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A Novel Reaction of Cyanate with Dehydroascorbate

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It is generally known that carbamyl phosphate, a precursor of nucleotides, spontaneously degrades to cyanate in organisms. In the present study, we revealed the presence of an alkaline-labile cyanate-derivative in *Solanum tuberosum* L., which released cyanate in alkaline solution, and elucidated that it was a reaction product of cyanate with dehydroascorbate. The reaction mechanism was revealed as follows: dehydroascorbate existing as a hemiacetal form in neutral solution reacts with cyanate to produce ultimately a spiro-compound consisted of hemiacetal and oxazolidone rings. The structure of the end product was identified by spectroscopic and X-ray analyses using a synthetic compound. This fact demonstrates that this reaction is a novel metabolic pathway of endogenous cyanate in organisms.

Key words cyanate; dehydroascorbate; carbamyl phosphate; carbamylated dehydroascorbate derivative; plant

Cyanate is a biological substance produced by the spontaneous degradation of carbamyl phosphate[1–6] as well as urea.[7–9] Especially, the fact that carbamyl phosphate is synthesized as a precursor of nucleotides in almost all organisms demonstrates that the production of cyanate commonly occurs in them. Regarding the enzymatic metabolism of cyanate, an expression of cyanate hydrolase termed cyanase has been reported only in bacteria,[10–13] but the metabolic fate of an endogenous cyanate in other organisms, including mammals and plants, has not been clear. In the case of mammals, cyanate produced from urea is an uremic toxin, so it is thought to be involved in several symptoms of uremia.[14] It has been reported that the long-term administration of cyanate as a drug therapy for sickle-cell disease was accompanied by side-effects such as cataract.[15,16] However, the real mechanisms of these effects are not clear.

One of characteristics of cyanate is to be electrophilic, and thus it is known as a carbamylating agent.[17–26] Due to its chemical reactivity, cyanate was studied as a toxic substance. Indeed, sodium cyanate is used as a herbicide.[27,28] It quickly blights plants just after attaching to leaves and stems, but the mechanism of this phenomenon is unclear. So far, two *in vivo* reactions of endogenous cyanate with biological substances, carbamylation of amino groups and that of sulfhydryl groups, have been revealed by quantitative techniques for end products.[20–23] During these studies, we found a novel substance, an alkaline-labile cyanate-derivative (ALCD) with a mass of 100—1000 Da, present in plants. This finding suggested a novel metabolic pathway of cyanate in organisms. In the present study, we elucidated the characteristics of the reaction product and its production mechanism.

Experimental

Materials Ascorbic acid and sodium cyanate were purchased from Wako Pure Chemicals (Japan). Dehydroascorbic acid was purchased from Aldrich Chem. Co. (U.S.A.). All other chemicals were of reagent grade. TSK gel SAX was obtained from Tosoh Co. (Japan). A standard solution of cyanate was prepared from sodium cyanate which had been recrystallized from ethanol.

Apparatus Melting points were measured on a Yanagimoto micromelting point hot-stage apparatus. ^{1}H- and ^{13}C-NMR spectra were obtained by a JNM-A500 spectrometer from Japan Electron Optical Laboratory (JEOL) equipped with a DEC VAX station 3200 computer system, a process controller and an array processor. Chemical shifts are shown on the δ (ppm) scale with tetramethylsilane (TMS) as an internal reference.

Preparation of ALCD Tubers of *Solanum tuberosum* L. were grated and then centrifuged at 3000 × *g* for 30 min. The supernatant (600 ml) was mixed with 150 ml of 10 mM NaOCN, and the mixture was incubated at 37 °C for 1 h. After the solution was concentrated *in vacuo*, it was passed through an Amberlite CG-120 column (H$^+$-form, 2.2 cm i.d. × 20 cm). The eluate was then applied to an Amberlite CG-400 column (Cl$^-$-form, 2.2 cm i.d. × 20 cm). The column was washed well with water, and ALCD was eluted by 0.2 M NaCl. Fractions containing ALCD were pooled and then applied to the gel-permeation HPLC using Asahipak GS-320 and GS-520 (Asahi Chemical Industry Co., Japan), successively. The chromatographic conditions are as follows: column, Asahipak GS-320 and GS-520; eluent, H_2O (1.2 ml/min); detection, 204 nm. The cyanate derivative fraction was then applied to the anion-exchange HPLC. The chromatographic conditions are as follows: column, TSK gel SAX (4 mm i.d. × 150 mm); eluent, 25 mM NaCl (1.2 ml/min); detection, 204 nm. In order to get rid of salt, the ALCD fraction was passed through a Dowex 50W-X8 (H$^+$-form, 2.2 cm i.d. × 10 cm), and then the eluate was dried *in vacuo*. The residue was submitted to structural analyses.

Syntheses of Carbamylated Dehydroascorbate Derivative (CDA) Dehydroascorbate was prepared from ascorbic acid by oxidation using cupric ion. Ascorbic acid powder (52 g) was added to 2 l of 0.15 M copper(II) acetate solution, and then the reaction solution was stirred vigorously at room temperature. After standing for 10 min, 1 l of 0.75 M sodium cyanate solution was added dropwise to the solution, and the reaction mixture stood overnight with stirring. The reaction mixture was filtered with filter paper (Advantec Toyo, 51A) three times, and the filtrate was passed through Dowex 1-X8 (Cl$^-$-form, 50—100 mesh, 5 cm i.d. × 10 cm). The eluate was concentrated to 200 ml by evaporation, and the solution was added dropwise to 800 ml of 99% ethanol. The precipitation of CDA was obtained by filtration. The precipitation was dissolved in water, and the volume was adjusted to 200 ml. CDA in the solution was recrystallized by mixing it with 800 ml of 99% ethanol. The recrystallization of CDA was achieved totally five times. The yield of CDA was more than 70%. CDA has the molecular formula $C_7H_8NaNO_8$, and this crystal contained two molecules of water of crystallization per one molecule of CDA. The result of the elemental analysis was as follows: calculated for $C_7H_8NaNO_8 \cdot 2H_2O$: C, 28.70; H, 4.09; N, 4.78; Na, 7.85; O, 54.61. Found: C, 28.70; H, 3.89; N, 4.55; Na, 7.70; other elements, 55.16.

Determination of Cyanate Cyanate was measured sensitively and specifically by the post-column HPLC established by us.[29] The chromatographic conditions were as follows: column, TSK gel SAX (4 mm i.d. × 50 mm); eluent, 0.3 M NaCl. The detection was based on the indophenol reaction for ammonia which was released from cyanate.

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Determination of ALCD The ALCD was determined as follows: the tubers, stems, and leaves of *Solanum tuberosum* L. were grated or brayed and then centrifuged at 3000 × *g* for 30 min. The supernatant was mixed with an equal volume of 1.0 M NaOH and then the mixture was ultrafiltered with Micropartition system MPS-1 (Amicon Co. U.S.A.). The filtrate was submitted to HPLC for cyanate quantitation.

Determination of CDA CDA was determined by HPLC equipped with a UV detector. The chromatographic conditions were as follows: column, TSK gel SAX (4 mm i.d. × 150 mm); eluent, 25 mM NaCl (1.2 ml/min); detection, 204 nm. CDA was eluted at 12—13 min. The determination of CDA was not interfered with ascorbate, dehydroascorbate or 2,3-diketo-gulonate.

Results and Discussion

Characteristics of ALCD in *Solanum tuberosum* L. When the tubers, leaves and stems of *Solanum tuberosum* L. were treated with an alkaline solution (finally 0.5 M NaOH), the release of a small amount of cyanate was observed. This finding indicates the presence of ALCD in each. The analytical results of ALCD and free cyanate in them are shown in Table 1. The gel-permeation chromatographic study using Asahipak GS-320 indicated that the molecular mass of ALCD was 100—1000 Da. To identify ALCD, it was submitted to the analytical methods for cyanate derivatives, including carbamyl phosphate, *S*-carbamyl compounds, *N*-carbamyl compounds and cyanate–metal complexes, and thus it was determined to be none of them (the results are not shown). From these results, ALCD was observed to be a novel cyanate derivative, and is thought to be a reaction product of endogenous cyanate with a biological substance in plants. To address this issue, we spiked the grated tubers with cyanate and examined the disappearance of cyanate. As shown in Fig. 1, the half-life of cyanate is approximately 5 min, suggesting that if endogenous cyanate is produced, it is quickly transformed to ALCD by a reaction with a certain substance in *Solanum tuberosum* L.

Table 1. ALCD in *Solanum tuberosum* L.

	Stems and leaves	Tubers
ALCD	2.9 ± 0.3 nmol/g[a]	3.8 ± 0.5 nmol/g[a]
Cyanate	N.D.	N.D.

[a] Wet weight. N.D., not detected.

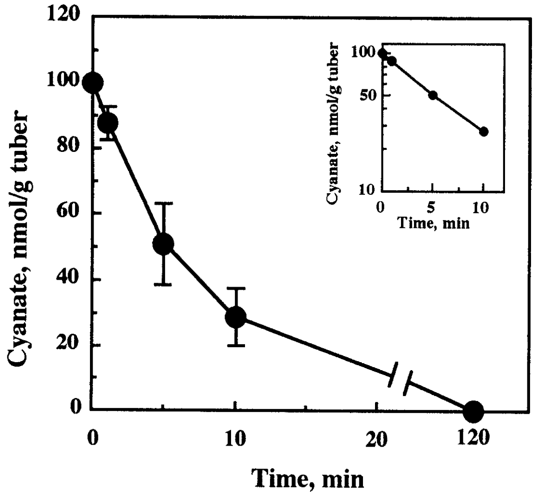

Fig. 1. Time Course of Cyanate in Grated Tubers of *Solaneum tuberosum* L.

The grated tubers (2.0 g) was spiked with 20 μl of 10 mM sodium cyanate and the mixture was incubated at 20 °C. The mixture was made up to 5 ml with 0.1 M phosphate (pH 7.4). After ultrafiltration with Micropartition system MPS-1 (Amicon Co., U.S.A.) at 4 °C, the filtrate was submitted to HPLC.

Preparation and Isolation of ALCD A large quantity of ALCD was isolated from the juice of tubers of *Solanum tuberosum* L., which was spiked with cyanate and incubated at 37 °C for 1 h, through successive treatments: Amberlite CG-120, Amberlite CG-400, Asahipak GS-320, Asahipak GS-520, and TSK gel SAX. Through these treatments, ALCD was isolated as a single peak on the anion-exchange HPLC (detected at 204 nm). The chromatogram is shown in Fig. 2. To get rid of salt, the isolated ALCD solution was passed through Dowex 50W-X8 (H^+-form, 2.2 cm i.d. × 10 cm), and the eluate was dried *in vacuo*. ALCD (H^+-form) was dissolved in D_2O and the solution was submitted to NMR spectroscopy. In addition, after substituting D_2O for dimethyl sulfoxide (DMSO)-d_6, the solution was submitted to NMR spectroscopy. The ^{1}H-NMR spectra of the cyanate derivative are shown in Fig. 3. These show that ALCD is a carbohydrate having four hydrogen atoms unsubstitutable for deuterium. From these results, we proposed ascorbate or dehydroascorbate as a candidate for it.

Reaction of Cyanate with Dehydroascorbate When cyanate was incubated with ascorbate and dehydroascorbate in neutral solutions at 37 °C, the disappearance of cyanate was observed in dehydroascorbate solution but not in ascorbate solution (Fig. 4), suggesting that cyanate is reactive with dehydroascorbate. The reaction mixtures were submitted to separation analysis by anion-exchange HPLC. Chromatograms are shown in Fig. 5. The reaction product was eluted at the same retention time as ALCD (Fig. 2).

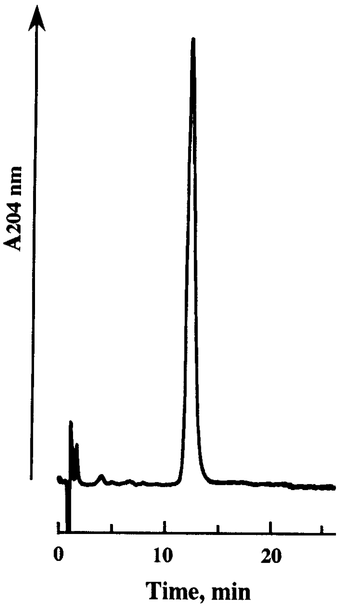

Fig. 2. Chromatogram of ALCD Isolated from the Cyanate-Treated Juice of Tubers of *Solanum tuberosum* L.

The freshly prepared juice of tubers of *Solanum tuberosum* L. (600 ml) was mixed with 150 ml of 10 mM NaOCN, and the mixture was incubated at 37 °C for 1 h. The resultant cyanate derivative was isolated by the chromatographic techniques described in the experimental section. The chromatographic conditions are as follows: column, TSK gel SAX (4 mm i.d. × 150 mm); eluent, 25 mM NaCl (1.2 ml/min); detection, 204 nm.

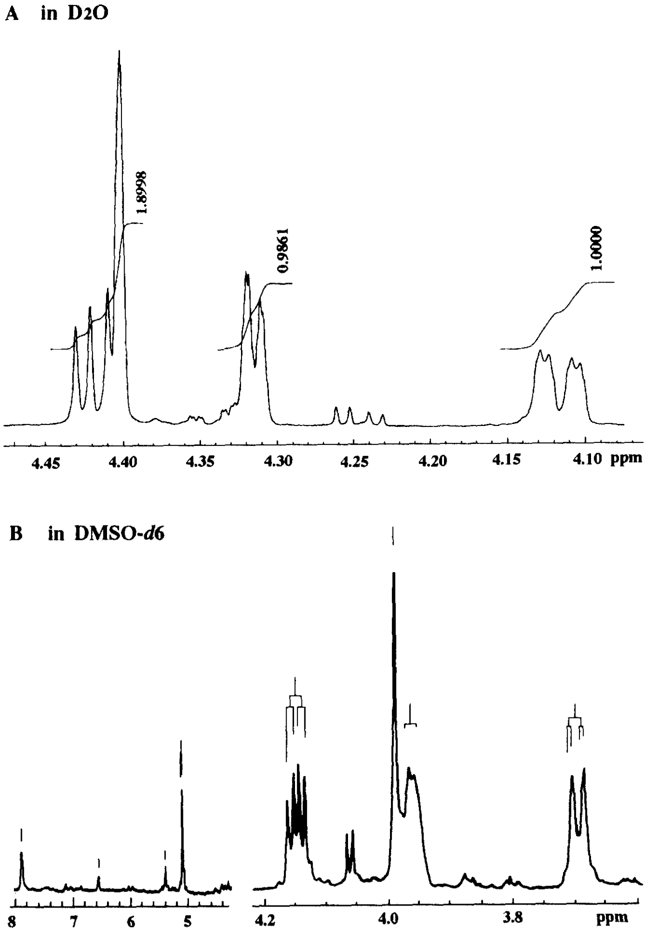

Fig. 3. 500 MHz ^{1}H-NMR Spectra of the ALCD

Structural Studies of CDA Ascorbate was oxidized by molecular oxygen in the presence of a cupric ion, and the produced dehydroascorbate reacted with cyanate under neutral conditions of pH 7.0—7.5. The reaction product was eluted at the same retention time on the anion-exchange HPLC as ALCD, and was degraded to cyanate in an alkaline solution (0.5 M NaOH) similarly to ALCD. The product termed CDA was crystallized in 80%(v/v) ethanol. Synthetic CDA was obtained as colorless cubes, mp 132—135 °C. The negative FAB-MS spectrum showed a negative molecular ion peak $[M-Na]^-$ at m/z 234. Together with the elemental analysis data, CDA has the molecular formula $C_7H_8NNaO_8$, indicating that dehydroascorbate reacts with one molecule of cyanate. The NMR spectra (as shown in Fig. 6) showed the presence of two carbonyl carbons, two acetal functions, and three hydroxylated carbons. The ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum revealed a C_4–C_5–C_6 moiety as existed in the starting material. All the protons and carbons could be assigned using heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra. The long-range connectivity between H-6 and the C-3 acetal carbon indicated the function of a cyclic acetal function between the 6-OH and C_3 carbonyl group in dehydroascorbate. The NH proton showed connectivities between C_3, C_2 and the amide carbon, indicating a spiro-acetal structure. It was shown that the synthetic CDA was identical to ALCD by comparison of

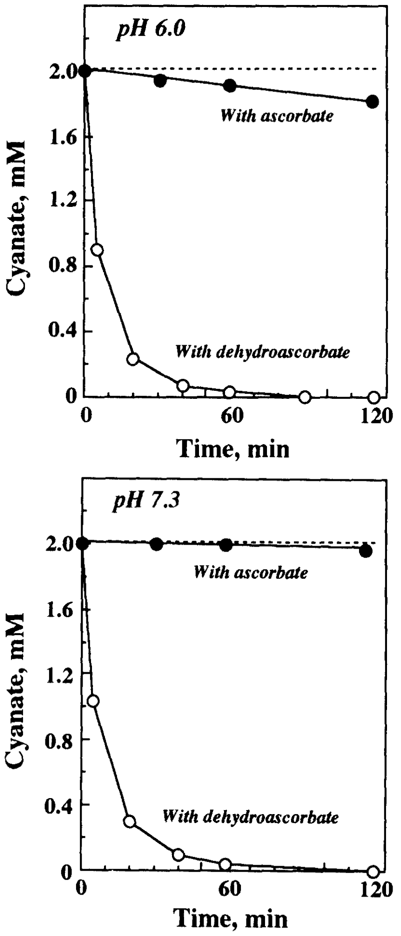

Fig. 4. Reaction of Cyanate with Ascorbate and Dehydroascorbate under Physiologic Conditions

Ascorbate (2.5 mM) and dehydroascorbate (2.5 mM) were respectively incubated with 2 mM cyanate in acetate buffer solution (10 mM, pH 6.0) or in Tris-acetic acid buffer (20 mM, pH 7.3) at 37 °C. The reactions were monitored by the disappearance of cyanate.

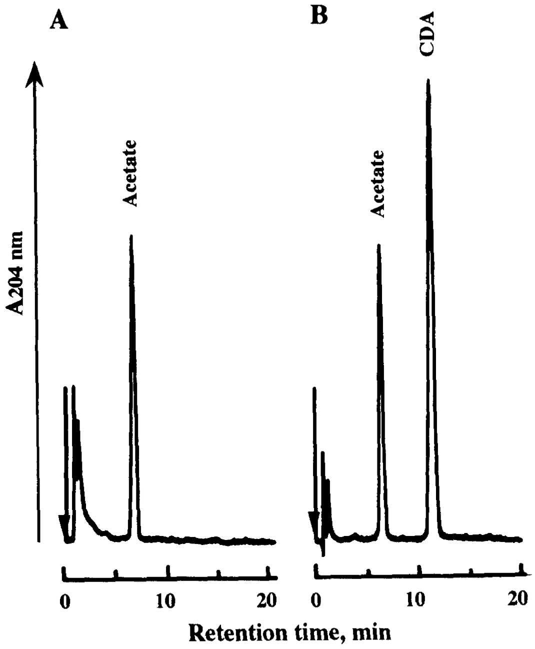

Fig. 5. Chromatograms of the Product Obtained by the Reaction of Cyanate with Dehydroascorbate

Cyanate (2 mM) was mixed with 2.5 mM dehydroascorbate in Tris–acetate (20 mM, pH 7.3), and the mixture was submitted to HPLC before (A) and after (B) incubation at 37 °C for 1 h. The chromatographic conditions are as follows: Column, TSK gel SAX (4 mm i.d. × 150 mm); eluent, 50 mM NaCl (1.0 ml/min); detection, 204 nm.

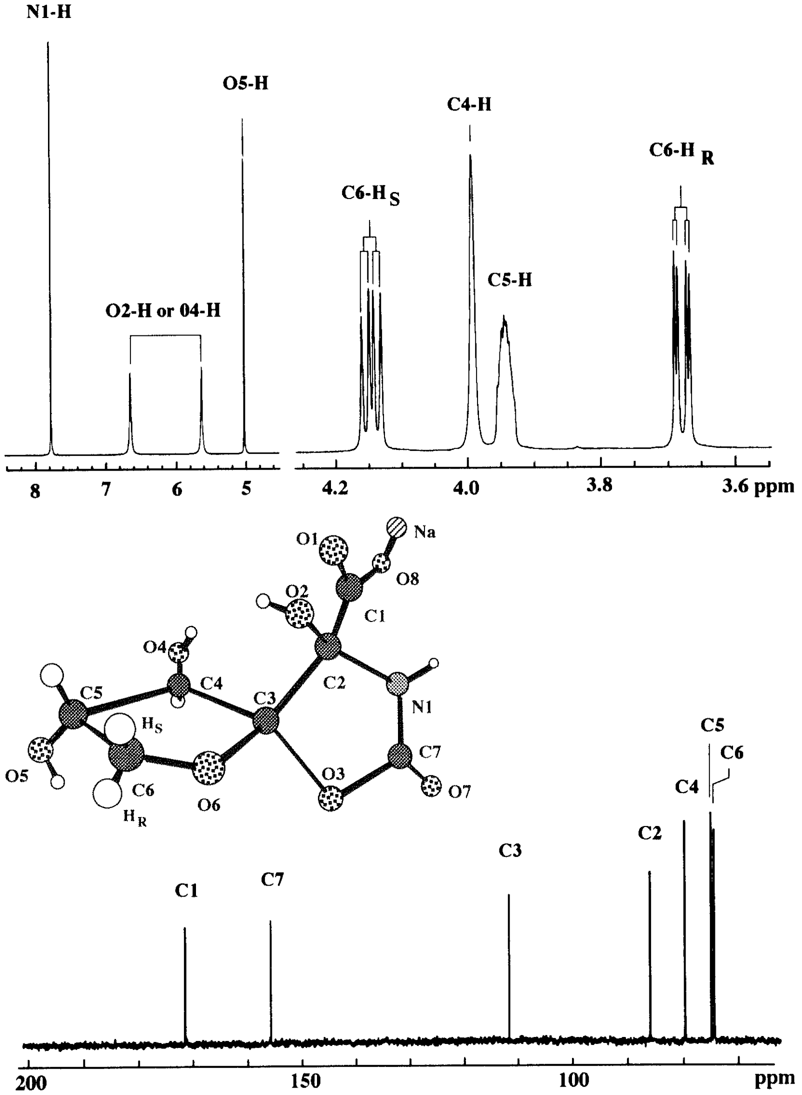

Fig. 6. 500 MHz ^{1}H-NMR and ^{13}C-NMR Spectra of CDA (Sodium Salt) in DMSO-d_6

Perspective view of CDA with atomic numbering is presented in the figure.

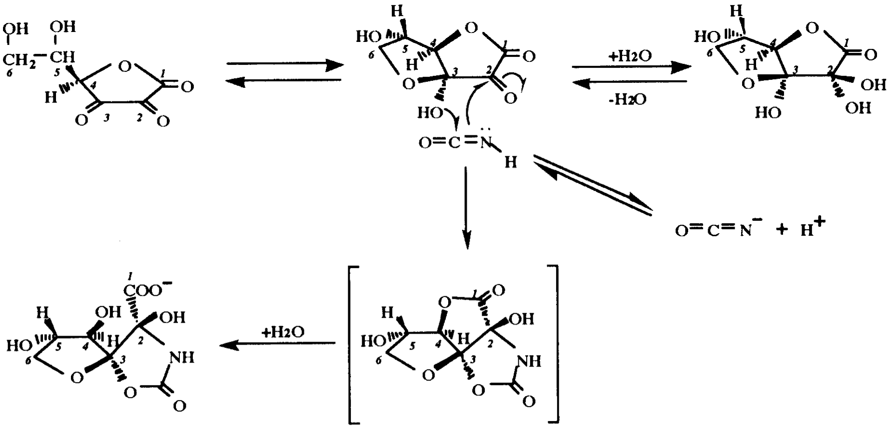

Fig. 7. Proposed Reaction Mechanism of Cyanate with Dehydroascorbate Producing CDA

their ^{1}H-NMR spectra.

A definitive structure of CDA was established by X-ray crystallographic analysis. Crystal data are as follows: lattice parameters, $a = 10.609(4)$, $b = 13.885(2)$, $c = 7.553(2)$ Å; $V = 1112.54$ Å^3; $Z = 4$; $D_{\text{calc}} = 1.732$ g/cm^3. A molecule of CDA found in an asymmetric unit is displayed in Fig. 6. Furthermore, X-ray crystallographic data indicated that this crystal contained two water molecules of crystallization per one CDA molecule (results were not shown).

Reaction Mechanism of Cyanate with Dehydroascorbate Dehydroascorbate in aqueous solution was elucidated to exist as 3,6-anhydro-L-xylohexurono-1,4-lactone hydrate, which was a hydrated hemiacetal formed by cyclization between the C_6 hydroxyl group and the C_3 carbonyl group of dehydroascorbate.[30] Cyanate may react with a hydroxyl group on an anomeric carbon (C_3) of this bicyclic compound, followed by a carbamyl group attack on the electrophilic C_2 carbon, resulting in the formation of an oxazolidone ring as shown in Fig. 7. The lactone ring in the tricyclic compound was finally hydrolyzed into the bicyclic spiro-compound.

Dehydroascorbate is produced from ascorbate in the redox system, and then it is reduced into ascorbate enzymatically or non-enzymatically. The reaction of cyanate with dehydroascorbate progresses rapidly and irreversibly under physiologic conditions of pH. In the human body, it is generally known that cyanate is produced by the spontaneous degradation of urea as well,[20,21] and the production of cyanate was thought to be considerable in patients with uremia.[22] The present study suggests that the resultant cyanate might react with dehydroascorbate and transform it into CDA, which no longer has anti-scurvy activity.

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