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Release of Bases from Deoxyribonucleic Acid by Ascorbic Acid in the Presence of Cu^{2+}

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When deoxyribonucleic acid (DNA) was treated with ascorbic acid (AsA) in the presence of Cu^{2+} under aerobic conditions, significant releases of cytosine, thymine, guanine and adenine from the DNA molecule occurred. Addition of catalase or chemical scavengers of hydroxyl radical to the reaction mixture prevented the release of the bases. Monoesterified phosphate groups were found in the reaction mixture, indicating that the cleavage of DNA had occurred. The total amount of the bases released was almost equivalent to the amount of monoesterified phosphate groups formed by the reaction of DNA with AsA and Cu^{2+}. These results indicate that the release of the bases from DNA molecule was easily caused by activated oxygen, possibly by hydroxyl radicals and/or hydrogen peroxide, formed by the autooxidation of AsA in the presence of Cu^{2+}.

Keywords——deoxyribonucleic acid; hydroxyl radical; adenine; guanine, cytosine; thymine; ascorbic acid

The autooxidation of ascorbic acid (AsA) involves free radicals and is accelerated by Cu^{2+}.[1] The fragmentation of deoxyribonucleic acid (DNA) by the free radicals formed by autooxidation of AsA has been extensively studied.[2-5] Recently, sequence-specific cleavage of DNA by AsA and Cu^{2+} has been reported.[6] However, little is known about the release of free bases from DNA by AsA. We have reported that the destruction of nicotinamide adenine dinucleotide by AsA and Cu^{2+} occurred mainly by cleavage of N-glycoside linkages by free radicals produced during the autooxidation of AsA in the presence of Cu^{2+}.[7] The possibility arises that free bases may be released from the DNA molecule by the reaction with AsA and Cu^{2+}.

The present paper describes the DNA-base-releasing action of AsA in the presence of Cu^{2+}.

Experimental

Materials——Special grade L-AsA was obtained from Wako Pure Chemical Industries; DNA from calf thymus (type I), catalase from bovine liver and superoxide dismutase (SOD) from bovine blood were from Sigma Chemical Co. Acid phosphatase from sweet potato, which was able to catalyze the hydrolysis of both 3′- and 5′-adenylic acids, but not that of phosphodiesters, was purified according to the method of Uehara *et al.*[8] All other reagents used were of the highest purity available.

Reaction with AsA and Cu^{2+}——The reaction mixture contained the following components in 6 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.3): DNA $A_{260}=4.3$, AsA 25 mM and Cu^{2+} 0.5 mM. The incubation was carried out at 37 °C with shaking in air as the gas phase unless otherwise stated. The reaction was terminated by addition of 30 mM ethylenediaminetetraacetic acid (EDTA) at pH 6.0.

Assays for Bases, Monoesterified Phosphate Groups and Phosphorus——Bases released from DNA were determined by high-performance liquid chromatography (HPLC) under the conditions described in the figure captions. Total phosphorus was determined after digestion of the sample in H_2SO_4–$HClO_4$ mixture.[9] Orthophosphate (Pi) was determined by the method of Chen *et al.*[10] Monoesterified phosphate groups formed in

DNA were calculated from the amount of Pi released enzymatically by using acid phosphatase as follows. The reaction mixture containing DNA, AsA and Cu^{2+} was passed through a Sephadex G-25 column equilibrated with 0.1 M acetate buffer (pH 5.8) containing 10 mM EDTA. Purified acid phosphatase (10 μg/ml) was added to the DNA fraction thus obtained, and the mixture was incubated for 24 h at 25 °C. The release of Pi was time-dependent and an incubation time of approximately 16 h was required for release of the maximum amount of Pi from the AsA-treated DNA.

Results and Discussion

Release of Bases from DNA

Figure 1 shows a chromatographic pattern of the reaction mixture containing DNA, AsA and Cu^{2+}. Significant amounts of cytosine, thymine, guanine and adenine were detected in the reaction mixture. No significant production of nucleosides and mononucleotides was observed. Under the chromatographic conditions used, DNA-bases were well separated from each other and also from their nucleosides and mononucleotides, as shown in Fig. 1-B. Although cytidine, thymidine, guanosine and adenosine as standard compounds are not shown in the figure, the retention times were 23, 31, 36 and 43 min, respectively. The production of bases from DNA by the reaction with AsA and Cu^{2+} was time-dependent, as shown in Fig. 2. Adenine was found to be released more effectively than thymine, cytosine or guanine. After 30 min of incubation of DNA with AsA and Cu^{2+}, the total amount of bases released from DNA amounted to approximately 10% of bases in the initial DNA. Production of bases was also found to occur slightly with AsA without Cu^{2+}, but not with Cu^{2+} alone. Although free thymine, cytosine, adenine and guanine were destroyed to some extent by AsA and Cu^{2+} under the present experimental conditions, the destruction was almost completely prevented by the presence of DNA (not shown). Therefore, the further destruction of the bases released seems not to occur significantly through this reaction.

Effects of Oxygen and Radical Scavengers on the Production of Bases

Under anaerobic conditions (nitrogen gas bubbling), no significant production of the bases from DNA was observed, indicating that molecular oxygen was essential for the base-releasing action of AsA and Cu^{2+}. It is known that activated oxygen species, such as hydroxyl radicals, superoxide radicals and hydrogen peroxide, are formed in the autooxidation of AsA. Table I shows the effect of various scavengers of hydrogen peroxide, superoxide radicals and hydroxyl radicals on the production of the bases from DNA by treatment with AsA and Cu^{2+}. Hydroxyl radical scavengers,[11-13] such as thiourea, potassium iodide and thiocyanate,

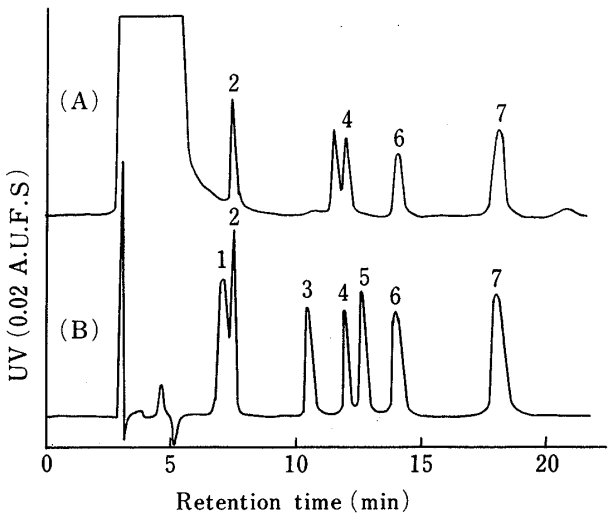

Fig. 1. High-Performance Liquid Chromatogram of the Reaction Mixture

(A) DNA (A_{260}=4.3) was incubated at 37 °C with AsA (25 mM) and Cu^{2+} (0.5 mM) in 6 ml of HEPES buffer (0.1 M, pH 7.3) with shaking. After incubation for 10 min, 50 μl of the reaction mixture was diluted 4 times with 40 mM EDTA and subjected to HPLC.

(B) Fifty microliters of solution containing approximately 0.06 nmol each of standard compounds.

HPLC was done with an ultraviolet absorbance monitor at 260 nm under the following conditions: column, stainless steel column (4.6×250 mm i.d.) packed with Cosmosil ($_5C_{18}$) resin; mobile phase, 0.17 M acetic acid containing 0.17 M NaCl and 0.5 mM 1-heptanesulfonate; flow rate, 0.8 ml per min; column temperature, 30 °C.

A.U.F.S., absorbance unit full scale.

Peaks: 1=2′-deoxycytidine 5′-monophosphate, thymidine 5′-monophosphate; 2=cytosine; 3=2′-deoxyguanosine 5′-monophosphate; 4=thymine; 5=2′-deoxyadenosine 5′-monophosphate; 6=guanine; 7=adenine.

TABLE I. Effect of Radical Scavengers on the Release of Bases from DNA by Ascorbic Acid in the Presence of Cu^{2+}

Scavenger added	Concentration (mM)	Relative base release (%)
None	—	100
Thiourea	1.0	11
	0.1	79
Sodium thiocyanate	10	2
	1.0	13
Potassium iodide	10	22
Sodium benzoate	10	82
Sodium formate	100	78
D-Mannitol	100	83
Ethanol	100	91
Catalase	50 μg/ml	18
	10 μg/ml	50
Superoxide dismutase	50 μg/ml	88
Bovine serum albumin	50 μg/ml	87

Reaction mixtures contained DNA (A_{260}=4.3), AsA (25 mM), Cu^{2+} (0.5 mM), and the specified amounts of various scavengers in 6 ml of HEPES buffer (0.1 M, pH 7.3). Incubation was carried out at 37 °C for 30 min with shaking.

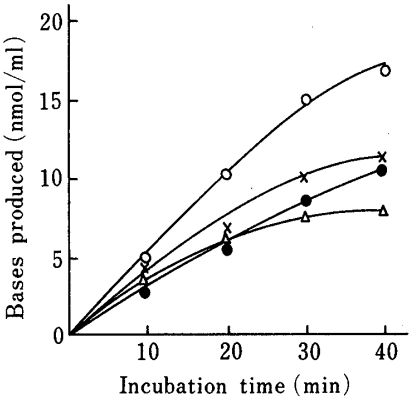

Fig. 2. Production of Cytosine, Thymine, Adenine and Guanine from DNA by Reaction with Ascorbic Acid and Cu^{2+}

DNA was incubated with AsA and Cu^{2+} under the conditions described in the legend to Fig. 1. Aliquots were withdrawn at the indicated times and assayed as described in the text.

●, cytosine; ×, thymine; ○, adenine; △, guanine.

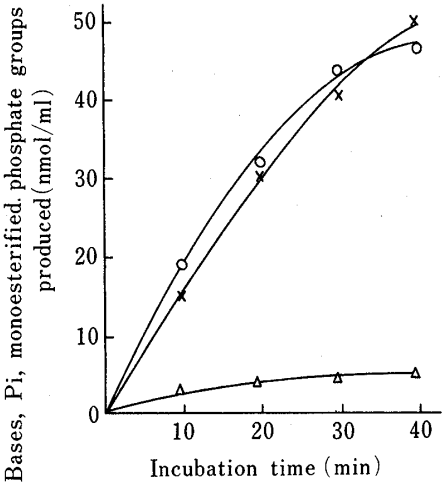

Fig. 3. Production of Bases, Pi and Monoesterified Phosphate Groups by the Reaction of DNA with Ascorbic Acid and Cu^{2+}

DNA was incubated with AsA and Cu^{2+} under the conditions described in the legend to Fig. 1. At the indicated times, the reaction mixtures were assayed as described in the text.

○, monoesterified phosphate groups; ×, bases (cytosine + thymine + adenine + guanine); △, Pi.

effectively prevented the release of the bases from DNA. Catalase was also found to be effective, but SOD was not. These results indicate that activated oxygen species formed during aerobic oxidation of AsA are responsible for the release of the bases from DNA by AsA and Cu^{2+}.

Production of Monoesterified Phosphate Groups

To determine the extent of cleavage of the DNA molecule by AsA and Cu^{2+}, the

formation of monoesterified phosphate groups was determined. The release of a significant amount of Pi from AsA-treated DNA, but not from intact DNA, by acid phosphatase was found. This finding indicates that significant cleavage of DNA is caused by the reaction with AsA and Cu^{2+} under the present experimental conditions. When Pi in the reaction mixture was measured, we also observed the production of Pi from DNA by the reaction with AsA and Cu^{2+}. However, the amount of Pi produced was less than 10% of that of monoesterified phosphate groups formed. The relationships among the total amount of the bases, the amount of monoesterified phosphate groups, and Pi produced during the course of the reaction of DNA with AsA and Cu^{2+} are summarized in Fig. 3. The total amount of the bases released was almost equivalent to the amount of monoesterified phosphate groups formed, indicating that the extent of base-release was similar to that of cleavage of DNA.

From the above results, we concluded that not only the cleavage of DNA but also the release of bases from DNA was caused by free radicals, probably by hydroxyl radicals and/or hydrogen peroxide, produced during the aerobic oxidation of AsA in the presence of Cu^{2+}.

Significant destruction of the bases in DNA in addition to the release of free bases from DNA by the reaction with AsA and Cu^{2+} was also found to occur (not shown). Therefore, it is not clear whether or not the AsA–Cu^{2+} system under the present experimental conditions creates single-strand breaks in DNA at the positions of the bases released, though it is known that the base release by hydroxyl radicals is followed by cleavage of phosphodiester bonds *via* cyclization or β-elimination.[14]

Recently, superoxide radicals generated from KO_2 have been found to release specifically three bases (uracil, cytosine and adenine, but not guanine) from ribonucleic acid.[15] However, adenine was most susceptible to release from DNA by hydroxyl radicals generated in the present AsA–Cu^{2+} system, though cytosine, thymine and guanine were also released (Fig. 2). Further studies on the specificity of base-releasing action of superoxide radicals and hydroxyl radicals seem desirable.

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