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RAPID INACTIVATION OF BACTERIOPHAGE T7 BY ASCORBIC ACID IS REPAIRABLE

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Treatment of bacteriophage T7 with ascorbic acid resulted in the rapid accumulation of single-strand breaks in the DNA with double-strand breaks appearing only after incubation times of 20 min or longer. The single-strand breaks were responsible for a rapid inactivation of the phage as assayed by immediate plating of the phage-bacteria mixture on nutrient agar. Incubation of the phage-bacteria mixture in liquid medium prior to plating allowed a host cell reactivation process to repair the nicks and reactivate the phage. Non-reversible inactivation of the phage was a slower process which could be correlated with the appearance of double-strand breaks in the phage DNA. Host cell reactivation of the phage was also manifested in the phenomena of delayed lysis and delayed appearance of the concatemeric DNA replication intermediate.

Introduction

Ascorbic acid oxidation products have an inactivating effect on avian Rous sarcoma virus [1] and many bacteriophage including RNA containing MS2 [2] and R17 [3], single-strand DNA containing δA [4] and double-strand DNA containing J1 [5]. While the inactivation was attributed to free radical products of ascorbate oxidation, it remained for Morgan et al. [6] to show that it was hydroxyl radicals generated from hydrogen peroxide produced during ascorbate oxidation which were responsible for the formation of single-strand breaks in the nucleic acids. Such radicals had previously been implicated as a cause of strand cleavage [7,8]. Presumably other modifications to bases of the ribose-phosphate chain also occur but are undetectable by sucrose gradients.

Although the inactivation of single-stranded DNA and RNA containing phage could be explained by the introduction of single-strand nicks, this was not the case for double-stranded DNA containing phage where single-strand nicks normally are not lethal because of their rapid rejoining in most cell systems [9]. The reported rapid rates of inactivation of phage J1 by ascorbate were inconsistent with the single-strand nicking mechanism and suggested that ascorbate inactivated phage by another mechanism or that the assay for inactivation was inaccurate.

This report confirms that the primary result of ascorbate treatment of whole phage assayable using sucrose gradients is the introduction of single-strand breaks in the DNA with double-strand breaks accumulating only at longer times. It is also shown that T7 phage, like J1 phage [5], was rapidly inactivated by ascorbate but by a process which was repairable, and that non-repairable inactivation occurred much more slowly than previously reported.

Methods

The bacterial strains used were *Escherichia coli* B23, a prototroph, and *Escherichia coli* B5975, a *rec* A mutant from the Yale culture collection. Cultures growing at 37°C in either minimal salts medium [10] supplemented with 16.7 mM glucose

or 'LB' medium [11], containing 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g sodium chloride per liter, were infected at a multiplicity of infection (m.o.i.) of 1 with wild-type T7 bacteriophage. Cell density was followed using a Klett-Summerson colorimeter with a blue filter. Uniformly ^{32}P-labeled T7 phage were prepared as described by McDonell et al. [12]. Appropriately diluted solutions of T7 phage were titered using the double-layer technique of Adams [13] except where the bacteria-phage mixture was incubated for 30 min at 37°C prior to mixing with the top agar and plating. Phage inactivation experiments with ascorbic acid were carried out as described by Murata and Kitagawa [5]. Prior to alkaline sucrose gradient analysis, the ascorbate reaction with ^{32}P-labeled T7 phage was stopped by adding 1 mM EDTA and chilling. After the addition of 0.2 M NaOH, the mixture was warmed to 37°C for 5 min and loaded on a linear 5–20% alkaline sucrose gradient in 0.25 M NaOH/0.1 M NaCl/1 mM EDTA. The gradients were run in polyallomer tubes in a Beckman SW50.1 rotor at 45000 rev./min for 2.5 h. Fractions were collected and radioactivity quantitated by Cerenkov counting [14]. Prior to neutral sucrose gradient analysis, ^{32}P-labeled T7 phage was treated as described for alkaline sucrose analysis but with the NaOH treatment replaced by incubation at 65°C for 2 min to release the DNA. Linear 5%–20% neutral sucrose gradients were prepared in 20 mM Tris-HCl (pH 7.6)/0.1 M NaCl/50 mM EDTA and centrifuged in a Beckman SW41 rotor at 35000 rev./min for 2.5 h. Fractions were collected and Cerenkov radiation determined [14].

Results

Effect of ascorbate on phage DNA

The involvement of hydroxyl radicals as the active species in DNA fragmentation during ascorbate treatment suggested that single-strand nicks should have been the principal result of ascorbate treatment. Because the rapid rate of activation of J1 phage by ascorbate was inconsistent with single-strand nicks being the only damage caused by ascorbate, a combination of alkaline sucrose gradients and neutral sucrose gradients was used to distinguish between single- and double-strand breaks introduced during ascorbate treatment. As expected, alkaline sucrose gradients revealed a very rapid accumulation of nicks in T7 phage DNA during incubation of the whole phage with ascorbic acid (Fig. 1). Neutral sucrose gradients revealed that no double-strand breakage had occurred even after a 10 min ascorbate treatment, indicating that the DNA secondary structure was intact despite many single-

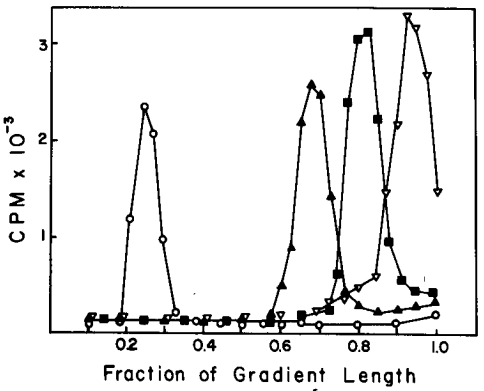

Fig. 1. Alkaline sucrose gradient analysis of ^{32}P-labeled DNA from T7 phage treated for various times with 0.57 mM ascorbic acid. Following the procedure described in Methods, the phage were untreated (○) or were treated with ascorbic acid in LB medium for 10 (▲), 20 (■) or 30 min (▽) prior to release of the DNA and centrifugation. Sedimentation was to the left.

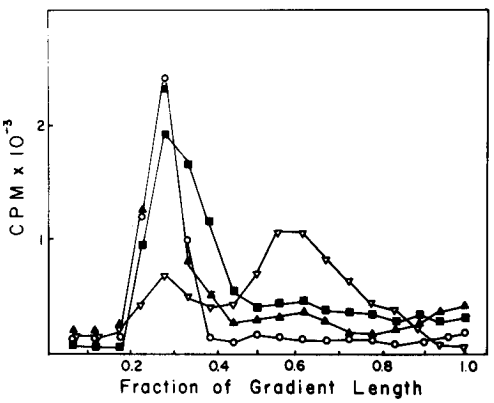

Fig. 2. Neutral sucrose gradient analysis of ^{32}P-labeled T7 DNA from phage treated for various times with 5.7 mM ascorbic acid. Following the procedure described in Methods, the phage were untreated (○) or were treated with ascorbic acid in LB medium for 10 (▲), 20 (■) or 30 min (▽) prior to release of the DNA and centrifugation. Sedimentation was to the left.

strand nicks (Fig. 2). Double-strand breaks began to appear after 20 min of incubation and most of the DNA had been fragmented by 30 min, probably the result of extensive and overlapping single-strand nicking. Quantitation of the single-strand nicks from the alkaline sedimentation profile [15] revealed that greater than 20 nicks, the upper limit for this calculation, had been introduced in 5–10 min of incubation with 0.57 mM ascorbate. For comparison, double-strand breaks appeared only after 20 min incubation with 5.7 mM ascorbate. Electrophoresis on agarose gels provided similar results wherein ascorbate had no apparent effect on phage DNA unless a heat-denaturation step was included prior to electrophoresis to disrupt the secondary structure. As expected, catalase (200 μg/ml) prevented the formation of nicks in the DNA even during a 20 min incubation with 5.7 mM ascorbate (Table I), confirming the role of hydrogen peroxide as an intermediate in the nicking process. Therefore, the rapid phage inactivation rates observed by Murata and Kitagawa [5] must be explained in terms of lethal single-strand breaks, an explanation inconsistent with the conclusions of Freifelder [9].

Rate of inactivation of bacteriophage T7 by ascorbic acid

The rates of inactivation of T7 bacteriophage by various concentrations of ascorbic acid were determined using the standard double-layer plating technique and, as shown in Fig. 3a, they were found to be similar to the rates reported for J1 phage inactivation [5]. However, when the same experiments were repeated with a procedural modification which allowed an incubation period in liquid medium of 30 min prior to layering the bacteria-phage mixture over the bottom agar layer, the rates of inactivation were significantly reduced (Fig. 3b). The phage were rapidly inactivated by ascorbate, consistent with the earlier report, but this initial inactivation was reversible during an incubation period in liquid medium. This is a manifestation of host cell reactivation which will repair, among other types of DNA damage, single-strand nicks, the principal lesion caused by ascorbic acid. Longer treatment times or treatment with high ascorbate concentrations inactivated the phage by a mechanism which was not reversible during the liquid incubation period. Significantly, double-strand breaks were observed in the DNA under the same conditions. The use of a *rec* A strain as the plating host resulted in a slightly higher rate of inactivation and a poorer recovery of activity during the liquid incubation phase (Fig. 4).

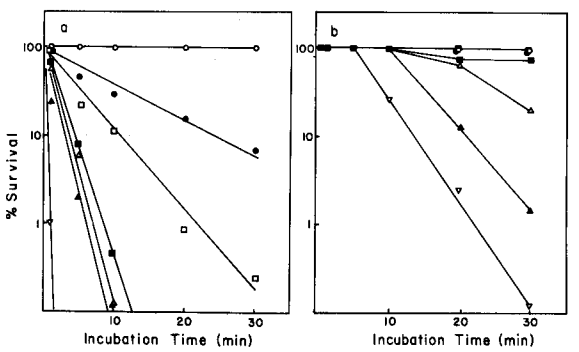

Fig. 3. Inactivation of bacteriophage T7 by ascorbic acid assayed using *E. coli* B23. Phage were incubated in LB medium for various times with ascorbic acid at zero (○), 0.057 (●), 0.285 (□), 0.57 (■), 2.85 (△), 5.7 (▲) or 28.5 mM (▽) prior to stopping the reaction by dilution and to mixing with bacteria. In part a, the bacteria-phage mixture was immediately layered on nutrient agar plates, while in part b, the bacteria-phage mixture was incubated for 30 min at 37°C before layering on agar plates.

TABLE I

EFFECT OF ASCORBIC ACID AND CATALASE ON THE FORMATION OF SINGLE-STRAND NICKS IN ^{32}P-LABELED T7 DNA

The number of nicks were calculated after Litwin et al. [15] from the DNA sedimentation profiles in alkaline sucrose gradients. Incubation of the phage with ascorbate and catalase was in medium (LB medium in Ref. 11).

Ascorbate concn. (mM)	Catalase concn. (μg/ml)	Number of nicks after incubation for (min)				
		0	1	5	10	20
5.7	—	0	5	16	>20	>20
5.7	200	0	0	0	0	0
—	200	0	0	0	0	0

Delayed lysis of E. coli by ascorbate-treated T7 phage

When cultures of *E. coli* growing in the pres-

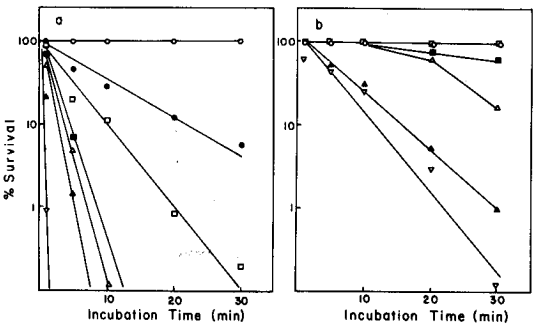

Fig. 4. Inactivation of bacteriophage T7 by ascorbic acid assayed using *E. coli* B5975 (*rec* A). The conditions were as described in the legend to Fig. 3.

ence of various concentrations of ascorbic acid were infected with T7 phage, the time of lysis was delayed in approximate proportion to the ascorbate concentration (Table II). The burst size was the same in all cases where lysis had occurred. In order to ascertain that this delay in lysis was not the result of ascorbate affecting bacterial metabolism [16] but was another manifestation of host cell reactivation, bacteria growing in the absence of ascorbate were infected with phage which had been pretreated with ascorbic acid. Cell lysis was detectable as a sharp decline in absorbance. As shown in Fig. 5, the onset of lysis was delayed by ascorbate pretreatment of the phage, but the time

TABLE II

EFFECT OF EXOGENOUS ASCORBIC ACID ON CELL LYSIS BY T7 PHAGE

Infection was carried out at a multiplicity of infection (m.o.i.) of 1 in the presence of the indicated concentrations of ascorbic acid in medium (LB medium in Ref. 11). Infection was allowed to proceed for 5 min in the presence of ascorbic acid. Any unabsorbed phage were removed by centrifugation and the cells were resuspended in fresh medium warmed to 37°C and containing ascorbic acid.

Ascorbic acid concn. (mM)	Lysis time (min)	Burst size
0	35	160
5.7	60	160
19.25	135	150
28.5	>290	160
57.0	no lysis	—

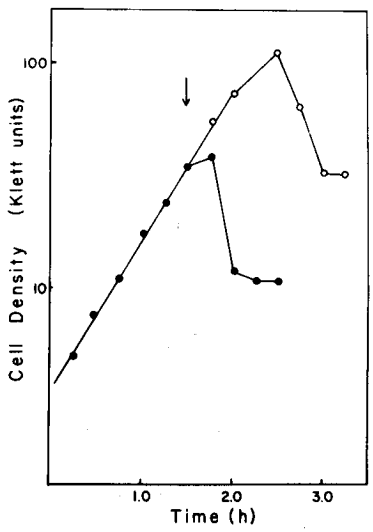

Fig. 5. Effect of the pretreatment on T7 phage with ascorbic acid on the time of lysis of *E. coli* B23. Bacterial cultures growing in LB medium were infected at a multiplicity of infection of 1 with T7 phage that had not been pretreated with ascorbic acid (●) and that had been pretreated with 2.85 mM ascorbic acid for 10 min in LB medium (○). Following ascorbate treatment, the phage were diluted to stop the reaction and immediately added to the bacterial culture at the time indicated by the arrow.

required for completion of lysis, once initiated, was not affected. The times of lysis of *E. coli* by T7 phage pretreated with various concentrations of ascorbate for various times are compiled in Table III and show that as the treatment time or ascorbate concentration increased, so did the length of the delay. The burst size was the same in all cases except after longer treatment times and the progeny phage were similar to the parental phage in causing normal lysis after 30–40 min. The presence of viable mutants resulting from DNA damage was not investigated. The nicks introduced during the ascorbate treatment were fully repaired during the delay period prior to lysis, a period equivalent to the liquid incubation period prior to titering on agar plates in the inactivation experiments. When a *rec* A strain was used as the host, the delay was longer and pretreatment of the phage for times longer than 10 min prevented lysis of the host, another indication of *rec* A involvement in the host cell reactivation process (Table III).

TABLE III

EFFECT OF PRETREATMENT OF T7 PHAGE WITH ASCORBIC ACID ON THE TIME OF LYSIS

T7 phage was treated in medium (LB medium in Ref. 11) with various concentrations of ascorbate, diluted 100-fold and immediately used to infect (m.o.i. of 1) cultures of *E. coli* growing in LB medium. n.l., no lysis.

Strain	Ascorbate concn. (mM)	Pretreatment time (min)	Lysis time (min)	Phage titer in lysate ($\times 10^{-10}$)
B23	0.57	0	40	2.0
		5	50	2.3
		10	60	2.0
		20	80	2.0
		30	n.l.	—
B23	2.8	0	40	3.0
		5	90	2.3
		10	105	2.8
		20	160	3.0
		30	n.l.	—
B23	28.5	0	40	3.0
		5	90	0.8
		10	n.l.	—
B5975	2.8	0	40	2.0
		5	90	2.0
		10	115	0.02
		20	n.l.	

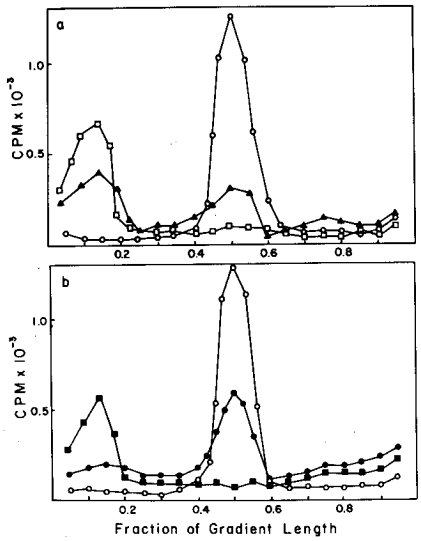

Fig. 6. Effect of ascorbic acid on the appearance of the concatemeric DNA intermediate of T7 phage DNA replication. The procedure was as described by Hausmann and LaRue [22] and Frankel [23] with extracts of infected cultures being analyzed on neutral sucrose gradients. ^{32}P-labeled T7 phage DNA was centrifuged as a control (○). Sedimentation was to the left. a. Aliquots of cells infected with untreated phage were removed 5 (▲) or 15 min (□) after infection, lysed and centrifuged. b. Aliquots of cells infected with phage treated for 5 min with 2.8 mM ascorbate in LB medium were removed 30 (●) or 60 min (■) after infection, lysed and centrifuged.

Effect of ascorbic acid on T7 DNA replication

As a further confirmation that repair of the DNA was involved in the delayed lysis phenomenon, the effect of ascorbate on T7 phage DNA replication was investigated. When untreated phage were the infecting agents, a high-molecular-weight concatemeric DNA characteristic of T7 DNA replication [17] appeared within 5 min but, when ascorbate-treated phage were used, longer times were required for appearance of the concatemeric intermediate (Fig. 6). This delay in the appearance of the replication intermediate can be correlated to the delay in lysis and is another manifestation of host-cell reactivation repairing the DNA such that the replication process can proceed.

Discussion

Rapidly formed single-strand nicks in DNA were the principal lesions introduced by the hydroxyl radicals created during ascorbate oxidation. Other modifications to bases and the ribose-phosphate backbone may have occurred but these were not assayable on sucrose gradients. The rapid inactivation of T7 phage and J1 phage [5] by ascorbate must be attributed to such single-strand breaks and whatever other modifications may have occurred in such a short time, because double-strand breaks appeared slowly, only after extensive nicking had occurred. Repair of the lesions causing the rapid inactivation occurred when the ascorbate-treated phage were incubated with bacteria in liquid medium. The liquid incubation or liquid holding recovery period provides time for DNA repair processes which can repair several types of damage to both host and phage DNA [18–21]. This repair of phage DNA would not take place when cell growth was immediately initiated on agar plates giving rise to the apparent rapid inactivation following ascorbate treatment [5]. The

phenomena of delayed lysis and delayed appearance of the concatemeric DNA replication intermediate were other manifestations of the need for such DNA repair. The wild-type *E. coli* B strain was clearly active in carrying out the repair process, host-cell reactivation, on ascorbate-treated phage while the *rec* A mutant was less effective and it is not known whether *Lactobacillus* strains [5] are capable of host cell reactivation. Therefore, there are two possible explanations for the rapid inactivation of J1 phage as reported by Murata and Kitagawa [5]: 1, a liquid incubation period prior to plating the bacteria-phage mixture was not carried out, making DNA repair impossible; and, 2, the *Lactobacillus* strain used was defective in some aspect of host cell reactivation. This work cannot distinguish between these two possibilities.

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