Antivirus Action of Acrolein, Glutaraldehyde and Oxidized Spermine

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SUMMARY

The inactivation of bacteriophages and myxoviruses by oxidized spermine, acrolein and glutaraldehyde has been studied. Oxidized spermine inactivated T5 and not T2 coliphages, while acrolein was more effective against T-even phages. Myxoviruses also differed in their sensitivity to the various aldehydes. This was determined by plaque counting or by assaying the haemagglutination activity after propagation in embryonated eggs.

High-molecular (condensation?) products, which were formed when spermine was oxidized for several hours by small quantities of enzyme also inactivated viruses.

These experiments stress the uniqueness of oxidized spermine as a virucidal agent, and do not support the hypothesis that its antivirus activity is due to the formation of acrolein as a spontaneous degradation product.

INTRODUCTION

Amine oxidase, purified from bovine plasma, catalyses the oxidation of the naturally occurring polyamines spermine, \( \text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2 \), and spermidine, \( \text{NH}_2(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2 \) (Tabor & Tabor, 1964; Bachrach, 1970). Previous studies indicated that the iminodialdehyde

\[
\text{O} \quad \text{C}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{C}/\text{O}
\]

and the monoaldehyde

\[
\text{H} \quad \text{C}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{C}/\text{H}
\]

are the primary oxidation products of spermine and spermidine, respectively (Tabor, Tabor & Bachrach, 1964a). These aldehydes appear to be unstable (Alarcon, 1964, 1966, 1968, 1970; Tabor et al. 1964a; Tabor et al. 1964b), mainly when incubated at elevated temperatures and alkaline pH (Kimes & Morris, 1971a). One source of this instability seems to be a \( \beta \)-elimination reaction to form acrolein

\[
\left( \text{CH}_2 = \text{CHC}/\text{O} \right)
\]

at 20 to 30 % yield (Alarcon, 1970), and other condensation reactions to yield polyiminoaldehydes (Kimes & Morris, 1971a). It has been shown that oxidized spermine inactivates certain bacterial viruses, mainly coliphages of the T-odd series (Bachrach, Tabor & Tabor, 1963; Bachrach & Leibovici, 1965, 1966; Fukami et al. 1967; Oki et al. 1968, 1969; Yamada et al. 1968), and is also toxic for plant (Bachrach et al. 1965) and animal viruses (Katz et al. 1966).
The toxicity of oxidized spermine for different tumour cells has also been reported (Alarcon, Foley & Modest, 1961; Halevy, Fuchs & Mager, 1962; Israel, Rosenfield & Modest, 1964; Bachrach, Abzug & Beikerkunst, 1967; Higgins et al. 1969). Alarcon (1964) explained the cytotoxicity of oxidized spermine in terms of acrolein formed by its degradation and showed that the ID50 of acrolein for S-180 cells in culture was $3 \times 10^{-2}$ #mole/ml., compared with $1.5 \times 10^{-2}$ #mole/ml. for oxidized spermine. Recent studies (Kimes & Morris, 1971 b) also indicated that acrolein inhibits nucleic acid synthesis in bacteria, as does oxidized spermine (Bachrach & Persky, 1969) or its analogue (Bachrach & Rosenkranz, 1969).

This study compares the antiviral activity of oxidized spermine with that of acrolein and the dialdehyde glutaraldehyde. It is shown that the virucidal activity of oxidized spermine is distinct from that of the other aldehydes tested. Thus, the biological activity of oxidized spermine is not simply explained by spontaneous degradation to acrolein.

**METHODS**

**Chemicals.** Spermine tetrahydrochloride was obtained from Fluka Ag, Buchs, Switzerland. Glutaraldehyde 25 % (v/v) was supplied by K and K laboratories, Plainview, New York, U.S.A. Acrolein (stabilized by hydroquinone) was the product of BDH Chemicals Ltd, Poole, England. Catalase as obtained from Boehringer and Soehne, Mannheim, W. Germany.

Serum amine oxidase was prepared according to Tabor, Tabor & Rosenthal (1954), omitting the last calcium phosphate gel step. The preparation was purified 150- to 160-fold to a specific activity of 100 spectrophotometric units/mg. (Tabor et al. 1954). Spermine was oxidized in a Warburg flask containing 0.2 ml. of amine oxidase (110 units, unless otherwise stated); 1.0 ml. of o.2 M-tris HCl buffer (pH 7.0 to 7.2); 0.1 ml. of catalase (750 units); 1.2 ml. of physiological saline (NaCl 0.85 %, w/v) and 0.5 ml. of spermine (10 #moles).

Condensation products, formed during the oxidation of spermine, were removed from low molecular weight components by filtration through Diaflo UM-2 or UM-05 ultrafiltration membranes (Amicon, Den Haag, Holland), which retained compounds with a molecular weight above 1000 or 500, respectively.

**Viruses and biological materials.** Coliphages and their host (Escherichia coli b) were used as described previously (Bachrach & Leibovici, 1965). Bacteria were grown in glycerol lactate medium (Hershey & Chase, 1952) and phages assayed by the agar layer plating method (Adams, 1959). Phages were inactivated by incubating 0.2 ml. quantities of phage suspensions (usually $10^9$ p.f.u., in glycerol lactate medium) at 37° for 90 min. with 0.2 ml. portions of the inactivating agents, or with nutrient broth in the control experiments.

The PR 8 strain of influenza A virus, and Newcastle disease (NDV) (strain v) were those used previously (Bachrach & Don, 1971). They were inactivated by incubating virus suspensions with equal volumes of inactivating agents, or nutrient broth in the control experiments. After intermittent shaking at 37° for indicated times, the infectivities of the viruses were assayed either by plaque counting or by determination of haemagglutination activity after propagation in embryonated eggs.

**Assay of haemagglutination activity.** Samples (0.2 ml.) of virus suspensions were injected into the allantoic cavities of 9- to 12-day-old chick embryos (4 to 6 eggs/sample). Allantoic fluids were harvested after 48 hr and tested for virus multiplication by haemagglutination assay. Serial twofold dilutions in phosphate-buffered saline (PBS), pH 7.2, were made in plastic trays (Linbro Chemical Co, New Haven, Connecticut, U.S.A.) by the microtitre technique (Sever, 1962). Thereafter, 0.05 ml. of 0.5 % washed chicken red blood cells in
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physiological saline (pH 7.2), was added to each well. After standing at room temperature for 60 min., the results were read (Salk, 1944), and the activity taken as the reciprocal of the highest dilution of virus suspension showing complete haemagglutination.

Cells. Monolayer cultures of primary chick embryo fibroblasts (CEF) were prepared by digestion of 10-day old decapitated chick embryos with 0.25 % trypsin. Plastic tissue culture plates (60 mm.) were seeded with cells suspended in medium 199 supplemented with 5 % inactivated calf serum, 100 units/ml. penicillin, and 100 μg./ml. streptomycin. Confluent monolayers were obtained after incubation for 48 hr at 37° in an atmosphere of 5 % CO₂ in air.

Plaque titration. Newcastle disease virus (v strain) was titrated on CEF monolayers. After washing with PBS each monolayer was inoculated with 0.2 ml. of virus suspension. After adsorption for 40 min. at 37°, monolayers were overlaid with 5 ml. of 1 % agar in Eagle's minimal medium containing 2 % calf serum (final concentration) and antibiotics. Plaques were counted after incubation in 5 % CO₂ and air at 37° for 3 days.

RESULTS

Inactivation of coliphages

To compare the antiphage activity of oxidized spermine with that of acrolein, each compound was incubated at 37° for 90 min. with various phages, whose infectivity was then tested by the agar layer method. Oxidized spermine (1.65 μmoles/ml.-prepared by oxidation with 110 units of enzyme) inactivated T₅ phages, as observed by the reduction of infectivity by 4.0 log units (Table I). The T₂ phages were not sensitive. This observation confirms previous findings (Bachrach & Leibovici, 1965).

The effect of acrolein on coliphages was markedly different: T-even phages were more sensitive than T₅ phages (Table I).

Table 1. Antiphage activity of oxidized spermine and acrolein

<table>
<thead>
<tr>
<th>Inactivating agent</th>
<th>T₂</th>
<th>T₄</th>
<th>T₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, phage only</td>
<td>1.7 × 10⁸</td>
<td>6.0 × 10⁸</td>
<td>8.7 × 10⁸</td>
</tr>
<tr>
<td>Oxidized spermine, 1.65 μmoles/ml.</td>
<td>1.7 × 10⁸</td>
<td>5.9 × 10⁸</td>
<td>6.9 × 10⁸</td>
</tr>
<tr>
<td>(slow oxidation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrolein: 4.0 μmoles/ml.</td>
<td>1.3 × 10⁹</td>
<td>5.6 × 10⁹</td>
<td>8.6 × 10⁹</td>
</tr>
<tr>
<td>2.0 μmoles/ml.</td>
<td>1.5 × 10⁹</td>
<td>1.2 × 10⁹</td>
<td>1.7 × 10⁹</td>
</tr>
<tr>
<td>1.0 μmoles/ml.</td>
<td>3.0 × 10⁹</td>
<td>2.8 × 10⁹</td>
<td>5.4 × 10⁹</td>
</tr>
</tbody>
</table>

Phages (0.2 ml.) were incubated at 37° for 90 min. with equal volumes of inactivating agents or nutrient broth and then assayed by the agar layer method.

Inactivation of myxoviruses

We have shown (Bachrach & Don, 1970, 1971) that certain myxoviruses (influenza virus (PR₈), NDV and Sendai virus) are inactivated by oxidized spermine. It was therefore of interest to determine whether inhibition was specific to oxidized spermine or common to a number of other aldehydes and dialdehydes. In the following experiment we compared the sensitivity of these myxoviruses to spermine, acrolein and glutaraldehyde

\[
\left(\overset{O}{\text{H}}\overset{\text{C(CH₃)}}{\text{O}}\overset{\text{C}}{\text{H}}\right)
\]

a dialdehyde which inactivates some animal viruses (Sabel, Hellman, McDade, 1969; Kremzner & Harter, 1970). Influenza virus (PR₈ strain) or NDV (v strain) was incubated at
37° for 24 hr with the drugs at various concentrations, and virus infectivity was tested by inoculation into embryonated eggs, followed by titration of haemagglutinin in the allantoic fluids. Even though haemagglutinin production was not always proportional to the amount of virus inoculated, the activity of a drug could be estimated from the formation of haemagglutinins after injection of undiluted reaction mixtures into chick embryos. Absence or

Table 2. Effect of various aldehydes on the infectivity of myxoviruses

<table>
<thead>
<tr>
<th>Inactivating agent (µmole/ml.)</th>
<th>Haemagglutination activity as % of untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Newcastle virus</td>
</tr>
<tr>
<td>Oxidized spermine: 0.4</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>(slow oxidation): 0.2</td>
<td>82.0</td>
</tr>
<tr>
<td>Glutaraldehyde: 3.3</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>1.65</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Acrolein: 4.0</td>
<td>0.1</td>
</tr>
<tr>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td>1.0</td>
<td>8.0</td>
</tr>
<tr>
<td>0.5</td>
<td>20.0</td>
</tr>
<tr>
<td>0.25</td>
<td>61.0</td>
</tr>
</tbody>
</table>

Virus (192 HAU/0.5 ml.) in nutrient broth was incubated with the reagents at 37° for 24 hr. Samples were then injected into embryonated eggs and the haemagglutination activities determined at 48 hr as a measure of virus multiplication.

reduction of haemagglutinins may be due to inactivation, but haemagglutinins may be induced normally by inactivated viruses. Oxidized spermine prepared by oxidation with 110 units of enzyme for 4 hr and used at a concentration of 0.4 µmole/ml., inactivated both NDV and influenza viruses (Table 2) and no haemagglutinins were detected after propagation in embryonated eggs. Oxidized spermine at lower concentrations (0.2 µmole/ml.), partially inactivated influenza PR 8 and NDV; the respective haemagglutination activities were 82 and 11 % of the control levels.

Glutaraldehyde, at 1.65 µmoles/ml. and acrolein at 2.0 µmoles/ml., were less effective since residual infectivity of virus was detected (Table 2).

To study the antivirus activity of the different aldehydes through the rates of inactivation, suspensions of NDV (v strain, 5120 HAU/ml.) were incubated with different concentrations of drug and the infectivity was determined by plaque assay at various times. As expected, oxidized spermine (prepared by oxidation with 110 units of enzyme for 4 hr), at a concentration of 1.65 µmoles/ml., inactivated NDV viruses by at least 5 log. units within 5 hr at 37° (Fig. 1). As our preparation of oxidized spermine contained 0.10 µmole/ml. of acrolein (determined by the m-aminophenol method, Alarcon, 1968), we examined the effect of this concentration of acrolein on the infectivity of NDV. Acrolein at 0.1 to 0.3 µmole/ml. (Fig. 1) had only a slight effect on the infectivity of the viruses and excluded the possibility that the toxicity of oxidized spermine was due to the spontaneous formation of acrolein.

Formation of high-molecular weight condensation products

Kimes & Morris (1971 a) emphasized the lability of oxidized spermine and demonstrated a half-life at 37° of approximately 42 min. It was also shown that condensation products were formed during the incubation of oxidized spermine at neutral pH. Our preliminary
Antivirus action of acrolein, glutaraldehyde and oxidized spermine experiments indicated that considerable amounts of these condensation products accumulated when a small quantity of serum amine oxidase (110 units) was used for oxidation over several hours. However, rapid oxidation (20 min.) by 1500 units of enzyme, produced no appreciable accumulation of condensation products.

To find out whether high-molecular weight products influenced the inactivation of viruses by oxidized spermine, the latter was filtered through a Diaflo UM-2 ultrafiltration membrane to remove compounds of a molecular weight higher than 1000. When spermine was slowly oxidized with 110 units of enzyme most of the activity which inactivated T5 phages was retained by the Diaflo membrane (Table 3) and was therefore associated with a molecular weight above 1000. When spermine was oxidized for 20 min. with 1500 units of enzyme, the antivirus activities of the Diaflo filtrates and upper fluids were similar (Table 3). It is also apparent (Table 3) that acrolein was not retained by a Diaflo UM-05 ultrafiltration membrane which retained compounds of molecular weight above 500.

The formation of high-molecular weight condensation products, after prolonged incuba-
tion (3 hr) of spermine with 110 units of enzyme, was also demonstrated with NDV. Again, at least half of the activity was retained by the Diaflo membrane, since the filtrate and the upper fluid reduced the infectivity of NDV to a similar extent (Table 4). Less high molecular condensation products were formed when spermine was oxidized for 20 min. with 1500 units of enzyme. This was demonstrated, when the antivirus activities of the Diaflo filtrates and upper fluids were compared (Table 4). It thus appears that high molecular weight products are formed during the incubation of oxidized spermine at 37° and that these products inactivate T5 phages and Newcastle disease virus.

Table 3. Inactivation of T5 coliphages by oxidized spermine and acrolein

<table>
<thead>
<tr>
<th>Inactivating agent</th>
<th>Phage infectivity (p.f.u./ml.) following treatment* with inactivating agent in</th>
<th>Phage infectivity before inactivation (p.f.u./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfiltered sample</td>
<td>Upper fluid</td>
</tr>
<tr>
<td>Oxidized spermine:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:65†</td>
<td>9.0 × 10^5</td>
<td>1.9 × 10^5</td>
</tr>
<tr>
<td>1:65§</td>
<td>1.2 × 10^6</td>
<td>1.8 × 10^7</td>
</tr>
<tr>
<td>Acrolein:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>1.6 × 10^5</td>
<td>—</td>
</tr>
<tr>
<td>4.0</td>
<td>2.2 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td>2.0</td>
<td>1.5 × 10^7</td>
<td>—</td>
</tr>
</tbody>
</table>

* Inactivating agents, 5.0 ml., were filtered through Diaflo membranes to pass 2.5 ml. Samples of the unfiltered sample filtrate or upper fluid were incubated at 37° with equal volumes (0.2 ml.) of T5 phage. After 90 min. the number of viable phage was determined by plating.
† Slow oxidation—110 units of enzyme, oxidation time 4 hr.
§ Filtered through a Diaflo UM-2 ultrafiltration membrane.
II Filtered through a Diaflo UM-05 ultrafiltration membrane.

Table 4. Inactivation of Newcastle disease virus by various preparations of oxidized spermine

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>Control</th>
<th>Slow oxidation</th>
<th>Fast oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude</td>
<td>Filtrate</td>
<td>Crude</td>
</tr>
<tr>
<td>0</td>
<td>2.2 × 10^7</td>
<td>1.1 × 10^7</td>
<td>1.3 × 10^7</td>
</tr>
<tr>
<td>1½</td>
<td>2.2 × 10^7</td>
<td>2.0 × 10^5</td>
<td>1.4 × 10^6</td>
</tr>
<tr>
<td>3</td>
<td>2.2 × 10^7</td>
<td>5.5 × 10^4</td>
<td>9.5 × 10^4</td>
</tr>
</tbody>
</table>

Oxidized spermine (3.0 ml.) prepared by oxidation with either 110 units of serum amine oxidase (slow oxidation) or with 1500 units (fast oxidation) was filtered to dryness through Diaflo UM-2 ultrafiltration membranes. Membranes were washed with 3.0 ml. PBS, and filtrates, upper fluids and unfiltered samples were incubated with NDV for times indicated. The infectivities of the virus suspensions were then determined by plaque assay.

DISCUSSION

It is accepted that spermine is oxidized to the iminodialdehyde

\[
\text{O}^\circ \text{H}^\circ \text{C(CH}_2\text{)}_2\text{NH(CH}_2\text{)}_2\text{NH(CH}_2\text{)}_2\text{C}^\circ \text{O}{\text{H}}^\circ
\]

but the fate of this labile compound is uncertain. Evidence has accumulated that acrolein is one of the degradation products of spermine, but its yield is never higher than 0.3 μmole/μmole, even in the presence of the trapping agent m-aminophenol (Alarcon, 1970). If the toxicity of oxidized spermine resides in the acrolein produced, then the latter ought to be at
least twice as active, on a molar basis, as oxidized spermine. The results described in this paper show that the antivirus activity of oxidized spermine differs quantitatively and qualitatively from that of either acrolein or glutaraldehyde. Alarcon et al. (1961), demonstrated the cytotoxicity of acrolein, but in their experiments oxidized spermine at half the molar concentration showed similar effects. It is to be expected that aldehydes are toxic for microorganisms and mammalian cells. Thus Carvajal & Carvajal (1957) reported that \( \beta \)-propyl-\( \gamma \)-butylalimine

\[
\text{CH(CH_2)_3NH(CH_2)_4C\text{--O}}
\]

was inhibitory for tubercle bacilli and Boyland (1940) showed that heptaldehyde and acrolein were toxic for mammalian cells. These results do not imply that the activity of these compounds is related to that of oxidized spermine.

It appears that the action of oxidized spermine does not resemble that of the other aldehyde tested. This hypothesis is supported by the observation that the inactivation of myxoviruses by oxidized spermine was temperature-dependent and negligible at 4°C (Bachrach & Don, 1971); in contrast, glutaraldehyde inactivated NDV and influenza PR 8 even at 4°C.

Our results strongly suggest that oxidized spermine gives rise to polymers which are at least as active as the iminodialdehyde (monomer?). We hope to identify this polymer as the result of condensation of the iminoaldehydes or as a copolymer which contains acrolein or putrescine. This question is of special interest as iminoaldehydes, or their condensation products, may provide a new class of antivirus and antitumour agents.

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REFERENCES


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