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Free radical mediated interaction of ascorbic acid and ascorbate/Cu(II) with viral and plasmid DNAs

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Abstract. Previous studies indicate that ascorbic acid, when combined with copper or iron cleaves several viral DNA. ln this study, we generated the ascorbate radical anion electrochemically in a simple chemical environment without the participation of a metal ion. This solution possesses viral DNA scission activity. Ohe absence of catalytic metal ions [Fe (Ill) and Cu(II)] in the incubation medium was evidenced by metal chelating agents such as desferrioxamine and EDTA. Ohe radical quenching at high EDTA concentration was attributed to ionic strength of EDTA rather than metal chelation. Ohe effects of antioxidants, radical scavangers, catalase, superoxide dismutase and some proteins on DNA cleavage have been tested. Cleavage may not arise directly from ascorbate free radical but the reaction of the radical form of ascorbate with oxygen may produce the actual reactive species. Aerobic oxidation of ascorbate itself strictly requires transition metal catalysts, however electrochemically produced ascorbyl radical avoided the kinetic barrier that prevented direct oxidation of ascorbic acid with oxygen and eliminated the need for the transition metal ion catalysts.

Keywords. Ascorbate/Cu(II); ascorbic acid; ascorbyl radical; DNA damage; free radicals; λ.-DNA.

1. Introduction

Reactive oxygen species are important in carcinogenesis, diseases and aging through oxidative damage of DNA (Halliwell and Aruoma 1991). Hydroxyl radical (·OH) can be derived from these species in the presence of transition metals such as copper and iron. Ascorbic acid as a reducing agent and radical scavenger provide effective protection against reactive oxygen species. However, ascorbic acid is a multifaceted compound that can act as a source of free radicals. The variety of biological reactions in which ascorbic acid participates passes through a free radical intermediate called ascorbyl radical ($A^{\cdot -}$) (Bielski *et al* 1971; Bielski 1982; Halliwell and Gutteridge 1990; lyanagi *et al* 1985).

The involvement of $A^{\cdot -}$ in biological reactions is extensive. Animal and plant cells contain an NADHdependent semidehydroascorbate reductase (EC 1.6.5.4) to remove this radical (Arrigani *et al* 1981). $A^{\cdot -}$ is one of the most stable radicals that can be detected by electron spin resonance (ESR) spectroscopy (lyanagi *et al* 1985). Despite their low reactivity, weakly reactive radicals have been suggested to pose a considerable threat to biomolecules since they can react more selectively with targets at critical cell locations and may diffuse to some distance from the site of their generation (Fucs *et al* 1990).

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Copper and iron are the two most effective transition metal ions examined that cleaved several viral DNAs in the presence of ascorbate (Chiou 1983, 1984; Paul *et al* 1987; Shamberger 1984; Wang and Ness 1989). True autoxidation of ascorbate in the absence of catalytic metals is a very slow process ($K \ll 6 \times 10^{-7}$ s^{-1}) due to spin restriction of oxygen (Buettner 1988, 1990; Miller *et al* 1990). In this respect, transition metals serve as a bridge for the reaction of ascorbic acid and oxygen through the *d* orbital of the metals. On the other hand, the radical form of ascorbate can react with oxygen with a rate constant on the order of 10^2 M^{-1} s^{-1} (Frohlinde 1983) and produce oxygen radicals.

ln this study, we have generated ascorbyl radical electrochemically without the participation of metal ions (Önal *et al* 1990). We have shown that this solution possesses viral DNA cleaving activity. lt does not mean that the ascorbyl radical itself makes DNA scission but the ascorbyl radical may react with trace amount of oxygen in the system and converted into a more reactive species. At a chemical level it is very probable that this simple chemical solution may be an alternative to Fe(II)-EDTA as a footprinting system for DNA-binding ligand studies. The footprints obtained with molecules smaller than DNase I define more precisely the binding site of the protein (Tulius *et al* 1987).

2. Materials and methods

2.1 *Materials*

Ultra-pure X.-DNA and agarose were obtained from Bathesda Research Laboratories. Supra-pure L-ascorbic acid, cupric chloride, sodium chloride, catalase (EC 1.11.l.6.), tryptophan, thiourea, EDTA, tert-butyl alcohol, reagent grade sodium perchlorate and boric acid were from Merck. Glutathione reductase [(GSSGR) (EC 1.6.4.2.)], bovine serum albumin, glutathione and Tris were from Sigma, Dithiothreitol and DL-cysteine hydrochloride were from BDH. Mannitol was from Riedel. Deionized water is used throughout the experiments (absence of metal ions was confirmed by atomic absorption spectroscopy).

2.2 *Generation of ascorbyl radical*

$A^{\cdot-}$ was generated from ascorbate in deionized water by constant potential electerolysis under nitrogen atmosphere. The electrolysis cell consisted of a Pt-bead working electrode. Pt-wire counter electrode and a saturated calomel electrode as reference electrode. A potentiostat-function generator couple was used to programme the potential. Reagent-grade sodium perchlorate or suprapure sodium chloride was used as the supporting electrolyte. Prior to electrolysis, a cyclic voltammogram of the AsA was obtained. Electrolysis potentials were selected as the oxidation peak potential of AsA obtained by cyclic voltammetry (Önal *et al* 1990).

2.3 *Detection of* $A^{\cdot-}$ *by ESR*

A Varian-E band ESR spectrometer was used to detect $A^{\cdot-}$. For this purpose an *in situ* electrolysis flat cell was fitted with two Pt-wires, one from the top as the

working electrode and the other from the bottom as the counter electrode. Silver wire, used as reference electrode was also inserted from the top (enal *et al* 1990).

2.4 *Exposure of viral DNA to* $A^{\cdot-}$

λ-DNA (1 μg) sample was incubated with freshly generated $A^{\cdot-}$ solution (generated from 15 mM ascorbate, pH 3-5) at 37°C for 10 min in a final volume of 10μl. The stability of $A^{\cdot-}$ during this incubation after the scission of electrolysis is demonstrated by ESR measurement. The reaction mixtures were applied to slab gel electrophoresis after the addition of 100 mM EDTA, 35% (w/v) sucrose and 0·025% (w/v) bromophenol blue as the tracking dye in 5 × TBE buffer (loading buffer). The reaction is terminated by the addition of these components. Control experiments were performed simultaneously by using the same reaction medium not subjected to electrolysis. Another control included the electrolyzed reaction medium without ascorbate.

2.5 *Exposure of λ-DNA to ascorbate/Cu (II) mixture*

λ-DNA (1 μg) was added to freshly prepared 1 mM ascorbic acid solution containing $CuCl_2$ (final concentrations 5, 10, and 30 μ,M) in deionized water in a final volume of 10 μl The mixture was incubated at 37°C for 10 min. The reaction was stopped by the addition of 4 μl loading buffer and electrophoresed in 2% (w/v) agarose gel (Chiou *et al* 1985).

2.6 *Addition of reductants, radical scavengers, metal chelator and proteins on λ-DNA scission reaction*

Reductants [glutathione (GSH), cysteine (Cys) and dithiothreitol (DTT)], radical scavengers [tryptophan (Trp), thiourea, mannitol and tert-butyl alcohol], metal chelator (EDTA) were added to the reaction mixtures at the final concentrations of 2 mM. The proteins [catalase, denatured catalase, glutathione reductase (GSSGR) and bovine serum albumin (BSA)] were added to the reaction mixtures at the final concentrations of 5 μM. The denatured catalase was prepared by autoclaving for 10 min at10 lb/in^2. The reaction mixtures were incubated at 37°C for 10 min.

2.7 *DNA slab gel electrophoresis*

DNA gel electrophoresis was carried out on horizontal submarine slab gel apparatus according to Chiou (1983) with minor modifications. The reaction mixtures were electrophoresed in 2% (w/v) agarose gel at a constant current of 100 mA for about 3 h in 1 × TBE running buffer. After electrophoresis, the gel was stained for 10 min in 0·5 μg/ml ethidium bromide and examined with 254 nm UV light.

2.8 *Incubation of plasmid DNA witm* $A^{\cdot-}$

Plasmid DNA (7·2 kb pMK3) (Sullivan *et al* 1984) was isolated from overnight *E. coli* culture (Chowdhury 1991). Plasmid DNA (0·5 μg) was incubated with the radical solution at 37°C for 15 min.

3. Results

The electron spin resonance spectra of the electrochemically generated ascorbyl radical gives the characteristic doublet of the radical as shown in figure 1. The radical is generated by the electrolysis of ascorbic acid in deionized water under N_2. This radical is present in its anionic form ($A^{\cdot-}$) in the 0-13 pH range (Bielski 1982). The radical in the medium increases as the amount of ascorbate is raised. In our previous report, the presence of the radical during electrolysis under nitrogen and its presence during the subsequent experiments has been confirmed by the ESR measurements (Önal *et al* 1990).

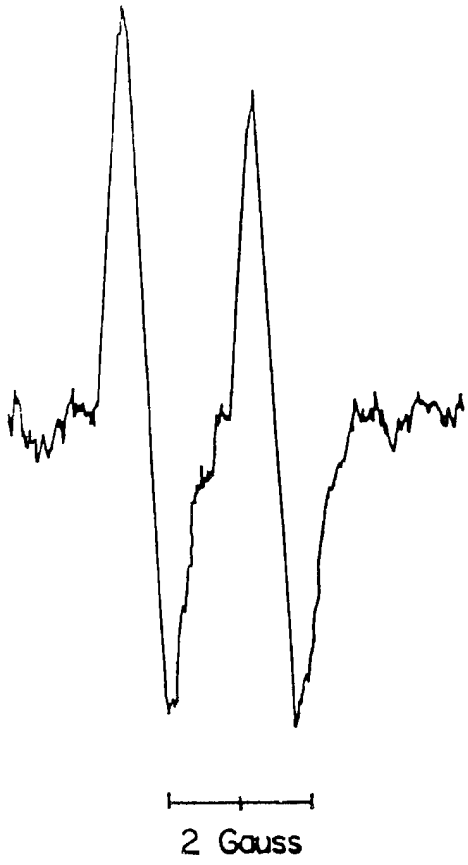


Figure 1. ESR spectrum of $A^{\cdot-}$ obtained during the cictrolysis of 15 mM AsA at +0·4 V vs Ag/Ag^{+}.

The gel banding patterns of X-DNA incubated with freshly generated radical solution at 37°C for 10 min are shown in figure 2. Lane 1 shows the control λ-DNA. The other control lanes include the λ-DNA incubated with the electrolyzed reaction medium without ascorbate (lanes 2, 6) and nonelectrolyzed reaction medium with ascorbate (lanes 3, 7). No detectable DNA modification occurred in these controls. λ-DNA was treated with the radical generated by the electrolysis of ascorbate ranging

from 5–15 mM initial concentrations. The threshold concentrations of ascorbate to generate $\dot{A}^-$, to produce damage to viral DNA was 15 mM ascorbate when NaCl was used as the supporting electrolyte (lane 5). When NaClO4 was used instead of NaCl, the threshold concentration of ascorbate to produce DNA damage was 7·5 mM ascorbate and continued with 10 mM ascorbate (lanes 9–10).

ln order to check the cleavage reaction, we used pMK3 plasmid DNA (super-coiled) and incubated the plasmid DNA with the standard radical solution at 37°C for 15 min. Figure 3 shows the cleavage pattern for this reaction. The supercoiled plasmid DNA is converted to the linear form and degradated.

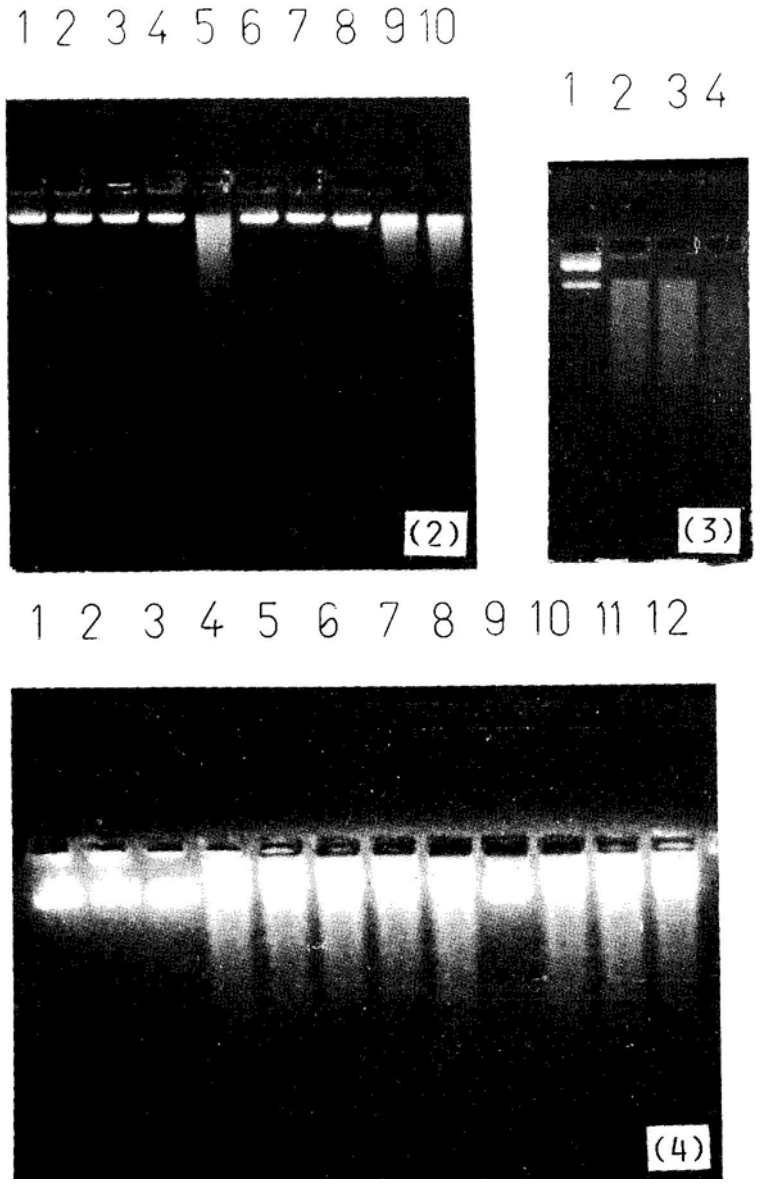
The effects of some antioxidants, radical scavengers and metal chelators on the radical cleavage reaction are presented in figure 4. 1M EDTA is the only effective reagent examined in quenching the cleavage reaction (figure 4, lane 9). Below this concentration EDTA was found ineffective of the radical.

Three kinds of proteins were evaluated for DNA cleavage protection assay. One is an antioxidant enzyme glutathione reductase. The other is a heme containing protein, catalase which is also an antioxidant enzyme. The third is serum albumin a widely used protein in radical cleavage reactions. lt is well known that ascorbate inhibits catalase. No protection against the DNA-scission was observed in presence of denatured catalase and catalase in the ascorbyl radical cleavage medium (figure 5, lanes 6, 7). GSSGR and serum albumin in final concentrations of 5 μ.M completely abolished the DNA-scission activity of the radical (figure 5, lanes 4, 5).

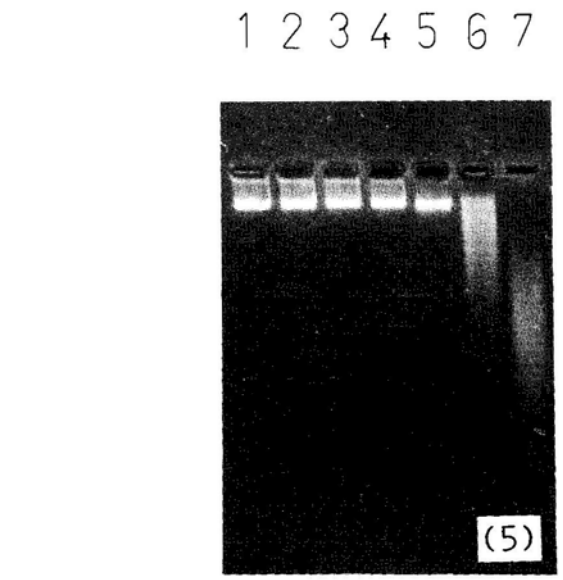
We have compared the DNA-scission activities of ascorbyl radical and ascorbate in the presence of Cu(II). Figure 6 shows the effect of ascorbate/Cu(II) on λ-DNA together with the effects of proteins on the system. Ascorbic acid cleaves λDNA extensively as the concentration of Cu(II) raises (figure 6a, lanes 2-4). ln contrast to the action of ascorbyl radical cleavage of 2-DNA, both catalase and denatured catalase abolished the DNA scission activity of ascorbate plus copper (figure 6b, lanes 2, 3). At comparable concentrations (5 μM each) GSSGR and BSA were effective in quenching this cleavage reaction (figure 6b, lanes 4, 5). The cleavage reaction of ascorbate/Cu(II) is given in figure 6b, lane 6.

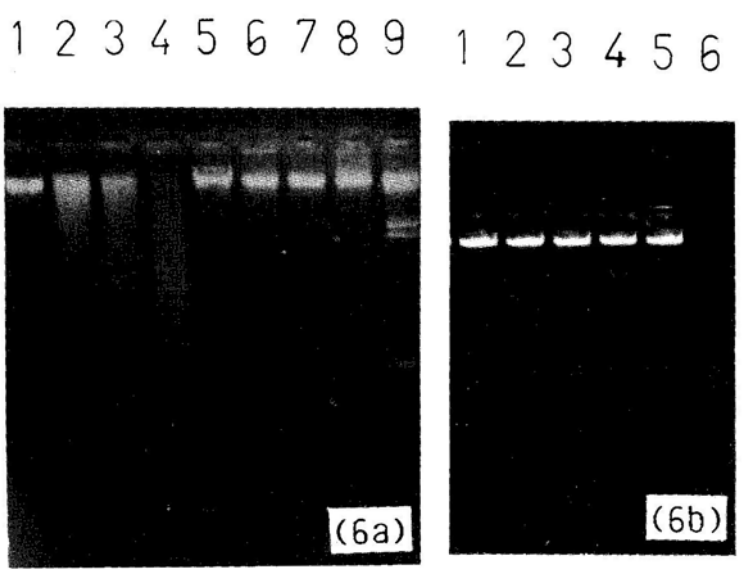
4. Discussion

lt is well known that ascorbic acid is beneficial and harmful depending on the sensitive balance of its concentration. Although ascorbyl radical is reported to be harmless and non toxic the concentration of the radical in the cells is critical (Bielski 1981; Halliwell *et al* 1990). Ascorbyl radical is presumably worth removing *in vivo* like hydroxyl and superoxyl radicals since the enzyme that scavenges this radical is ubiquitious in plant and animal cells. The results of the present study show that ascorbyl radical generated in a simple chemical environment devoid of reactive primary radicals can induce viral DNA damage. The diffuse smeared patterns of λ-DNA treated with ascorbyl radical (figure 2) indicate the random nature of cleavage reaction as observed with DNase treated DNAs. ln this DNA cleaving solution ascorbyl radical itself may not be the species that cleaves. lt has been reported that ascorbyl radical reacts with 02 to produce superoxide (O_2^-) with a rate constant of the order of 10^2 s^{-1} s^{-1} (Frohlinde 1983). At saturated reactant concentrations the rate of ascorbyl radical reaction with oxygen is approximately 50% of the rate of disproportionation of the radical. Thus it is apparent that the only significant uncatalyzed reaction of dioxygen in the incubation medium is with ascorbyl radical,



Figures 2–4. (**2**) λ.-DNA damage induced by A . Lane l: 1 g λ-DNA (control); lane 2: Electrolysis medium containing NaCI as supporting electrolyte; subsequent additions were; lane 3: 15 ma AsA before electrolysis; lane 4: 7·5 mM AsA after 30 min electrolysis; lane 5: 15 ma AsA after 30 min electrolysis. Lane 6: Electrolysis medium containing $NaClO_4$ as supporting electrolyte: subsequent additions were; lane 7: 15 ma AsA before electrolysis; lane 8: 5 mM AsA after 30 min electrolysis; lane 9: 7·5 ma AsA after 30 min electrolysis; lane 10: 15 ma AsA after 30 min electrolysis. (**3**) lncubation of pMK3 plasmid DNA with A⁻· Lane 1: 0·5 mg plasmid DNA (control); subsequent additions were; lane 2: radical solution from 5 ma AsA; lane 3: from 10 mM AsA and lane 4: from 15 mM AsA. The incubation was at 37°C for 15 mM in final concentration of 10μ1. **(4)** The effect of antioxidants, radical scavengers and metal chelator on λ.-DNA damage by A . Lane l: 1 .μg λ-DNA; subsequent additions were; lane 2: electrolysis medium containing NaCI as supporting electrolyte without AsA; lane 3: 15 ma A5A before electrolysis; lane 4: 15 ma AsA after 30 min electrolysis; lanes 5–12: 15 mM AsA after 30 min electrolysis and GSH (2 ma), Cys (2 ma), Trp (2 ma), DOT (2 mM), EDTA (2 ma), thiourea (2 ma), mannitol (2 mM) and tert-butyl alcohol (2 ma), respectively.





Figures 5 and 6. (**5**) Ohe effect of proteins on λ-DNA cleavage by A . Lane l: 1μg λ-DNA; subsequent additions were; lane 2: electrolysis medium containing NaCI as supporting electrolyte without AsA; lane 3: 15 ma AsA before electrolysis; lanes 4–7 contain addition of GSSGR (5 μM), BSA (5 μM), denatured catalase (5 μM)and catalase (5 pM) after 30 minutes eletrolysis of 15 ma AsA, respectively. (**6a**))λ-DNA damage of ascorbic acid/Cu(II) system. Lane 1: 1 mg λ-DNA; subsequent additions were; lane 2: 1 ma ascorbic acid, 5 μM $CuCl_2$; lane 3: 1 mM ascorbic acid, 10 μM $CuCl_2$; lane 4: 1 mM ascrobic acid, 30 μM $CuCl_2$: lane 5: 1 mM AsA; lane 6: 5 μM $CuCl_2$; lane 7: 10 μM $CuCl_2$; lane 8: 30 μM $CuCl_2$ and lane 9: *Hind*III digested λ-DNA was used as molecular weight marker. (**6b**) Ohe effects of proteins on λ-DNA damage induced by AsA/Cu(II). Lane 1: 1 μg λ–DNA (control); subsequent additions were; lane 2–5: 1 μM AsA/ 30 μM $CuCl_2$ and additions of 7·5 μg/10 μl of GSSGR, BSA, denatured catalase and catalase (in final concentrations of 5 μM) respectively; lane 6: 1 mM AsA and 30 AM $CuCl_2$.

generating oxygen radical reactions. Another radical generated in this system is probably the hydroperoxyl radical ($HO_{2'}$), which is produced by the protonation of superoxide. The pKa of $HO_{2'}$ is 4.7– 4.8 (Fucs *et al* 1990). ln our radical generating system the pH is 3·5. There is no clear evidence yet that $HO_{2'}$ plays a cytotoxic role in biological systems but is less polar than $O_2^{\cdot-}$ and somewhat more reactive.

The absence of metal ions in this reaction medium was evidenced by the following facts. Chiou *et al* (1985) reported that 1-2 μM EDTA would be enough to remove these trace metal ions. Here, we have 100 μM EDTA in the incubation medium which is prepared in deionized water. Also of concern is the possibility of DNA contamination by trace metals. The' use of ultrapure λ-DNA and the intact DNA band in the control experiment performed by incubating λ-DNA with the same reaction medium not subjected to electrolysis exclude this possibility. The nature of the salt used as the supporting electrolyte in the production of $A^{\cdot-}$ influenced the effective DNA cleavage concentration of ascorbate. Perchlorate potentiates the effects of ascorbate as compared to chloride. This difference may arise from the fact that sodium chloride was ultrapure and perchlorate was reagent grade. Addition of electron donors like GSH, Cys, DTT and .OH radical scavengers such as thiourea, Trp, tert- butyl alcohol and mannitol at comparable concentrations did not have any discernable effect on the ascorbyl radical cleavage of λ-DNA. However, addition of 2 mM EDTA quenched the scission reaction (figure 4, lane 9). The results presented in this report suggest that this is an ionic strength effect of EDTA rather than metal chelation. Similar effect of added salt (EDTA) on ascorbyl radical between pH 3·3-9·0 was observed in a previous report (Bielski 1982). Our results support that the added salt increased the rate of reaction between two radical anions so the radical reaction is quenched by the following scheme:

$$2A^{\cdot-} + H^+ \rightarrow AH^- + DHA$$

AH^-:Ascorbate, DHA : Dehydroascorbate.

The effect of inhibition of catalase appeared qualitatively distinct in the case of ascorbyl radical and ascorbate/Cu(II) cleavage of λ-DNA. Both active and denatured catalase were ineffective to inhibit the ascorbyl radical cleavage reaction of λ-DNA. lt is well known that ascorbate inhibits catalase (On 1967) and it has been reported that ascorbyl radical may be the main species that inhibit catalase (Davidson and Kettle 1986). Since catalase is a heme containing enzyme, it can also be argued that ascorbate in combination metal ions, in this case, iron from catalase possesses DNA-scission activity (Chiou 1984). The protective mechanism of the effect of GSSGR and BSA should be further investigated.

In the case of corbate/Cu(II) cleavage of λ-DNA, catalase, denatured catalase, glutathione reductase and serum albumin were effective in quenching the cleavage reaction (figure 6b, lanes 2-5). These proteins quench the cleavage reaction by binding the Cu(II) in the system. Ascorbate by itself does not cleave λ-DNA and at this concentration (1 mM) does not inhibit catalase. Catalase action seems to be not enzymatic since denatured catalase also effectively abolished the cleavage reaction. Moreover, the proteolytic activity of ascorbate/Cu(II) system may be involved in this reaction (Chiou 1983).

This study focusses on the action of ascorbyl radical on the degradation of viral DNA. Since ascorbyl radical is less hazardous and more stable than other free radicals, its reactivity may be considered in the development of free radical

generating antiviral drugs. Therefore it is of biochemical and chemical interest to study the interactions of ascorbyl radical with biomolecules.

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