Note

NAD⁺-Braking Action of Ascorbic Acid in the Presence of Cu²⁺Sadaki FUJIMOTO, Susumu ISHIMITSU
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In the autooxidation of ascorbic acid (AsA), the reaction involves free radicals and accelerated by cupric ions.¹⁾ Free radicals formed by autooxidation are involved in a number of biologically important reactions such as destruction of methionine²⁾ and phenylalanine,³⁾ degradation of hyaluronic acid,⁴⁾ release of peptides and amino acids from proteins,⁵⁾ inactivation of enzymes,^{6,7)} and cleavage of DNA.^{8,9)}

We have found that NAD⁺ is also destroyed with loss of its coenzyme activity by AsA in the presence of Cu²⁺, and that free radicals formed by the autooxidation of AsA are responsible.

NAD⁺ and reaction products were measured using a Hitachi 675-TG high performance liquid chromatograph. A stainless steel column, 4.6×250 mm, packed with Hitachi Cosmosil (5C₁₈) resin, was attached to the apparatus. The column temperature was kept constant at 12°C. The mobile phase was 0.17 M acetic acid, 0.17 M NaCl and 0.5 mM 1-heptanesulfonate, and the flow rate was 0.8 ml per min.

When NAD⁺ (1.0 mM) was incubated in acetate buffer (0.2 M, pH 5.5) containing AsA (100 mM) and Cu²⁺ (1.0 mM) at 37°C with shaking, destruction of NAD⁺ was observed, as was analyzed by high performance liquid chromatograph (HPLC). This destruction was time dependent. After 30 min of incubation, the concentration of NAD⁺ was reduced to approximately 45% of the initial value. Only a 6% reduction, however, was observed with AsA without Cu²⁺, and no significant reduction was observed with Cu²⁺ alone. When the remaining NAD⁺ was measured by its coenzyme activity by the method of Ciotti and Kaplan¹⁰⁾ with yeast alcohol dehydrogenase through the reaction of NAD⁺ with AsA and Cu²⁺, the destruction curve of NAD⁺ was similar to that obtained by the analysis with HPLC. The destruction of NAD⁺ by the reaction with AsA and Cu²⁺ was pH dependent, and the rate of destruction was much faster at lower pH: at pH 6, 7, and 8, the rates were approximately 96, 58, and 49%, respectively, of that at pH 5. Considering the stability of NAD⁺, we did all the experiments at pH 5.5.

Under anaerobic conditions with nitrogen gas bubbling

through the reaction mixture, no significant destruction of NAD⁺ was observed, indicating that molecular oxygen was essential for the destruction. It is known that activated oxygen, such as hydrogen peroxide, hydroxyl radicals, and superoxide radical species, are formed in the autooxidation of AsA. Table I shows the effects of various scavengers for hydrogen peroxide, superoxide radicals, and hydroxyl radicals on the destruction of NAD⁺ by the reaction with AsA and Cu²⁺. Hydroxyl radical scavengers^{11~13)} such as thiourea, potassium iodide, thiocyanate, and cysteine, effectively prevented the destruction of NAD⁺. Although cysteine is not a specific scavenger for hydroxyl radicals, it does act as one.¹⁴⁾ Our results indicate that free radicals formed during aerobic oxidation of AsA are responsible for the destruction of NAD⁺.

To identify the products formed by the reaction of NAD⁺ with AsA in the presence of Cu²⁺, the reaction mixture was chromatographed as described above. A typical chromatographic pattern of the reaction mixture is shown in Fig. 1. Significant quantities of adenine and nicotinamide were detected in the reaction mixture. In addition, trace amounts of AMP, adenosine, and nicotinamide ribonucleoside were also detected. When Pi in the reaction mixture was measured by the method of Chen *et al.*,¹⁵⁾ we found production of Pi from NAD⁺ by the reaction with AsA and Cu²⁺. The relationships

TABLE I. EFFECTS OF RADICAL SCAVENGERS
ON THE DESTRUCTION OF NAD⁺ BY ASCORBIC
ACID IN THE PRESENCE OF Cu²⁺

Reaction mixtures contained 1.0 mM NAD⁺, 100 mM ascorbic acid, 1.0 mM Cu²⁺, and the specified amount of various scavengers in 10 ml of 0.2 M acetate buffer (pH 5.5). Incubation was at 37°C for 30 min with shaking.

Scavenger added	Concentration (mM)	NAD ⁺ remaining (%)
None	—	45
Thiourea	10	100
	1.0	91
	0.1	52
Cysteine	10	100
	1.0	95
Potassium iodide	10	96
	1.0	53
Sodium thiocyanate	10	99
	1.0	72
Sodium formate	50	61
D-Mannitol	50	56
Dimethyl sulfoxide	50	61
Tiron	50	64
Catalase	100 µg/ml	56
Superoxide dismutase	100 µg/ml	47
Bovine serum albumin	100 µg/ml	48

FIG. 1. High Performance Liquid Chromatogram of the Reaction Mixture.

(A) After reaction of NAD^+ with AsA and Cu^{2+} for 30 min under the conditions described in the legend of Fig. 2, 50 μl of the reaction mixture diluted 10 times with 10 mM ethylenediaminetetraacetic acid was used for HPLC.

(B) Fifty μl of solution containing approximately 0.6 nmol each of standard compounds was used for HPLC. HPLC was done with an ultraviolet absorbance monitor at 260 nm under the conditions described in the text. Peaks: 1 = nicotinamide mononucleotide; 2 = nicotinic acid; 3 = nicotinamide; 4 = AMP; 5 = nicotinamide ribonucleoside; 6 = adenine; 7 = NAD^+ ; 8 = adenosine.

FIG. 2. Destruction of NAD^+ and Production of Adenine, Nicotinamide, and Pi by the Reaction of NAD^+ with Ascorbic Acid and Cu^{2+} .

NAD^+ (1.0 mM) was incubated at 37°C with AsA (100 mM) and Cu^{2+} (1.0 mM) in 10 ml of 0.2 M acetate buffer (pH 5.5) with shaking. Aliquots were withdrawn at the indicated times and assayed as described in the text.

●, NAD^+ ; △, nicotinamide; ○, adenine; ×, Pi.

between the destruction of NAD^+ and the production of adenine, nicotinamide, and Pi during the course of the reaction are shown in Fig. 2. It was found that both adenine and nicotinamide produced after 30 min of reaction corresponded to about 20% of the decomposed NAD^+ , and the Pi was about 2%.

The above results suggest that the destruction of NAD^+ by AsA in the presence of Cu^{2+} happens mainly by cleavage of *N*-glycoside linkages in NAD^+ molecules by free radicals, probably by hydroxyl radicals, produced during the aerobic oxidation of AsA in the presence of Cu^{2+} , and that the rate of cleavage of *N*-glycoside linkages is much faster than that of phosphoric ester linkages including ribose-phosphate by free radicals.

Although DNA scission by AsA in the presence of Cu^{2+} has been extensively studied,^{8,9} there is no description of the DNA base-releasing activity of AsA. Our results here suggest the possibility that bases are released from DNA molecules easily before the scission of DNA by the reaction with AsA and Cu^{2+} . Exploration of this possibility is now in progress.

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