kind of heterocyclic base. Furthermore, polyA exist in the single stranded form at pH $\geqslant 6$. In a study of the radiation damage of the polymer chains light scattering and viscosity measurements were used to investigate possible degradations (work currently being prepared for publication). However, molecular-weight determination could not be used for chain breaking with the ascorbate/Cu(II) system because the process is time dependent, with the conductivity increasing to a limiting value in a certain time limit. The objective here was to illustrate the potential deleterious effect of a common antioxidant, namely ascorbic acid, on a model system and to elucidate its mechanism.

EXPERIMENTAL

The potassium salt of poly(adenylic acid) (polyA) was purchased from Boehringer Mannheim. As measured by low-angle laser light scattering, the weight-average molecular weight of polyA used in these experiments was $\sim 6.0 \times 10^5$ g mol^{-1}. Supra-pure ascorbic acid and

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cupric chloride were obtained from Merck. Triple-distilled water was used in the preparation of polyA, ascorbic acid and $CuCl_2$ solutions.

Conductivity measurements were carried out in a thermostated bath, having a double temperature control, which maintained the temperature to $\pm 0.1°C$. The conductivity of polyA solutions ($60 mg l^{-1}$)/Cu^{2+} (0.36 mM) were measured by using a Schott Gerate conductivity meter (model Konduktometer CG 854), and a Schott Gerate cell (model LF 1100 T, cell constant $K = 1.05 cm^{-1}$), immediately after mixing with ascorbic acid (5 mM) solution. The pH values of the solutions were adjusted to 7.2 by adding NaOH. The measurements were carried out for 30 min at each temperature. The pH of the reaction mixture did not change significantly throughout the measurements.

The electron spin resonance (e.s.r.) spectrum of the ascorbate/Cu(II) mixture was recorded on a Varian E band ESR spectrometer by using a quartz-flat cell.

RESULTS AND DISCUSSION

Ascorbic acid is generally regarded as an antioxidant and for this reason is used as a food preservative. However, there are certain conditions where the ascorbate can

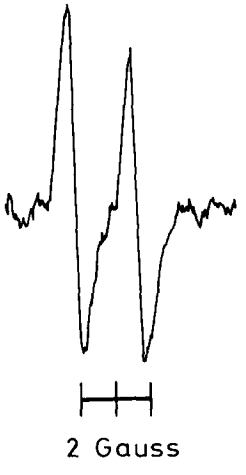

Figure 1 E.s.r. spectrum of the ascorbate anion radical obtained from an ascorbate (5 mM)/Cu(II) (0.36 mM) mixture at room temperature

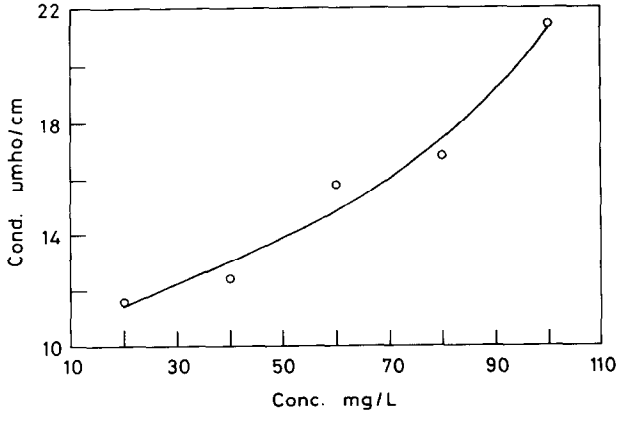

Figure 2 Concentration dependence of the initial conductivity of polyA solutions

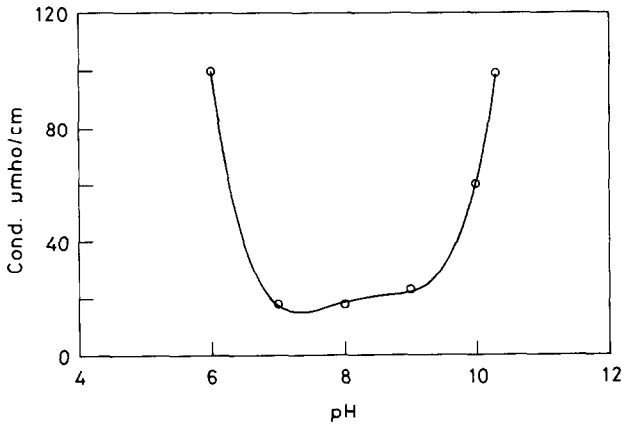

Figure 3 The pH dependence of the initial conductivity of polyA solutions

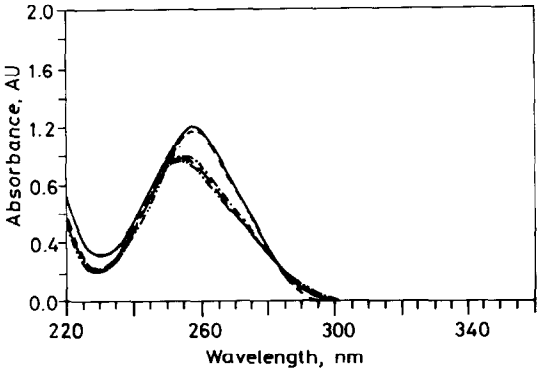

Figure 4 Variation of the u.v. spectrum of an aqueous polyA solution with pH: (—) pH 7.8; (– – –) pH 6.9; (– · –) pH 6.09; (— —) pH 6.03; (· · —) pH 5.94

contribute to oxidative damage, i.e. the cleavage of DNA[4].

The autoxidation of ascorbic acid in the presence of transition metals such as Cu(II) involves the formation of ascorbyl radical intermediates[14]. The presence of the ascorbyl anion radical in the ascorbate/Cu(II) mixture has been confirmed by e.s.r. measurements[15,16]. The spectrum displays a doublet, which is characteristic of the ascorbyl anion radical (as shown in *Figure 1*).

The strand-break activity of the ascorbate/Cu(II) mixture on polyA was investigated by monitoring the conductivity increase upon incubation of an aqueous polyA solution with the ascorbate/Cu(II) mixture. The concentration dependence of the initial conductivity of the polyA solution was studied by measuring the conductivity of the aqueous polyA solutions with various concentrations of polyA. The results are shown in *Figure 2*. As seen from this figure the initial conductivity increases with increasing polyA concentration.

Prior to this investigation of the strand-breaking activity of the ascorbate/Cu(II) mixture, the pH dependence of the conductivity was also studied. As shown in *Figure 3*, the conductivity of the polyA solution increases sharply below pH 6 and above pH 9. The increase above pH 9 could be due to hydrolysis of the polyA[17], resulting in smaller segments and a high degree of dissociation. The conductivity increase below pH 6 could be due to formation of a double stranded helix. It is well known

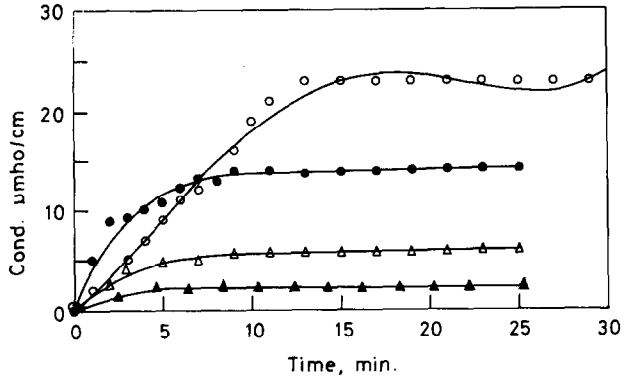

Figure 5 Conductivity of aqueous polyA solutions (60 mg l^{-1}) at pH 7.2 with respect to time in the presence of ascorbate (5 mM)/Cu(II) (0.36 mM), measured at different temperatures: (△) 31°C; (○) 37°C; (●) 40°C. Conductivity was also measured at 37°C in the presence of t-BuOH (▲)

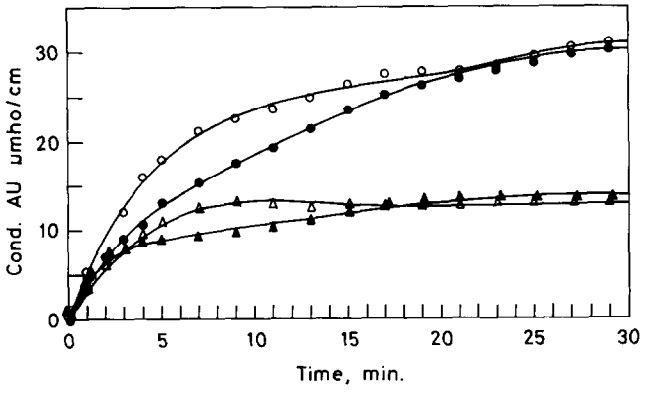

Figure 6 Conductivity of aqueous polyA solutions (60 mg l^{-1}) at pH 3.5 with respect to time in the presence of ascorbate (5 mM)/Cu(II) (0.36 mM), measured at different temperatures: (▲) 23°C; (△) 30°C; (●) 37°C; (○) 45°C

that below pH 6, polyA is protonated and a double stranded helix forms via a process of hydrogen bonding[18,19]. The formation of a double stranded helix below pH 6 was also shown from the shift of λ_{max} and a decrease in absorbency, as shown in *Figure 4*.

Since the conductivity of an aqueous polyA solution does not change significantly in the pH range from 6 to 9, the conductivity measurements were carried out at pH 7.2. The conductivity buildup immediately after incubating the polyA solution (60 mg l^{-1}) with ascorbic acid (5 mM) and Cu^{2+} (0.36 mM) was studied as a function of time at temperatures of 31, 37 and 40°C, and the results obtained are shown in *Figure 5*. Since conductivity measurements respond to the net effect of all ionic species in solution, the effects of additional counterions and co-ions also have to be taken into account[20]. Therefore, the conductivity of the polyA/Cu(II) mixture in the absence of any ascorbic acid was also monitored for ~30 min which showed no significant variation of the conductivity within the given time interval, except for an approximately initial doubling of the conductivity of the polyA solution due to additional ions.

Furthermore, in order to observe the effect of the ascorbate/Cu(II) system on the structure of polyA, we have also measured the conductivity buildup at pH 3.5,

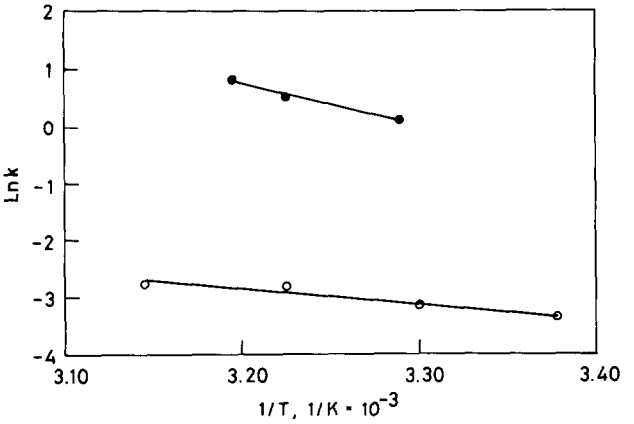

Figure 7 Arrhenius plots of aqueous polyA solutions (60 mg l^{-1}) in the presence of ascorbate (5 mM)/Cu(II) (0.36 mM): (○) pH 3.5; (●) pH 7.2

where polyA becomes double stranded, at temperatures of 23, 30, 37 and 45°C. The results are given in *Figure 6*.

Incubation of the full reaction mixture resulted in an immediate conductivity increase, which reached a plateau within 5–10 min. This increase in conductivity is a result of an increased dissociation of the polynucleotide due to the formation of strand breaks.

It was observed that under similar experimental conditions neither copper, nor ascorbate, when present alone, caused any significant conductivity changes within a period of 30 min. *Figures 5* and *6* also show that the rate of strand-break formation increases with increasing temperature in the initial period. When approaching its limiting value the trend in the change of conductivity with temperature is not regular. For instance, the limiting conductivity at 37°C becomes higher than at 40°C (*Figure 5*). Similar unusual trends are also observed in *Figure 6* for temperatures of 23 and 30°C. We are unable to suggest a satisfactory explanation for this at this present time. The rate constants for the strand-break formation are equal to the initial slopes of the conductivity *versus* time plots. It was found that the rate of strand-break formation did not change significantly when changing the initial concentration of polyA. A plot of the initial rates against $1/T$ is given in *Figure 7*. The apparent activation energies for strand-break formation were calculated and found to be 59.8 kJ mol^{-1} at pH 7.2 and 22.4 kJ mol^{-1} at pH 3.5 (obtained from the slopes of the Arrhenius plots).

It is known that cupric ion generates oxy radicals in the presence of oxygen via the Haber–Weiss cycle[21] (reactions 1–5):

$$AsA^- + Cu^{2+} \rightarrow AsA^{\cdot} + Cu^+ \quad (1)$$

$$AsA^- + O_2 \rightarrow AsA^{\cdot} + O_2^{\cdot -} \quad (2)$$

$$Cu^{2+} + O_2^{\cdot -} \rightleftharpoons Cu^+ + O_2 \quad (3)$$

$$2O_2^{\cdot -} + 2H^+ \rightleftharpoons H_2O_2 + O_2 \quad (4)$$

$$Cu^+ + H_2O_2 \rightleftharpoons \cdot OH + OH^- + Cu^{2+} \quad (5)$$

The presence of reducing agents such as ascorbic acid provides a continuous source of cuprous ions (equation (1)) which results in the generation of OH radicals (equation (5)). Thus, the OH radical is responsible for the strand-break formation. In this present study, we found

that addition of a chealating agent, such as EDTA, to the reaction medium prevented oxidation of the ascorbate, and consequently any buildup in the conductivity due to strand-break formation of polyA. This is because the EDTA complexes with Cu^{2+}, thus inhibiting the above reaction which terminates with the formation of the OH radical. These results show that the strand-break activity of the ascorbate/Cu(II) mixture could be mainly due to the formation of OH radicals through Fenton reactions[22]. In order to prove this, we have also measured the conductivity of the polyA–ascorbate/Cu(II) reaction medium in the presence of t-butyl alcohol (2 mM), which is known to be a good OH radical scavenger (at a temperature of 37°C). As can be seen in *Figure 5* the conductivity of the reaction medium did not increase appreciably in the presence of t-butyl alcohol, indicating that the main cause of the strand-break activity of the ascorbate/Cu(II) system on polyA is OH radicals. A small increase might be due to some OH radicals which are not retained by the t-butyl alcohol.

CONCLUSION

In this present work, we have found that ascorbic acid, a multifaceted compound, causes the formation of strand breaks in poly(adenylic acid) in the presence of copper(II). Although the autoxidation of ascorbic acid involves ascorbyl ion radicals, the hydroxyl radical is found to be the main cause of the formation of strand breaks. The apparent activation energy of the strand-break formation was found to be 59.8 kJ mol^{-1} at pH 7.2 and 22.4 kJ mol^{-1} at pH 3.5; the difference between the values of the activation energy at these two pHs can be due to either or both of the following: (a) double-strand formation at a pH of 3.5, and/or (b) an increase in the reaction rate to give more Cu^{+} (equation (1)) at a pH of 3.5. In the first case, the double-stranded molecule will extend much easily when compared to the single stranded chain. Therefore, the steric effect for the attack of the OH radical on the double stranded chains will be less. This will mean that strand-break formation is more likely to take place at a pH of 3.5, thus decreasing the activation energy. On the other hand, the reduction potential of Cu^{2+} will be higher in acidic solution, which will cause an increase in the formation of Cu^{+} at a pH of 3.5, when compared to that of at a pH of 7.2. As a result, the concentration of OH radicals will increase more at the lower pH, with consequently more positions for strand breaking being attacked.

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