Virus-Inactivating Effect of D-Isoascorbic Acid*

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Summary The effect of D-isoascorbic acid, an epimer of L-ascorbic acid, on viruses was investigated using a wide variety of bacterial viruses (phages) as model systems. D-Isoascorbic acid exerted an inactivating effect on all phages examined. The reaction mechanism of virus inactivation by D-isoascorbic acid was investigated using phage J1 as a model system. Bubbling oxygen through the reaction mixture and the addition of H₂O₂ or transition metal ions into the reaction mixture enhanced the phage inactivation by D-isoascorbic acid. In contrast, nitrogen bubbling and the addition of reducing agents, chelating agents or radical scavengers prevented phage inactivation. Experiments using specific radical scavengers, superoxide dismutase or catalase showed that OH· could be mainly responsible for phage inactivation by D-isoascorbic acid. These findings are similar to those obtained with L-ascorbic acid, and indicate that phage-inactivating activity is independent of the stereoisomerism with inversion of the hydroxyl group at carbon 5 of ascorbic acids.

Key Words D-isoascorbic acid, D-araboascorbic acid, erythorbic acid, virus inactivation, phage inactivation

The *in vitro* virus-inactivating effect of L-ascorbic acid has been well documented; the viruses reported to be inactivated by L-ascorbic acid include a number of animal viruses (coxsackie, echo, foot-and-mouth disease, herpes, influenza, polio, rabies, vaccinia, etc.), a plant virus (tobacco mosaic) and several bacterial viruses (*Bacillus*, *Escherichia*, *Lactobacillus*, *Pseudomonas*, *Shigella* and *Staphylococcus*) (1-8).

On the other hand, the effect of the D-configuration of ascorbic acid on viruses has not yet been studied. D-Isoascorbic acid (D-araboascorbic acid or erythorbic acid) is stereoisomer with inversion of the hydroxyl group at carbon 5. It has little antiscorbutic activity but is used as an antioxidant preservative in the food industry.

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We have therefore studied the effect of D-isoascorbic acid on viruses using a wide variety of bacterial viruses (phages) as model systems, and found that D-isoascorbic acid has an inactivating effect on phages. Subsequently, the mechanism of phage inactivation by D-isoascorbic acid was studied using phage J1 as a model.

This paper reports studies of the inactivating effect of D-isoascorbic acid on phages and of the reaction mechanism involved in the inactivation of phages by D-isoascorbic acid.

MATERIALS AND METHODS

Reagents. D-Isoascorbic acid (special grade) was obtained from Tokyo Kasei Kogyo Co., and catalase (from bovine liver, 36,000 units/mg protein) and superoxide dismutase (from bovine liver, 3,000 units/mg protein) were from Sigma Chemical Co. All other reagents were obtained from Wako Pure Chemical Industries and were of the highest grade available.

Phages. Phages examined are listed in Table 1. These 9 phages present a wide variety from the viewpoint of nucleic acid type, morphology, host bacteria and certain other characteristics.

Media and culture conditions. Nutrient broth was used for *Escherichia* and *Bacillus*, MRT medium (9) for *Lactobacillus*, and SSM medium (10) for *Pseudomonas*. *Escherichia* and *Bacillus* were incubated with shaking at 37°C, and *Pseudomonas* with shaking at 25°C. *Lactobacillus* was incubated without shaking at 37°C.

Assay of phages. Phages were assayed by the double-agar layer technique (11, 12).

Inactivation of phages. Tris-HCl buffer (0.02 M, pH 7.4) was used throughout the experiments. A concentrated D-isoascorbic acid solution was freshly prepared

Table 1. Bacterial viruses (phages) examined.

Phage	Nucleic acid	Morphology	Host bacteria
J1	Double-stranded DNA		<i>Lactobacillus casei</i> S1
T5			<i>Escherichia coli</i> B
T4			<i>Escherichia coli</i> B
M2			<i>Bacillus subtilis</i> Y
T3			<i>Escherichia coli</i> B
ϕ X174	Single-stranded DNA		<i>Escherichia coli</i> C
δ A			<i>Escherichia coli</i> K12 W3110 F ⁺
MS2	Single-stranded RNA		<i>Escherichia coli</i> K12 W3110 F ⁺
ϕ 6	Double-stranded RNA		<i>Pseudomonas phaseolicola</i> HB10Y

prior to each experiment. Phages ($1-4 \times 10^7$ PFU/ml) were incubated with D-isoascorbic acid in the buffer for 30 min at 37°C. Phage $\phi 6$ was incubated for 20 min at 25°C because of its relative instability at 37°C. The reaction was carried out in a standing test tube lightly covered with an aluminium cap; under these conditions D-isoascorbic acid underwent auto-oxidation. The reaction was terminated by dilution with a chilled diluent to below the effective D-isoascorbic acid concentration range. The diluent was a phosphate buffer (0.01 M, pH 7.0) containing 1 g NaCl, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.03 g gelatin per liter.

Others. Other experimental methods were the same as those described by Adams (11).

RESULTS

Effect of D-isoascorbic acid on phages

The effect of D-isoascorbic acid on phages was investigated using 9 phages. The results are shown in Table 2. D-Isoascorbic acid exerted an inactivating effect on all of the phages examined, although the phages exhibited different sensitivities to inactivation by D-isoascorbic acid. Sensitivities of the phages, except $\phi 6$, to D-isoascorbic acid were arranged, from the highest to the lowest, in the following order: T3, $\phi X174$, J1, MS2, M2, δA , T5, T4. These results indicate that D-isoascorbic acid has an inactivating effect on all types of phages.

When the inactivating potency of D-isoascorbic acid against each phage was

Table 2. Effect of D-isoascorbic acid on a wide variety of phages.

Phages ($1-4 \times 10^7$ PFU/ml) were incubated with D-isoascorbic acid in 0.02 M Tris-HCl buffer (pH 7.4) for 30 min at 37°C. The survival of the control without D-isoascorbic acid is taken as 100%.

Phage	Survival (%)		
	Concentration (M)		
	1×10^{-3}	3×10^{-4}	1×10^{-4}
J1	0-1	8-15	25-40
T5	40-60	60-80	90-100
T4	70-80	90-100	90-100
M2	5-10	30-40	60-80
T3	0-1	5-10	20-30
$\phi X174$	0-1	5-10	25-40
δA	30-50	60-80	90-100
MS2	0-1	10-20	40-60
$\phi 6^a$	40-60	60-80	90-100

^a Incubated for 20 min at 25°C.

compared with that of L-ascorbic acid, no significant differences in potency were seen between the two.

Inactivation of phage by D-isoascorbic acid

The time course of phage inactivation by D-isoascorbic acid was investigated using phage J1, a double-stranded DNA phage of *Lactobacillus casei*, as a model. Figure 1 shows the typical inactivation kinetics of the phage. The rate of phage inactivation was proportional to the concentration of D-isoascorbic acid and the length of incubation. The inactivation curves were initially shouldered but became linear after several minutes of incubation.

Reaction mechanism involved in phage inactivation by D-isoascorbic acid

With respect to the inactivation of phages by L-ascorbic acid, we have shown that oxygen free radicals generated during the auto-oxidation of L-ascorbic acid are involved in the inactivation and that the hydroxyl radical ($\text{OH}\cdot$) is directly responsible for inactivation (13). Therefore, with respect to phage inactivation by D-isoascorbic acid, the following experiments were carried out using phage J1 to examine whether the free radical mechanism is involved in the inactivation reaction.

1) *Effect of oxygen on inactivation.* As shown in Table 3, bubbling oxygen gas through the reaction mixture markedly enhanced phage inactivation by D-

Fig. 1. Inactivation curves of phage J1 by different concentrations of D-isoascorbic acid. The phage (4×10^7 PFU/ml) was incubated with D-isoascorbic acid in 0.02 M Tris-HCl buffer (pH 7.4) at 37°C . The concentration of D-isoascorbic acid: ---, 0 M; $\circ$, 3×10^{-5} M; $\bullet$, 1×10^{-4} M; $\triangle$, 3×10^{-4} M; $\blacktriangle$, 1×10^{-3} M.

Table 3. Effect of oxygen on inactivation of phage J1 by D-isoascorbic acid. The phage (4×10^7 PFU/ml) was incubated with 3×10^{-4} M D-isoascorbic acid for 30 min at 37°C. The survival of the control without D-isoascorbic acid is taken as 100%.

Incubated	Survival (%)
In normal atmosphere	10
In oxygen atmosphere ^a	0
In nitrogen atmosphere ^b	90–100
<i>In vacuo</i> ^c	90–100

^a Oxygen gas was bubbled through the reaction mixture. ^b Nitrogen gas was bubbled through the reaction mixture. ^c The reaction was performed in a Thunberg tube evacuated with a vacuum pump.

Table 4. Effect of transition metal ions or chelating agents on inactivation of phage J1 by D-isoascorbic acid.

The phage (4×10^7 PFU/ml) was incubated with 3×10^{-4} M D-isoascorbic acid and an additional reagent in the buffer for 30 min at 37°C. The survival of the control (D-isoascorbic acid-free, containing the reagent) is taken as 100%.

Addition	Concentration (M)	Survival (%)
None	0	10
Cu ²⁺	1×10^{-6}	0
	1×10^{-7}	0
Fe ²⁺	1×10^{-6}	— ^a
	1×10^{-7}	1–2
EDTA	1×10^{-5}	90–100
	1×10^{-6}	90–100
Citrate	1×10^{-3}	50–70
	1×10^{-4}	50–70

^a No data are available due to the phage-inactivating effect of the reagent itself.

isoascorbic acid. On the other hand, bubbling nitrogen gas completely prevented the inactivation. Also, no inactivation of the phage was seen *in vacuo*. These results indicate that molecular oxygen is required for phage inactivation.

2) *Effect of transition metals or chelating agents on inactivation.* As shown in Table 4, the addition of transition metal ions into the reaction mixture markedly enhanced phage inactivation. On the other hand, chelating agents prevented the inactivation; in particular, EDTA completely prevented it. These results indicate that a trace level of transition metal ions is required for phage inactivation.

3) *Effect of oxidizing or reducing agents on inactivation.* As shown in Table 5, the addition of oxidizing agents such as H₂O₂ and permanganate markedly enhanced phage inactivation. On the other hand, reducing agents prevented

Table 5. Effect of oxidizing or reducing agents on inactivation of phage J1 by D-isoascorbic acid.

See the legend to Table 4.

Addition	Concentration (M)	Survival (%)
None	0	10
H ₂ O ₂	1 × 10 ⁻⁴	0
	1 × 10 ⁻⁵	0
KMnO ₄	1 × 10 ⁻⁵	— ^a
	1 × 10 ⁻⁶	1–2
L-Cysteine	1 × 10 ⁻⁴	90–100
	1 × 10 ⁻⁵	70–90
Thioglycollate	1 × 10 ⁻⁴	60–80
	1 × 10 ⁻⁵	40–60
2-Mercaptoethanol	1 × 10 ⁻⁴	60–80
	1 × 10 ⁻⁵	50–60
Dithiothreitol	1 × 10 ⁻⁵	60–80
	1 × 10 ⁻⁶	60–80
Glutathione	1 × 10 ⁻⁵	60–80
	1 × 10 ⁻⁶	40–60

^a No data are available due to the phage-inactivating effect of the reagent itself.

Table 6. Effect of radical scavengers on inactivation of phage J1 by D-isoascorbic acid.

See the legend to Table 4.

Addition	Concentration (M)	Survival (%)
None	0	10
AET ^a	1 × 10 ⁻²	90–100
	1 × 10 ⁻³	60–80
MEA ^b	1 × 10 ⁻²	90–100
	1 × 10 ⁻³	50–70
HCOONa	1 × 10 ⁻¹	90–100
	1 × 10 ⁻²	60–80
KBr	1 × 10 ⁻¹	90–100
	1 × 10 ⁻²	60–80
KI	1 × 10 ⁻¹	90–100
	1 × 10 ⁻²	60–80
KSCN	1 × 10 ⁻¹	90–100
	1 × 10 ⁻²	40–60
DABCO ^c	1 × 10 ⁻¹	— ^d
	1 × 10 ⁻²	10–20

^a 2-Aminoethylisothiuronium. ^b 2-Mercaptoethylamine. ^c 1,4-Diazabicyclo[2.2.2]-octane. ^d No data are available due to the phage-inactivating effect of the reagent itself.

Table 7. Effect of catalase or superoxide dismutase on inactivation of phage J1 by D-isoascorbic acid.

See the legend to Table 4, but enzymes were used instead of reagents.

Addition	Concentration (units/ml)	Survival (%)
None	0	10
Catalase	100	90-100
	30	90-100
	10	60-80
	3	50-60
	1	40-50
SOD ^a	100	20-30
	30	10-20
	10	10

^aSuperoxide dismutase.

inactivation; in particular, cysteine completely prevented it. These results indicate that phage inactivation is accompanied by the auto-oxidation of D-isoascorbic acid.

4) *Effect of radical scavengers on inactivation.* Table 6 shows the effect of radical scavengers on the inactivation of phage. 2-Aminoethylisothiuronium (AET) and 2-mercaptoethylamine (MEA), general radical scavengers, completely prevented the inactivation, indicating the involvement of free radicals in phage inactivation. Also, sodium formate, potassium bromide, potassium iodide and potassium thiocyanate, specific scavengers for $\text{OH}\cdot$ (14), fully prevented the inactivation. The results indicate the involvement of $\text{OH}\cdot$ in inactivation.

On the other hand, 1,4-diazabicyclo[2.2.2]octane (DABCO), a specific quencher for $^1\text{O}_2$ (15), hardly prevented the inactivation, indicating little involvement of $^1\text{O}_2$ in phage inactivation.

5) *Effect of catalase or superoxide dismutase on inactivation.* As shown in Table 7, catalase (H_2O_2 dismutase) fully prevented phage inactivation. Superoxide dismutase, which catalyzes the dismutation of superoxide anion radical (O_2^-) to O_2 and H_2O_2 , did not markedly prevent the inactivation. These results confirm that $\text{OH}\cdot$, and not O_2^- or $^1\text{O}_2$, is mainly involved in phage inactivation and that it is directly responsible for the inactivation of phage by D-isoascorbic acid.

DISCUSSION

The *in vitro* virus-inactivating effect of L-ascorbic acid is well known (1-8). On the other hand, the effect of the D-configuration of ascorbic acid on viruses has not been previously studied.

From the results presented here, it is evident that D-isoascorbic acid as well as L-ascorbic acid has an inactivating effect on phages. Also, the results suggest that D-isoascorbic acid could be effective against all types of phages, since the phages

examined present a wide variety from the viewpoint of nucleic acid type, morphology, host bacteria and certain other characteristics.

The results of investigation on the reaction mechanism involved in the inactivation of phage by D-isoascorbic acid can be summarized as follows: 1) Molecular oxygen and transition metal ions are required for the inactivation. 2) The inactivation is accompanied by the auto-oxidation of D-isoascorbic acid. 3) Oxygen free radicals are involved in the inactivation. 4) $\text{OH}\cdot$ is the major reactive species in the inactivation. 5) H_2O_2 , at a concentration approximating that theoretically produced on the auto-oxidation of D-isoascorbic acid, shows no direct effect on the phage (16).

These results, taken together, indicate that the free radical mechanism is involved in phage inactivation and that $\text{OH}\cdot$ generated during the auto-oxidation of D-isoascorbic acid is directly responsible for the inactivation of phage by D-isoascorbic acid.

We speculate that the main pathway for the generation of $\text{OH}\cdot$ is as follows:

where DH_2 is a reduced form of D-isoascorbic acid, D an oxidized form of D-isoascorbic acid, and M a trace level of a transition metal ion. D-Isoascorbic acid plays a dual role; it converts the metal ion to its reduced state and forms, in the presence of the metal ion, H_2O_2 , which in turn reacts with the reduced metal ion to generate $\text{OH}\cdot$.

Comparing D-isoascorbic acid and L-ascorbic acid, no significant differences were seen in the inactivating potency against each phage. Also, the reaction mechanism involved in the inactivation of phage by D-isoascorbic acid was similar to that obtained with L-ascorbic acid. These findings indicate that the phage-inactivating activity is independent of the stereoisomerism with inversion of the hydroxyl group at carbon 5 of ascorbic acid.

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