Water as a Factor in the Photoinactivation of Vaccinia Virus

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SUMMARY

High concentrations of glycerol and glucose decreased the rate of photoinactivation of vaccinia virus by methylene blue. These results could not be attributed to changes in the viscosity of the medium or to the impairment of the initial dye sensitization of the virus. Inhibition of photoinactivation by glycerol was not wholly dependent on $O_2$ concentration. Lyophilization of dye-sensitized virus prevented its subsequent inactivation by exposure to light. These results are interpreted as indicating that water is involved in the photoinactivation process, although its role is as yet obscure.

INTRODUCTION

The photodynamic inactivation of vaccinia virus is inhibited by glycerol (Turner & Kaplan, 1965). Further observations on this effect reported here suggest that water or its elements are necessary for photoinactivation.

METHODS

Unless otherwise stated, virus preparation, virus assay, illumination, light and dark controls, etc., were as described by Turner & Kaplan (1965).

Reaction mixtures

Reaction mixtures contained purified virus with an initial titre of $10^5-10^6$ plaque-forming units (p.f.u.)/ml. and methylene blue at a concentration of $3 \times 10^{-5}M$. Since maximal dye-sensitization and subsequent inactivation occur at high pH, most reaction mixtures were buffered with 0.01M-2-amino-2-hydroxymethyl-propane-1:3-diol (tris) to pH 9.0, which is within the stability limits of vaccinia virus.

Inhibition of photoinactivation

Inhibition was tested by including in the reaction mixtures suitable concentrations of the various substances. Control solutions were tested for their effect on the virus alone and on the cell monolayers used for virus assay.

Drying of photosensitized virus

Method 1. Gelatin was incorporated into reaction mixtures which were then dried in the dark over $P_2O_5$ as pellets or films as described by Lea (1962) for the X-irradiation of bacteriophages.
**Method 2.** Purified virus with an initial titre of $10^8$ p.f.u./ml. was diluted and irreversibly sensitized with $3 \times 10^{-5} \text{M}$-methylene blue at pH 9·0 (Wallis & Melnick, 1964). Free dye was removed with ion exchange resin (analytical grade Dowex AG. 50 W-X8, 200-400 mesh, Na form). Unsensitized control virus was similarly treated. One ml. volumes were snap-frozen and freeze-dried overnight. All procedures were conducted under the safelight.

**Gassing with air or oxygen**

When vigorous gassing was required, a departure was made from the previous system of illumination. Instead of being exposed in thin layers in 9 cm. Petri dishes, reaction mixtures were dispensed in 10 ml. volumes in 6 x 1 in. test tubes placed against a reflector of aluminium foil. The required gas was bubbled vigorously through the mixtures for 30 min. before, and then throughout, the illumination period.

**RESULTS**

**Effect of glycerol on photoinactivation**

The rate of photoinactivation was substantially decreased by glycerol, proportionally to its concentration (Fig. 1). The effect was the same with virus in the presence of free dye and with presensitized virus freed of excess dye by ion exchange resin. Some substances which inhibit photoinactivation act either by competing with virus for dye or by preventing its necessary entry into or attachment to virus (Yamamoto, 1958; Turner & Kaplan, 1965). No changes in the absorption spectrum of methylene blue occurred when glycerol was added to a concentration of 40% (w/v), indicating that interactions of dye with glycerol were absent (Kay, Walwick & Gifford, 1964). If virus was mixed with dye in the presence of 40% (w/v) glycerol and then resuspended in glycerol-free buffer it was as photosensitive as virus sensitized in buffer alone. It seems, therefore, that glycerol does not inhibit the dye attachment stage of photoinactivation.

**Effect of oxygen on glycerol inhibition**

Since oxygen is essential for photoinactivation glycerol might inhibit by decreasing the amount of dissolved oxygen in the reaction mixtures. Under conditions of vigorous oxygenation concentrations of glycerol up to 20% (w/v) did not inhibit photoinactivation, suggesting that the effects might be due to changes in the solubility of $O_2$. However, the inhibitory effect of 40% (w/v) glycerol was unaffected in reaction mixtures gassed with either air or oxygen.

**Effect of viscosity on photoinactivation**

Solvent viscosity may influence the efficiency of photochemical reaction (Simons, 1959). Reaction mixtures were prepared in solutions of substances whose viscosities had been adjusted to that of 40% (w/v) glycerol. It is evident from Table 1 (Turner, 1966) that the inhibition of photoinactivation cannot be attributed solely to increases in viscosity.
Osmotic effects and photoinactivation

The results in Table 1 suggest that the osmotic effects of high-molarity solutions might influence photoinactivation by withdrawing bound or occluded water from the virus particle. It might be expected, therefore, that solutions of non-electrolytes

Fig. 1. Photoinactivation of vaccinia virus in graded concentrations of glycerol. ●, 40 % (w/v) glycerol; △, 20 % (w/v) glycerol; ○, 10 % (w/v) glycerol; □, buffer control.

Table 1. The effect of viscous suspending media on photoinactivation

<table>
<thead>
<tr>
<th>Additive to virus suspension in pH 7.0 buffer + 3 × 10⁻⁵ M-methylene blue at 18°C*</th>
<th>Relative viscosity</th>
<th>Inactivation rate (k min⁻¹) at 50 ft.-c. illumination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil (buffer only)</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>0.23 % (w/v) methocel (DOW, 4000 cps)</td>
<td>4.5</td>
<td>&gt; 1.7</td>
</tr>
<tr>
<td>10 % (w/v) polyethylene glycol (mol.wt./6000)</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>5 % (w/v) polyethylene glycol (mol.wt./20000)</td>
<td>4.5</td>
<td>&gt; 1.7</td>
</tr>
<tr>
<td>2.7 % (w/v) gelatin</td>
<td>4.5</td>
<td>0.6</td>
</tr>
<tr>
<td>1.0 % (w/v) starch</td>
<td>4.5</td>
<td>0.82</td>
</tr>
<tr>
<td>16 % (w/v) polyvinylpyrrolidone</td>
<td>4.5</td>
<td>0.18</td>
</tr>
<tr>
<td>60 % (w/v) glucose</td>
<td>4.5</td>
<td>0.17</td>
</tr>
<tr>
<td>40 % (w/v) glycerol</td>
<td>4.5</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Unilluminated suspensions in these media showed no photoinactivation and in the dilutions required for assay were without effect on the cells. They had no effect on the virus alone.
equimolar with glycerol would be similarly inhibitory, and in fact concentrations of glucose greater than 2M were as effective as equimolar concentrations of glycerol.

The effects of dehydration on photoinactivation

Dried films or pellets of mixtures of gelatin, virus and dye were not inactivated when exposed to light for periods far in excess of those required to inactivate control samples reconstituted in aqueous suspension. Since oxygen diffusion may have been retarded or light penetration obstructed by opaque dried material, the experiments were repeated with material prepared by the second procedure described under Methods. The dried product was barely visible and provided little obstruction to the passage of light or to the access of atmospheric oxygen. Air was admitted to the ampoules of dried dye-sensitized virus, which were then illuminated for different times at 50 ft.-c. After illumination each sample was reconstituted to its original volume with distilled water and titrated in parallel with control samples reconstituted to the liquid state before illumination and with unsensitized control virus. Lyophilized dye-sensitized virus was not inactivated by illumination for periods 6 times longer than that required for obvious photoinactivation in similar hydrated samples (Table 2) (Turner, 1966).

<table>
<thead>
<tr>
<th>Period (min.) of illumination at 50 ft.-c.</th>
<th>Log₁₀ p.f.u./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unsensitized virus</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
</tr>
<tr>
<td>0</td>
<td>6.51</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>120</td>
<td>ND</td>
</tr>
<tr>
<td>180</td>
<td>6.51</td>
</tr>
</tbody>
</table>

Reaction rate (k min.⁻¹) — — 0 0.33

ND = not done.

DISCUSSION

The inhibition of the photodynamic inactivation of vaccinia virus by glycerol has analogies in radiation biology. *Serratia marcescens* could be protected from X-irradiation damage by glycerol (Dewey, 1960). The concentrations of glycerol were lower than those required here, but protection was proportional to glycerol concentration and also independent of oxygen concentration. Protection was not correlated with competition for radiation-induced O₂ radicals. *Bacillus megaterium* spores were less sensitive to X-rays when suspended in 8.9M-glycerol (Webb & Powers, 1961). Neither of these authors was convinced that the protection resulted from dehydration of the test organisms. Wood (1959) suggested that high concentrations of 'non-injurious' chemicals like glucose and glycerol could protect yeast cells from X-rays either by
Water and virus photoinactivation

Dehydration of the cells or by binding cellular water necessary for some radiation effects. Vaccinia virus is hydrated in aqueous suspension and its state of hydration may be modified osmotically by chemicals such as glycerol and sucrose (Smadel, Pickels & Shedlovsky, 1938; McFarlane, MacFarlane, Amies & Eagles, 1939).

Aqueous solvent molecules contribute to inactivation by ultraviolet, X- and γ-irradiation. The role of water in dye-sensitized photodynamic action has received little attention. Spikes & Ghiron (1964) briefly mention photodynamic effects in a non-aqueous system of eosin Y and rat tendon. The present results with dye-sensitized lyophilized vaccinia virus suggest that water is essential; how it could be involved must be largely conjectural. It might act by promoting collisions between light-excited dye molecules and virus particles in suspension but could not be doing so with the preformed complexes of dye+virus. Dye+virus complexes may dissociate on lyophilization, or by the osmotic abstraction of water, and adequately hydrated particles may be essential for permanent dye attachment.

Free OH radicals are formed through the direct decomposition of water by radiation or indirectly through reactions in which water is involved. Ample evidence suggests that radicals of similar species are generated in photodynamic systems (Smith, Santamaria & Smaller, 1961; Simpson, Kirby-Smith & Randolph, 1963; Egerton, 1964). Whether such radicals are generated via water in photodynamic systems seems doubtful (Strauss & Nickerson, 1961; Grossweiner & Zwick, 1961; Postuma & Berends, 1966; Fridovich & Handler, 1960). However, illumination of dehydrated dye-sensitized virus was without effect and the results of photoinactivation of vaccinia virus with methylene blue indicate that either water must be present, or the virus particle adequately hydrated.

REFERENCES


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