J. Hyg., Camb. (1977), **78**, 199
Printed in Great Britain

Mechanisms of inactivation of bacteriophage φX174 and its DNA in aerosols by ozone and ozonized cyclohexene

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(*Received* 4 *August* 1976)

SUMMARY

The mechanisms of inactivation of aerosolized bacteriophage ϕX174 in atmospheres containing ozone, cyclohexene, or ozonized cyclohexene were studied by using ^{32}P-labelled phage. The inactivation of the aerosolized phage in clean air or in air containing cyclohexene is due to damage of the protein coat since the deoxyribonucleic acid (DNA) extracted from the inactivated phage retains its biological activity. Inactivation of the phage in air containing ozone is mainly due to protein damage whereas inactivation in air containing ozonized cyclohexene is due both to protein and DNA damage. Sucrose gradient analysis shows that aerosolized inactivated ϕX174 releases unbroken DNA. In contrast, the DNA from phage ϕX174 inactivated by ozonized cyclohexene is broken.

The inactivation of aerosolized phage ϕX174-DNA was studied in the same atmospheres using ^{32}P-labelled DNA. ϕX174-DNA aerosolized in clean air or air containing cyclohexene at 75% r.h. is inactivated by a factor of 2 in 30 min. The inactivated DNA is broken. Ozone as well as ozonized cyclohexene inactivates DNA very fast causing breaks in the molecule. This is in contrast with the intact bacteriophage in which ozone does not produce breaks in the DNA.

INTRODUCTION

Primary air pollutants undergo photochemical reactions giving various secondary pollutants, some of which are toxic. Much attention has been paid to ozone which is generally regarded as the most important photochemical toxicant in air pollution. Only a few papers deal with the toxicity of reaction products of ozone with primary or secondary air pollutants. Haagen-Smit *et al.* (1952) reported that symptoms of plant damage resembling those occurring in the field, could be produced in test plants by the reaction products of certain olefins with ozone. Arnold (1959) also described the toxic action of ozonized olefins. The properties of the so called 'open air factor' (OAF) (Druett & May, 1968) could be explained by assuming that OAF is identical with the reaction products of ozone and olefins. In laboratory experiments the behaviour of OAF and ozonized olefins proved to be the same (Dark & Nash, 1970) and indirect evidence has been obtained by relating the survival of *Escherichia coli* measured in the open air to the concentrations of ozone and hydrocarbons (de Mik & de Groot, 1977).

The finding that certain ozonized olefines are more toxic for some organisms than ozone itself warrants an investigation of the inactivation mechanism. In a previous paper (de Mik, de Groot & Gerbrandy, 1977) the effects of ozone, trans-2-butene, ozonized trans-2-butene, cyclohexene, and ozonized cyclohexene on the survival of aerosolized bacteriophage ϕX174 were described. This phage was selected for a study of the OAF-induced inactivation of a simple biological system containing only DNA and proteins as possible target molecules.

It was shown that phage ϕX174 sprayed from distilled water is very sensitive to ozonized cyclohexene. In order to investigate whether DNA or the proteins are primarily damaged the study presented in this paper was carried out.

MATERIALS AND METHODS

Labelling of the phage with ^{32}P

E. coli C was employed as the host bacterium. *E. coli* C was transferred from an overnight culture into a medium with a low phosphate concentration (X-medium) and cultured with shaking at 37 °C to a titre of approximately 10^9 cells/ml. 10 μCi $^{32}PO_4$ (Radiochemical Centre, Amersham, England), previously incubated with catalase in X-medium to remove peroxides, were added per ml of culture. The culture was infected with 5×10^9 plaque forming units (p.f.u.) of ϕX174 per ml and incubated for 45 min. The lysate was centrifuged 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 shaken vigorously 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, containing 2×10^{11} ϕX174 p.f.u./ml with a specific activity of 4×10^{-5} cts/min/p.f.u. was further purified by centrifugation for 3 h at 24000 rev./min (average 76000 *g*) in a 5–23 % (w/v) linear sucrose gradient (SW27 rotor of the Beckman Spinco L). 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 distilled water. The final stock thus obtained contained $6{\cdot}5 \times 10^9$ p.f.u./ml with a specific activity of $3{\cdot}4 \times 10^{-6}$ cts/min/p.f.u.

Bacteriophage assay

Viable phage was assayed by the agar layer method of Adams (1959). Bottom and top agar were prepared as described by Zahler (1958).

Phage ϕX174-DNA

After recovery of the purified phage from the sucrose gradient used in the final purification step, the preparation was dialysed against 0·025 M phosphate buffer (pH 7·0). The DNA was isolated by three successive extractions with phenol saturated with buffer as described by Sinsheimer (1959). The phenol was removed by extraction with ether and the ether was removed by bubbling nitrogen through the solution. The DNA was then dialysed against 0·025 M phosphate buffer

(ph 7·0) for 3 days in bags that had been boiled in distilled water for 5–10 min to reduce contamination of the solution with impurities from the bags.

For isolation of DNA from samples of aerosolized phage, the same procedure was used.

Assay of biological activity of φX174-DNA

The procedure described by Blok, Luthjens & Roos (1967) was closely followed. Spheroplasts were prepared according to the method of Guthrie & Sinsheimer (1963). The media used were the same as described by these authors. Samples of DNA were diluted to 0·5 μg/ml before incubation. Titrations were carried out in duplicate.

Gradient analysis

The phage or DNA samples were layered on top of a 5–23 % (w/v) linear sucrose gradient (1 M-NaCl + 0·02 M sodium citrate, pH 7). The gradients were centrifuged in the SW27 rotor of the Spinco L3 ultracentrifuge for 3·5 or 15 h at 24000 rev./min and 5 °C. After centrifugation the bottom of the tube was pierced with a needle through which carbon tetrachloride was introduced at a constant rate. Fractions were collected through a conical glass tube fitted over the upper end of the centrifuge tube (Van der Schans & Aten, 1969).

Radioactivity measurements

Phosphorus-32 was counted in a liquid scintillation counter (Nuclear Chicago type Mark I or Mark II) using Czerenkov radiation.

Aerosol equipment

Aerosols were generated with a spray gun type FK8 and stored in a double-walled tank of stainless steel with a volume of 2000 l of air (de Jong & Winkler, 1968). After each experiment the air was pumped out of the tank and replaced by sterile air of the desired r.h. and temperature. Phage φX174 was nebulized from distilled water whereas DNA was nebulized from 0·025 M phosphate buffer (pH 7·0). Aerosol samples were taken by using the lower stage of May's multistage liquid impinger (May, 1966) containing 10 ml collection fluid. Phage φX174 was collected in borate–versene buffer, whereas DNA was collected in 0·025 M phosphate buffer (pH 7·0). The sampling time was 5 or 10 min at a rate of 55 l/min.

Since the phage and DNA were labelled with radioactivity the fraction of survivors could be easily calculated by titrating the samples for biological activity and measuring the radioactivity.

Ozone was generated by passing pure oxygen over an ultraviolet lamp. The ozone was introduced continuously through a Teflon tube into the tank at a rate of 140 l/h throughout the exposure of the phage resulting in a steady increase during the experiment from about 20 to 80 parts/10^9. The ozone concentration in the tank was measured continuously during the experiment (see fig. 3 in de Mik *et al.* 1977).

Cyclohexene (BDH Chemicals, England) was evaporated simultaneously with the

aerosolization of the phage by introducing 20 μl of cyclohexene in the barrel of the spray gun, thus giving a concentration in the tank of 2·4 parts/10^6, under the assumption that all cyclohexene vaporizes. In the presence of cyclohexene part of the ozone was consumed by reaction with cyclohexene resulting in an equilibrium concentration of ozone of about 20 parts/10^9 as shown in fig. 5 of an earlier paper (de Mik *et al.* 1977).

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. X-medium was prepared by dissolving 1 g NH_4Cl, 2 g NaCl, 0·61 g $MgSO_4.7H_2O$, 0·5 g KCl, 6 g Tris (hydroxymethyl) aminomethane, 0·01 g gelatine, 30 g glycerol, and 150 ml of a 10% solution of dephosphorylized casamino acids per l. After sterilization 0·3 ml 1 M-$CaCl_2$ and 16 ml 0·01 M phosphate buffer (pH 7·1) were added per l.

RESULTS

Inactivation of phage $\phi X174$ in different atmospheres

Six ml of phage suspended in distilled water ($6{\cdot}5 \times 10^9$ p.f.u./ml with a specific activity of $3{\cdot}4 \times 10^{-6}$ cts/min) were sprayed in clean air at 70% r.h. Samples were taken from 1 to 6 min (sample 1) and from 30 to 40 min (sample 2) after the beginning of the exposure time. The surviving fraction is given in Fig. 1. The decay rate is low.

Again 4·5 ml of phage suspension was sprayed in clean air at 54% r.h., simultaneously with 20 μl of cyclohexene. The surviving fractions in the samples from 1 to 6 min (sample 3) and from 30 to 40 min (sample 4) are shown in Fig. 1. In another experiment the survival was measured in the presence of cyclohexene at 70% r.h. giving surviving fractions of 0·46 and 0·35 respectively, showing that cyclohexene does not decrease the survival at 70% r.h.

Again 6·4 ml of phage suspension was sprayed in clean air containing 40 parts/10^9 of ozone at 70% r.h. During the exposure of the phage ozone was supplied continuously resulting in a concentration increase up to 110 parts/10^9 at the end of the exposure time, as shown before. As was previously shown (de Mik *et al.* 1977), the inactivation rate of ϕX174 is practically independent of the ozone concentration above 30 parts/10^9. The surviving fractions in this atmosphere are given in figure 1 (sample 5 and sample 6). Evidently, the decay rate is appreciably increased by ozone.

Finally 6·5 ml of phage suspension was sprayed in clean air containing 40 parts/10^9 of ozone at 70% r.h., simultaneously with 20 μl of cyclohexene. Here also ozone was supplied continuously but owing to the reaction with cyclohexene the ozone concentration decreased until an equilibrium was reached between ozone supply and ozone consumption at about 20 parts/10^9 of ozone as shown before. The surviving fractions of the phage in the samples from 3 to 8 min (sample 7) and from 30 to 40 min exposure (sample 8) are shown in Fig. 1. A very high decay rate is observed.

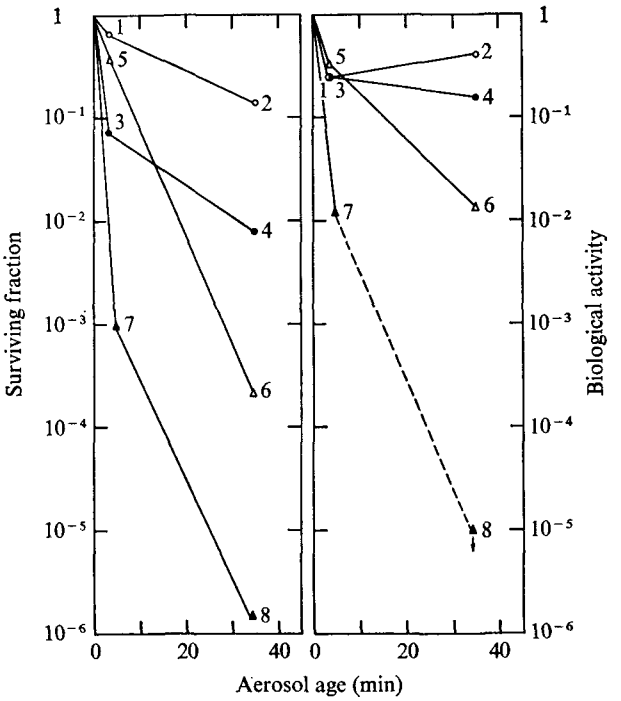

Fig. 1 Fig. 2

Fig. 1. Survival of bacteriophage φX174 sprayed from distilled water and collected in borate–versene buffer. ○——○, Clean air with a r.h. of 70%; ●——●, air containing 2·4 parts/10^6 of cyclohexene with a r.h. of 54%; △——△, air containing 40–110 parts/10^9 of ozone with a r.h. of 70%; ▲—▲, air containing 2·4 parts/10^6 of cyclohexene and 40–27 parts/10^9 of ozone with a r.h. of 70%. Samples1–8 were taken from 1–6, or 3–8 min and from 30 to 40 min.

Fig. 2. Biological activity of DNA extracted from the phage samples taken in the experiment of Fig. 1. The symbols and numbers correspond to those of figure 1. (↕ means that the fraction of the biological activity is lower than 10^{-5}).

Biological activity of the DNA of the collected phage φX174

Part of the samples 1–8 taken in the above-mentioned experiments (see Fig. 1) was used for the extraction of the DNA from the phage to measure the biological activity. The activity of the DNA obtained by extraction of the labelled phage stock was used as the reference and taken as 1. If the inactivation of the phage is only due to protein damage, the biological activity of the extracted DNA would be expected to be unaffected and nearly equal to 1. If, however, DNA is the target molecule, then the inactivation of DNA will be decreased, in proportion to the inactivation of the virus.

The biological activities measured in the various samples are given in Fig. 2. A comparison of these data with those presented in Fig. 1 shows that inactivation of the DNA is generally equal to or smaller than that of the virus.

Sedimentation of the aerosolized phage φX174 in sucrose gradients

Parts of the samples of phage φX174, the surviving fractions of which are given in Fig. 1, were centrifuged in a sucrose gradient for 3.5 h at 24000 rev./min. The

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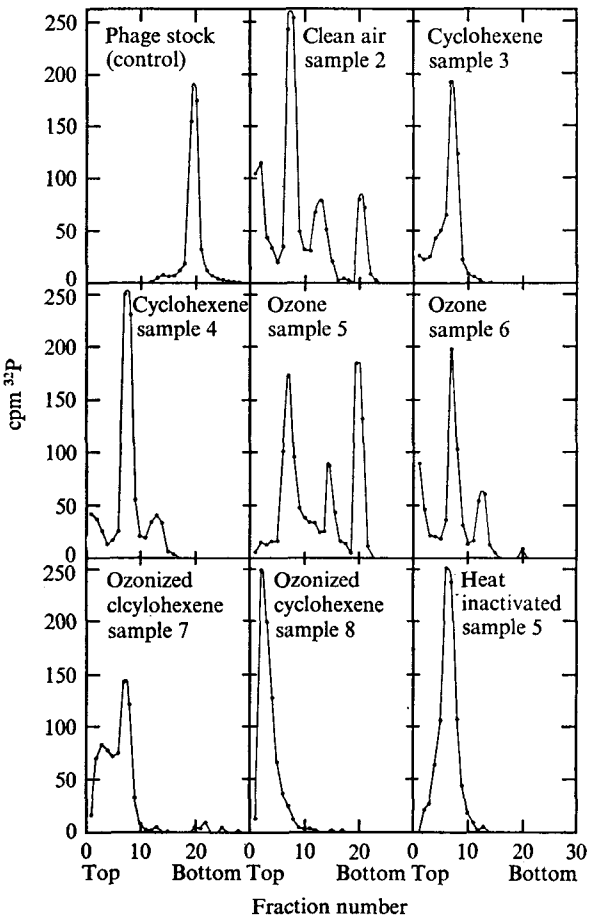

Fig. 3. Sucrose gradient analysis of radioactive phage ϕX174 before and after aerosolization from distilled water into different atmospheres and collected in borate–versene buffer. The sample numbers refer to the samples of which the surviving fractions are given in Fig. 1 and of which the biological activities of the DNA are given in Fig. 2. The samples were centrifuged for 3·5 h at 24000 rev./min.

radioactivity measurements of the different fractions obtained from the gradient are given in Fig. 3. The sample numbers refer to those in Figs. 1 and 2.

The sedimentation pattern of the phage stock is shown in Fig. 3 (top, left). All radioactivity was found at the site of intact phage and no free DNA was detectable as could be expected since the phage has been purified by centrifugation.

Most samples of aerosolized phage show radioactivity at the site of free DNA (fractions 6–9) and also intermediate peaks which are probably due to DNA with some protein attached. Sample 2 (clean air, 35 min) and sample 5 (ozone, 3·5 min) still contain radioactivity at the site of intact phage (fractions 20–25) but also appreciable quantities of free DNA and intermediates. The percentage activity at the site of intact phage in these gradients corresponds fairly well with the surviving fractions (sample 2: radioactivity 11%, survival 10%; sample 5: radioactivity 28%, survival 37%). Samples 3 and 4 (cyclohexene, 54% r.h., 3·5 and 35 min)

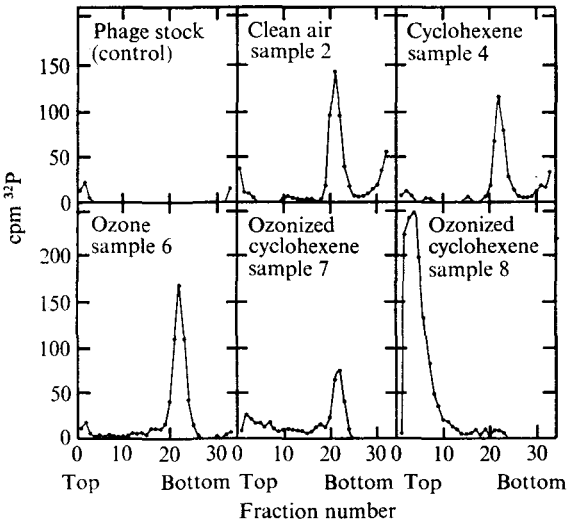

Fig. 4. Sucrose gradient analysis of the different samples (see Fig. 1) centrifuged for 15 h at 24000 rev./min.

contained mainly free DNA. In sample 6 (ozone, 35 min) all intact phage has disappeared. Apart from free DNA and protein-containing intermediates there is some radioactivity at the top of the gradient, which probably represents degraded DNA (see below). Sample 7 (ozone + cyclohexene, 6 min) contains both undegraded and degraded DNA whereas sample 8 (ozone + cyclohexene, 35 min) appears to contain degraded DNA only.

Evidence that the intermediate peaks are due to DNA with protein attached (samples 2, 4, 5 and 6) was obtained by heating a part of sample 5 for 30 min at 75 °C before gradient analysis. The result is shown in Fig. 3 (bottom, right). Obviously all radioactivity is measured at the site of free DNA probably due to release of protein.

To obtain further information about the size of the DNA in the samples, parts of these were centrifuged in a sucrose gradient for 15 h at 24000 rev./min. The sedimentation patterns of the samples 2, 4, 6, 7 and 8, together with that of the phage stock are given in Fig. 4. As has already been shown, the phage stock contains no free DNA. The radioactivity in the samples 2, 4 and 6 was found at the site of intact φX174-DNA, whereas the DNA of sample 8 still remained at the top of the gradient, which shows that the DNA was degraded. In sample 7 (Fig. 4, bottom, middle) part of the DNA was degraded and another part was still intact.

Inactivation of phage φX174-DNA in different atmospheres

To obtain further information about the mechanism of inactivation by ozone and ozonized cyclohexene, radioactively labelled DNA was sprayed from 0·025 M phosphate buffer (pH 7·0). The atmospheres in which the inactivation of DNA was measured were similar to those already mentioned for phage φX174. The r.h. of the air was 72–76 %. DNA was sampled at times 2–7 min, 15–20 min and 30–40 min

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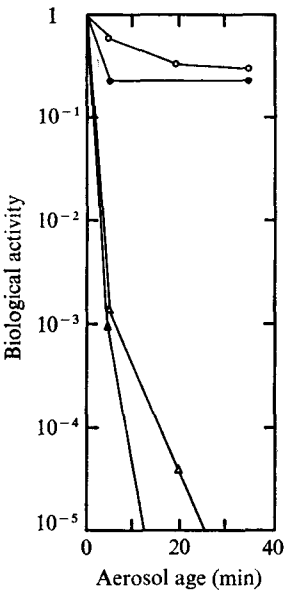

Fig. 5. Aerosol inactivation of ϕX174-DNA after spraying from 0·01 M phosphate buffer (pH 7·2). ○——○. Sprayed in clean air at 74% r.h.; ●——●, sprayed in air containing cyclohexene at 76% r.h.; △——△, sprayed in air containing ozone at 76% r.h.; ▲——▲, sprayed in air containing cyclohexene as well as ozone at 72% r.h. Phage was collected in phosphate buffer.

after the beginning of the exposure in 0·025 M phosphate buffer (pH 7·0). The biological activities measured in the samples are given in Fig. 5.

In clear air or air containing cyclohexene only, a slow inactivation was observed. On the other hand, a very fast inactivation was found in air containing ozone or ozonized cyclohexene. In contrast with the results obtained with phage ϕX174 (Figs. 1, 2) practically no difference in inactivation between these two atmospheres could be detected.

Sedimentation of the aerosolized DNA in sucrose gradients

The different samples were also analysed by centrifugation for 15 h at 24 000 rev./min. The results are summarized in Fig. 6. Before aerosolization the DNA showed only one peak (Fig. 6A). When nebulized in clean air (Fig. 6B) the DNA is partially degraded. However, the percentage radioactivity in the intact DNA-peaks does not vary during the exposure (45·9, 45·0 and 47·5% at 4·5, 17·5 and 35 min, respectively) and is comparable with the biological activity measured (60, 34 and 30%, Fig. 5). The same phenomenon was found when DNA was aerosolized in air containing cyclohexene (Fig. 6C). The percentage radioactivity in the peaks at the site of intact DNA was 37·5 and 26·8 at 4·5 and 35 min respectively, whereas the percentage biological activity was 23 in both cases (Fig. 5).

The sedimentation patterns of DNA exposed to ozone or ozonized cyclohexene are similar (Figs. 6D, E). In both atmospheres DNA is degraded. In contrast with the results obtained in clean air or air containing cyclohexene only (Figs. 6B, C), the percentage of degraded DNA is time-dependent.

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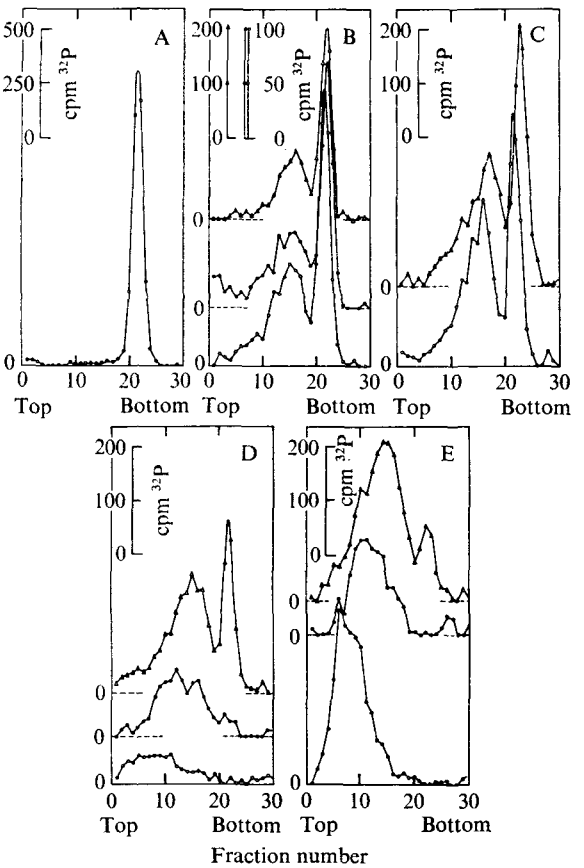

Fig. 6. Sucrose density gradient analysis of radioactive φX174-DNA before and after aerosolization. The DNA was sprayed and collected in 0·025 M phosphate buffer (pH 7·0). (A) DNA before aerosolization. (B) DNA sprayed in clean air at 74% r.h. (C) DNA sprayed in air containing cyclohexene at 76% r.h. (D) DNA sprayed in air containing ozone at 76% r.h. (E) DNA sprayed in air containing ozone and cyclohexene at 72% r.h. △——△, Sample taken at 2–7 min after aerosolization; ●——●, sample taken at 15–20 min; ○——○,, sample taken at 30–40 min.

To exclude the possibility that the air in the tank was contaminated with other olefins which may react with ozone so as to give the same effect as ozonized cyclohexene, the tank was filled with clean air and ozone was supplied for 20 h to burn up all olefins present. Even in this cleaned atmosphere, no difference was observed between the DNA damage inflicted by ozone or ozonized cyclohexene.

DISCUSSION

Inactivation in clean air

The inactivation of aerosolized viruses and phages in clean air has been studied by several investigators. Dubovi (1971) showed that the biological activity of the nucleic acids of phage φX174 and phage MS-2 was not damaged by atomization, aerosol storage, or collection, although he could not exclude the possibility of

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damage to the nucleic acid through severe distortions of the viral protein(s). Trouwborst & de Jong (1972), studying the inactivation of phage T_1 in aerosols, found free DNA in the samples which were subjected to sedimentation centrifugation suggesting that the protein coat becomes separated from the DNA. They supposed that the separation of coat protein and DNA did not occur in the gradient because the radioactivity (^{32}P) of the aerosol-inactivated T_1 phage was unadsorbable to bacteria before gradient analysis.

De Jong, Harmsen, Trouwborst & Winkler (1974), in studies of encephalomyocarditis (EMC) virus, observed that during virus inactivation in aerosols the virus RNA is released in a free, infectious form. On the other hand, the antigenic structure of the hemagglutinin seemed to be destroyed. The fact that pure proteins can be denatured by aerosolization was shown for the enzyme trypsin by Trouwborst (1971). These findings are strong indications that protein is the main target molecule for aerosol inactivation of lipid-free viruses.

Our results also suggest that in clean air the protein of the phage is damaged during exposure. The DNA extracted from recovered phage is biologically active (samples 1 and 2 in Figs. 1 and 2). The biological activity does not differ greatly from the activity of the DNA extracted from unexposed phage. As the spray-gun may produce some damage different from the damage occurring during exposure, the DNA extracted from sample 1 is perhaps a better reference for sample 2. In any case the biological activity of the DNA does not decrease during exposure.

Gradient analysis (sample 2 in Fig. 3) shows the separation of free DNA from the protein. This separation is incomplete as DNA with adhering protein is still present. The separation of DNA from the protein can hardly occur in the droplet nuclei in the aerosol. Furthermore the much stronger influence of ozone on free DNA (Fig. 5) than on phage (Fig. 1, sample 6) shows that the DNA in the phage is still partially protected by the protein. We therefore believe that the separation of DNA and protein occurs during collection and dehydration rather than during exposure.

When ϕX174-DNA is sprayed from phosphate buffer at 75% r.h., about 70% is inactivated (Fig. 5). The inactivated DNA is broken as shown in Figs. 6B and C. These breaks may be caused either by shear forces in the spray gun, although this circular DNA is not very sensitive to shear, or during dehydration. Break introduction by collection is unlikely since the DNA from the phage samples (Fig. 4, samples 2, 4 and 6) does not show any breaks.

Inactivation in air containing cyclohexene

It was shown in a previous paper (de Mik *et al.* 1977) that cyclohexene alone has no effect on the aerosol inactivation of phage ϕX174. It is unfortunate that the experiment with cyclohexene in this series was taken at 56% r.h. If it is, however, accepted that the lower survival in samples 3 and 4 in Fig. 2 is due to the lower relative humidity and not to the cyclohexene it is again clear that the damage during exposure is mainly due to protein damage. Comparison between samples 3 and 4 in Figs. 1 and 2 show that the biological activity of the DNA does not decrease during exposure. At this lower r.h. the separation of DNA and protein

during collection is more complete as shown by the gradient analysis (samples 3 and 4 in Fig. 3) where intermediate peaks are lacking.

Inactivation in air containing ozone

Ozone is known to be a strong oxidizing agent which reacts with many important biochemical substances. Proteins are inactivated directly by reaction of ozone with susceptible amino acid residues. Among the amino acids, the most susceptible are cysteine, tryptophan and methionine (Mudd, Leavitt, Ongun & McManus, 1969). The susceptibility of sulphydryl compounds to ozone was demonstrated by Menzel (1971) with the enzymes papain and glyceraldehyde 3-phosphate dehydrogenase. There is evidence that ozone may also react with DNA. Ozone has been shown to appreciably modify pyrimidine bases in *E. coli* nucleic acids (Prat, Nofre & Cier, 1969). Christensen & Giese (1954) studied the effect of ozone on nucleic acids, nucleotides, nucleosides, purines and pyrimidines in solution. The adsorption spectra of all these compounds were changed by ozone. The effects of ozone on nucleic acid were ascribed to its effects on the constituent purines and pyrimidines, each of which appeared to be individually affected.

Ozone affects the survival of phage φX174 as shown in Fig. 1. The survival decreases by a factor of 10^3–10^4 within 40 min. Such a high inactivation rate is typical for phage sprayed from distilled water in which no other compounds are present to react with ozone, thus protecting the phage.

The data show that ozone mainly inactivates the protein since the fraction of the biological activity of the extracted DNA after 30–40 min exposure time is 100 times higher than the surviving fraction. Compared with clean air there is, however, an appreciable decrease of the biological activity of the DNA. Part of the DNA seems to become exposed owing to the heavy damage to the protein coat.

In comparison with the effect of ozone on the phage, the effect on aerosolized phage DNA is dramatic (Fig. 5). Apart from being inactivated very fast, the DNA is also degraded as shown in Fig. 6D. It is concluded from these data that the protein coat protects the DNA when the phage is exposed to ozone. This is only possible if the DNA is not released during exposure, since otherwise the ozone would destroy its biological activity; this is not observed (Fig. 4, sample 6). This would imply that the DNA and protein are mainly separated during collection and rehydration. Although, at this ozone concentration, the protein protects the DNA of the phage, it obviously cannot prevent the phage from being damaged to some extent. It may be that, when part of the susceptible amino acids have reacted, the structure of the phage is altered in such a way as to allow ozone to react with DNA, causing some damage. This damage, however, although resulting in a decreased activity of the DNA does not give breaks as shown in gradient analysis.

Inactivation in air containing ozonized cyclohexene

Ozonized cyclohexene inactivates the phage as well as its purified DNA very fast due to both protein and DNA damage (Figs. 1, 2). Comparison of the results presented in Figs. 2 and 4 with those presented in Figs. 5 and 6E shows that the

protein hardly protects the DNA when the phage is exposed to ozone and cyclohexene. In these experiments a large excess of olefin was used. Assuming that the particles generated by the spray gun are surrounded by cyclohexene, the effect of ozone itself may be neglected (Arnold, 1959), especially during the first few minutes. The biological effect observed is thus due to the reaction product of ozone with cyclohexene. Whatever this product may be, it reacts very effectively with DNA.

Ozone has been recognized as a very toxic agent in the environment. This has led to a great number of papers dealing with the effects of ozone on micro-organisms, plants, animals and human beings. In comparison with ozone itself, the products of certain ozonized olefins must be categorized as very dangerous indeed. The question whether these products also react with the DNA of bacteria will be the subject of further studies.

The authors wish to thank K. C. Winkler and H. C. Bartlema for their stimulating discussions.

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