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Survival of aerosolized bacteriophage φX174 in air containing ozone–olefin mixtures

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SUMMARY

The effects of ozone and ozonized olefins on aerosol survival of bacteriophage ϕX174 were studied. The ozone concentrations used were between 0 and 110 parts/10^9, giving decay rates up to 0·03 min^{-1}. The olefins used were trans-2-butene and cyclohexene in concentrations of 500 parts/10^9 and 2·4 parts/10^6, respectively. Olefins alone have no effect, whereas in combination with ozone, decay rates of 0·1 min^{-1} and higher were obtained. The results are discussed in relation to the viricidal effect of open air.

INTRODUCTION

Open air has a bactericidal activity which is attributed to the so called open-air factor. There are strong indications that the bactericidal activity which affects vegetative bacteria but is inactive against bacterial spores is caused by reactive products of atmospheric ozone and olefins (Druett & May, 1968; de Mik & de Groot, 1977). Open air also has a viricidal activity (May, Druett & Packman, 1969; Benbough & Hood, 1971) and the reactive products of ozone and olefins are also viricidal (Druett & Packman, 1972). However, in the open air the bactericidal effect is variable from day to day, whereas the viricidal effect does not show such variation (Benbough & Hood, 1971). An explanation for this difference may be that airborne viruses and bacteria are inactivated by the same component in open air (the OAF) but that viruses are susceptible to lower concentration ranges (Benbough & Hood, 1971).

To study the mechanism of inactivation by OAF or ozone–olefin mixtures, viruses are more suitable than bacteria since they are less complex, containing only DNA or RNA and some proteins as possible target molecules.

The bactericidal and viricidal activities of the reaction products of ozone and olefins are most pronounced at high relative humidities (Druett & Packman, 1972). Therefore the viricidal activity can best be studied with a virus which is stable at high r.h. values. Since bacteriophage ϕX174 is stable at high r.h. (Dubovi, 1971), and since it has been the subject of numerous investigations, this phage was selected to study the inactivation by ozone–olefin mixtures.

MATERIALS AND METHODS

Bacteriophage stock

The bacteriophage ϕX174 and its host-bacterium *Escherichia coli* C used in this laboratory were provided by Dr R. L. Sinsheimer. The phage was propagated according to the modified method of Sinsheimer as described by van der Ent, Blok & Linckens (1965).

E. coli C was transferred from an 18 h culture into 3XD medium (Fraser & Jerrel, 1953), and was cultured under shaking at 37 °C to a titre of approximately 10^9 cells/ml. The culture was then inoculated with about 10^8 ϕX174 particles/ml and was incubated until complete lysis (1–2 h). The lysate was centrifuged (1500 ***g***) and the pellet of bacterial debris onto which the major part of the phage was adsorbed was resuspended in borate–versene buffer to 1/20 of its original volume. The suspension was vigorously shaken by hand or sonicated for 1 min in a MSE sonic oscillator with 6% chloroform and kept at 4 °C. After settling of the chloroform with most of the bacterial debris the upper fluid layer was carefully removed by pipetting, and centrifuged. The clear supernatant contained the ϕX174 particles in a concentration of approximately 10^{12} per ml.

The bacteriophage was further purified by centrifugation for 3 h at 24000 rev/min in a 5–20% (w/v) linear sucrose gradient, prepared as described by Van der Schans, Aten & Blok (1969). The gradients were centrifuged using the SW 27 rotor of the Beckman Spinco L or L2-50 ultracentrifuge. After centrifugation, the phage-containing fractions were collected and dialysed against two changes of borate–versene buffer for 24 h. Finally the phage was centrifuged for a second time in a sucrose gradient and dialysed against borate–versene buffer, 0·01% lysine or distilled water, depending on the requirements. The final stock thus obtained contained 10^{10}–10^{11} ϕX174 particles per ml.

Bacteriophage assay

Viable phage was assayed by the agar layer method (Adams, 1959). Volumes of 1 ml of suitable phage-dilutions were mixed in quadruplicate with 3 ml of melted Zahler top-agar (Zahler, 1958) at 45 °C and 0·5 ml of an *E. coli* C culture (5×10^8/ml) and poured into petri dishes containing Zahler bottom-agar (Zahler, 1958). Plates were incubated for 18 h at 37 °C.

Tracer organism

Spores of *Bacillus globigii* (syn. B. *subtilis* var. *niger*) were used as a physical decay tracer. The organism was grown and assayed as previously described (de Mik & de Groot, 1977).

Aerosol equipments

To measure the survival of phage ϕX174 in different atmospheres two systems were used: a small glass static system of about 50 l and a large metal static system of 2000 l.

The glass system consisted of a glass box sealed with a glass plate. Tubes and connexions were made of Teflon. Laboratory air was dried by silica gel and purified

by activated charcoal. A part of the air was humidified and mixed with dry air to the desired relative humidity (r.h.).

Ozonized air was produced by irradiating a part of the air with an ultraviolet lamp. By varying the ratio of ozonized and normal air, the ozone concentration could be changed. Ozone concentrations were recorded continuously with an ozone analyser (IG-TNO model G373; Lindqvist, 1972).

Trans-2-butene (Baker Chemical Co., USA) was diluted with conditioned air to a final concentration of 500 parts/10^9. Before an experiment the system was flushed for at least 1 h with the gas mixture to be used.

Aerosols were generated from a suspension (containing about 10^9 spores and 5×10^9 ϕX174 particles/ml) with a three-jet Collison nebulizer for 15 s. The aerosol was mixed with a small Teflon fan placed on the bottom of the box. The total numbers of organisms nebulized in the 50 l system were determined to be 10^7 for phage ϕX174 and 10^6 for *B. globigii* spores. After an exposure time of 30 min the aerosol was sampled for 5 min with a raised Porton impinger (May & Harper, 1957) (11·5 l/min) containing 10 ml collecting fluid. The air sampled was replaced by clean air with the same r.h. and temperature.

The large system was the same as that used by de Jong (1967) and de Jong & Winkler (1968). Aerosols were generated with a spray gun of the type FK-8 (de Jong, 1967) which aerosolizes 1 ml phage suspension (10^{10}/ml) in about 4 s. The aerosol was kept at 20 °C in the double walled system with a volume of 2000 l and was mixed by a fan. Samples were collected with a raised Porton impinger for 1 min, the sample size being 11·5 l of aerosol. With this system no tracer organism was used.

Ozone was generated by passing pure oxygen over an ultraviolet lamp. With a Teflon tube the mixture was introduced into the system continuously at a rate of 140 l/h throughout the exposure of the phage. The air in the system was monitored during the experiment with the ozone meter.

Cyclohexene (BDH Chemicals, England) was evaporated simultaneously with the aerosolization of the phage by dropping 20 μl of cyclohexene in the barrel of the spray gun, thus giving a concentration in the system of 2·4 parts/10^6.

Presentation of the results

When *B. globigii* spores were used as a physical tracer, the ratio between the numbers of plaque forming units of phage ϕX174 and colony-forming units of *B. globigii* spores in the spray suspension was taken as the 100 % value (average of titres measured before and after nebulization). Since phage ϕX174 and *B. globigii* spores are nebulized in a fairly constant ratio and since the spores do not suffer any decay during the exposure, the percentage survival of phage ϕX174 is given by the formula:

$$S_t = 100 \left(\frac{\phi X}{BG}\right)_t \left(\frac{BG}{\phi X}\right)_0,$$

where ϕXand BG are the counts of ϕX174 and *B. globigii*, respectively, and where the suffixes t and 0 refer to the sample at time t and the value derived from counts of the spray suspension.

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Table 1. *Effect of various compounds on the surface inactivation of bacteriophage* ϕX174: *survival measured in an impinger with medium through which sterile air was drawn for* 15 *min*

Medium		Survival
Distilled water		80·2%
Peptone water	1·0%	74·5%
Peptone water	0·01%	52·2%
D-ribose	0·01%	75·4%
Inositol	0·01%	81·8%
Glycine	0·01%	78·7%
Ca-acetate	0·01%	38·5%
Lysine	0·01%	85·0%

The decay rate k was calculated according to the formula $S_t = 100\, e^{-kt}$. The fraction of survivors was calculated as N_t/N_0, where N_t is the number of viable ϕX174 collected at time t and N_0 the expected number of viable ϕX174 in a corresponding sample volume at time 0, calculated from the total number of ϕX174 aerosolized.

Media

Borate–versene buffer was prepared by adding 8 ml of 0·1 M versene to 100 ml of a saturated (4 °C) solution of $Na_2B_4O_7$, pH 9·2.

Tryptone agar contained 1% tryptone (Difco), 0·5% NaCl and 1·5% agar (Difco). After sterilization glucose was added to a concentration of 1%.

Peptone water contained 1% peptone (Oxoid), pH 6·2.

RESULTS

Inactivation during spraying and collection

In an indirect type aerosolizer and in a liquid impinger the ϕX174 particles repeatedly find themselves in the interface between gas and liquid, which may result in 'surface inactivation' of the phage. Various substances were tested for their capacity to reduce surface inactivation to a minimum, when added to the phage suspension. This capacity was determined by measuring the survival percentage of a phage suspension in a liquid impinger through which sterile air was drawn at critical velocity for 15 min. Phage suspensions were obtained by diluting the borate-versene stock by a factor of 1000 or more with various diluents.

The results are listed in Table 1. These results show that only 0·01% peptone water and 0·01% Ca-acetate are unsuitable for spraying and collecting. With the other media the loss is relatively small.

Generally, borate–versene buffer is used to store phage ϕX174. Therefore, the suitability of this buffer as a collecting medium was compared with that of 1% peptone water and 0·01% lysine. The phage was aerosolized from 0·01% lysine in the large system at two different r.h. values (70 and 87%) and sampled in either borate–versene buffer, 0·01% lysine, or 1% peptone water. The survivals measured in these collecting fluids are presented in Table 2. The results obtained with these

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Table 2. *Influence of the sampling medium on the survival of phage* ϕX174 *sprayed from* 0·01 % *lysine and stored at* 70 *or* 87 % *r.h.*

Aerosol age (min)	Collecting fluid	Survival (%) 70 % r.h.	Survival (%) 87 % r.h.
2·5	0·01 % lysine	27	55
	1 % peptone	29	52
	Borate–versene	30	44
12	0·01 % lysine	17	43
	1 % peptone	24	43
	Borate–versene	22	48
32	0·01 % lysine	23	31
	1 % peptone	36	38
	Borate–versene	24	29

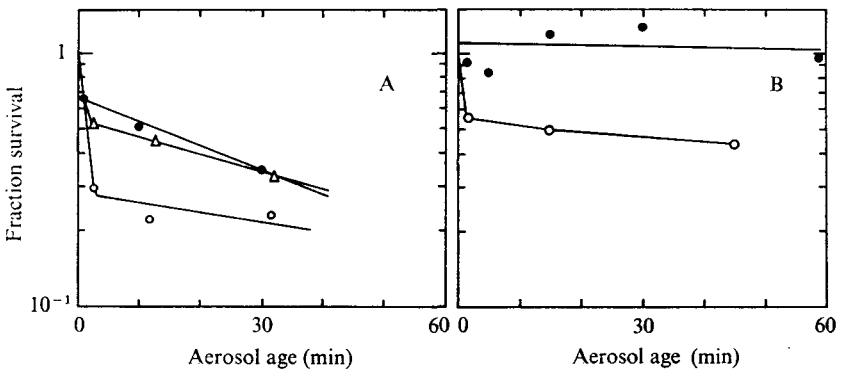

Fig. 1(A). Survival of bacteriophage ϕX174 sprayed from 0·01 % lysine at 94 % r.h. (●——●), 87 % r.h. (△——△), and 70 % r.h. (○——○) in the large system. Collecting medium: 1 % peptone water. (B) Survival of bacteriophage ϕX174 sprayed from 0·01 % lysine at 96 % r.h. in the presence of 2·4 parts/10^6 of cyclohexene (●——●) and sprayed at 75% r.h. in the presence of 500 parts/10^9 of trans-2-butene (○——○) in the large system.

collecting fluids are similar for this r.h. range. Consequently either of these may be used (depending on further analysis) without influencing the aerosol survival of the phage.

Survival of phage $\phi X174$ *in air with or without olefins*

The survival of phage ϕX174 sprayed from 0·01 % lysine into the large system at different r.h. values and collected in 1 % peptone water is shown in Fig. 1A. Most of the inactivation occurred during the first minutes. At 70 % r.h. similar results were obtained when phage ϕX174 was sprayed from distilled water or borate–versene buffer. Contamination of the atmosphere with 500 parts/10^9 of trans-2-butene or 2·4 parts/10^6 of cyclohexene had no effect on the inactivation (Fig. 1B.).

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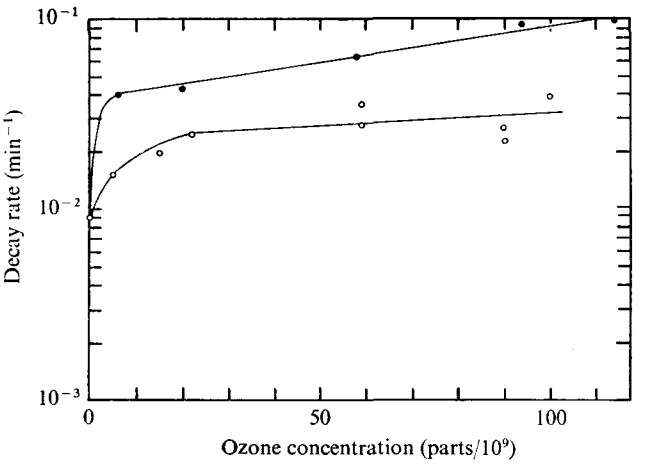

Fig. 2. Aerosol inactivation of phage ϕX174 as a function of the ozone concentration in the absence (○——○), and in the presence (●——●) of 500 parts/10^9 trans-2-butene at 96% r.h. (glass system, exposure time 30 min).

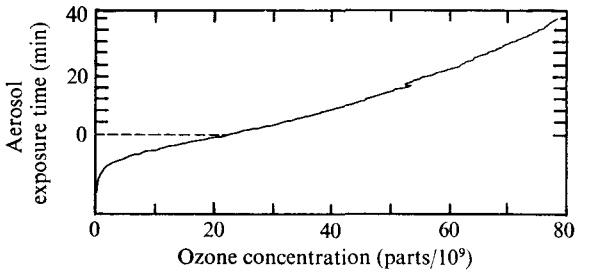

Fig. 3. Course of the ozone concentration before and during exposure of aerosolized phage ϕX174 to ozone alone in the large system.

Survival of phage ϕX174 in air containing ozone and in air containing ozone and olefins

The effect of various concentrations of ozone in the absence or in the presence of 500 parts/10^9 of trans-2-butene was measured in the glass system at 96% r.h. The phage together with *B. globigii* spores was sprayed from 0·01% lysine and collected in 1% peptone water after an exposure time of 30 min. The results are summarized in Fig. 2. The decay rate in clean air did not exceed 0·01 min^{-1}. Ozone alone only slightly increased the decay rate, reaching a maximum effect at 30 parts/10^9. In the presence of 500 parts/10^9 of trans-2-butene, the decay rate increased with increasing ozone concentration. This may be an indication that the inactivation by ozone and by ozonized butene are two different processes.

The effect of ozonized cyclohexene was studied in the large system. Since the wall consists of stainless steel, which inactivates ozone as well as the active products formed by reaction between ozone and cyclohexene, ozone was supplied continuously during the exposure of the phage. The course of the ozone concentration during the exposure of the phage to ozone alone is shown in Fig. 3. After filling the

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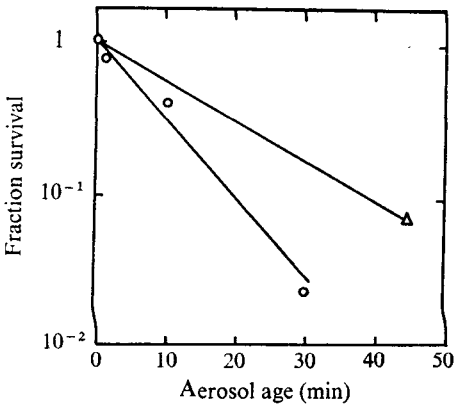

Fig. 4. Survival curves of phage ϕX174 aerosolized from 0·01 % lysine at 74 % r.h. in air containing 20–80 parts/10^9 of ozone in the large system.

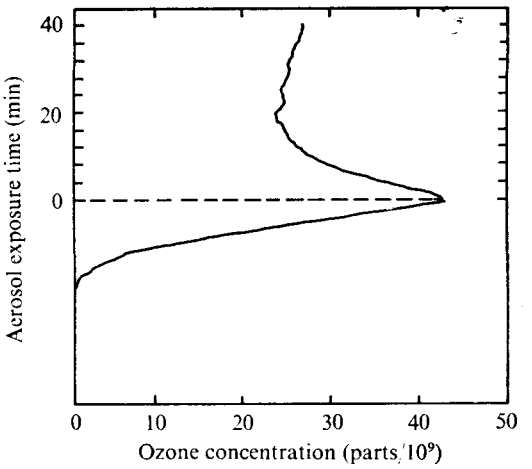

Fig. 5. Course of the ozone concentration before and during the exposure of aerosolized phage ϕX174 to ozone as well as cyclohexene in the large system.

system with conditioned clean air, u.v.-irradiated oxygen was supplied at a rate of 140 l/h. When the concentration had reached 23 parts/10^9, phage ϕX174 was aerosolized with the spray gun. During the 40 min of exposure the ozone concentration increased to about 75 parts/10^9. The survival curves of two such experiments in which phage was sprayed from 0·01 % lysine are given in Fig. 4. The decay rates of 4 other experiments varied between these two.

When phage was aerosolized together with 20 μl of cyclohexene into air with 40 parts/10^9 of ozone the course of the ozone concentration was different, as is shown in Fig. 5. In contrast with the continuous increase shown in Fig. 3 the ozone concentration rapidly decreased owing to the reaction with cyclohexene, until an equilibrium was reached between supply and consumption of ozone.

The effect of ozonized cyclohexene on the survival of phage ϕX174 sprayed from borate–versene buffer, 0·01 % lysine or distilled water at 70 % r.h. and collected

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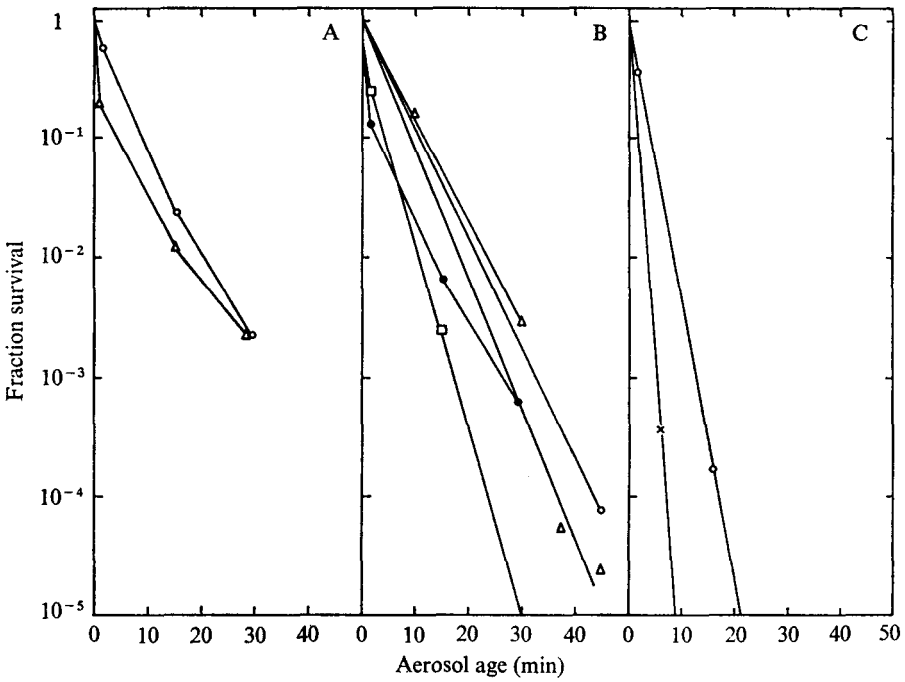


Fig. 6. Survival curves of phage ϕX174 aerosolized at 70% r.h. in air containing initially 30 parts/10^9 of ozone and 2·4 parts/10^6 of cyclohexene in the large system. Each line represents one experiment. (A) Sprayed from borate–versene buffer; (B) sprayed from 0·01% lysine; (C) sprayed from distilled water.

in 1% peptone water in a number of experiments is shown in Fig. 6A, B and C. The initial concentration of ozone was 30 parts/10^9, the initial cyclohexene concentration 2·4 parts/10^6.

The data show that spraying from borate–versene buffer and from 0·01% lysine give similar results, the phage being inactivated by a factor of more than 100 within 30 min. The inactivation rate of phage ϕX174 sprayed from distilled water is much higher. No phage was recovered after 20 min corresponding with a survival of less than 10^{-5}. These results suggest that both 0·01% lysine and borate–versene buffer act as a protecting agent.

DISCUSSION

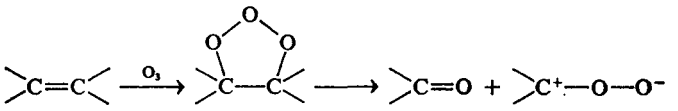
Lipid-free viruses are generally stable in moist air (de Jong, Trouwborst & Winkler, 1973) and the present data show that phage ϕX174 is no exception to this rule. Bacteriophage ϕX174 aerosolized in clean air is reasonably stable at relative humidities higher than 70%. In this r.h. range the percentage survivals in clean air of aerosols sprayed from borate–versene buffer, 0·01% lysine or distilled water do not differ greatly. Similar results are obtained, when the phage is collected in borate–versene, 0·01% lysine or 1% peptone water.

The aerosol survival of phage ϕX174 was also studied by Dubovi (1971), who atomized and collected the phage in borate–versene buffer. Although the aerosol

age in his experiments was only 7·5 min, the results agree with ours since most of the inactivation occurs in the first few minutes, as is shown in Fig. 1.

Ozone inactivates aerosolized phage ϕX174, the maximum effect being obtained at an ozone concentration of 30 parts/10^9. At higher concentrations the decay rate remained constant (figure 2). A similar phenomenon has been described for the inactivation of Semliki Forest virus in the open air (Benbough & Hood, 1971). The decay rates measured in the large system in the presence of ozone are higher than those measured in the glass system. An explanation may be that the air in the large system was contaminated with atmospheric olefins due to insufficient cleaning of the air. This may also explain why there was more variation in the results obtained with the large system than in those obtained with the glass system. On the other hand, *B. globigii* spores which were used as a physical tracer in the glass system but not in the large system may have interfered by consuming part of the ozone.

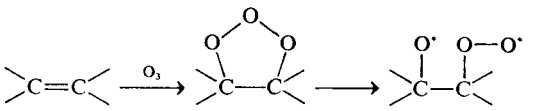
The effect of ozonized olefins on bacteria has been described earlier (Dark & Nash, 1970). It is shown in this study that the product(s) formed by the reaction between ozone and olefins have also a viricidal activity. The gas phase ozone–olefin reactions are very complex and produce a variety of products. Several extensive product studies and a number of absolute and relative reaction rate studies have been made, because these reactions play a key role in the formation of photochemical smog (Leighton, 1961). Generally, the Criegee reaction scheme originally proposed to explain solution ozonolysis reactions is also accepted for gas phase reactions. In this scheme the initial reaction is the formation of an ozonide, followed by decomposition to a carbonyl product and a zwitterion product (Scheme 1). Subsequent zwitterion reactions include various rearrangements



Scheme 1

additions and dimerizations, which are proposed to explain the various products observed (alkanes, acids, esters, alcohols, ketenes, CO, CO_2 and H_2O).

Recently a new mechanism for gas phase ozone–olefin reactions has been proposed in terms of a biradical mechanism (O'Neal & Blumstein, 1973), which is shown in Scheme 2. The biradical can react in a variety of ways.



Scheme 2

One or more of the products formed has a bactericidal or viricidal activity or both, although it is not yet clear whether this is a zitterion, a radical or some other product. We only know that it is very unstable.

Trans-2-butene added to ozone increases the decay rate as shown in Fig. 2, whereas butene alone has no effect. These data strongly suggest that the mechanisms of inactivation by ozone and ozonized butene are different.

The present data also show that ozonized cyclohexene is a very strong viricidal agent. Apparently the phage can be protected to some extent by adding lysine or borate versene to the spraying medium. Probably this compound reacts with the active product, thus protecting the phage. Since it is unknown which product formed by the ozonolysis of butene or cyclohexene is the active one, it is very difficult to explain the inactivation mechanism. It seems possible, however, to investigate whether DNA or proteins or both are the main target molecules of phage inactivation. This will be the subject of another study.

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