The Photosensitivity of Semliki Forest and Other Viruses

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SUMMARY

Semliki Forest virus was inactivated by exposure to daylight or artificial light. The active wavelengths were in the region 3300 to 4700 Å. Inactivation was reduced by calf serum and completely prevented by cysteine or anaerobic conditions. Light destroyed the infectivity of the virus-associated RNA at the same rate as that of whole virus; it did not affect the reaction of the virus in immunodiffusion tests nor the infectivity of previously extracted RNA. Inactivation was probably the result of photo-oxidation of the viral nucleic acid which was sensitized by some naturally occurring pigment. Although riboflavin and vitamin A were able to increase the photosensitivity of Semliki Forest virus, the evidence presented indicates that neither of these substances was the natural photosensitizer.

Sindbis, Murray Valley encephalitis, influenza and rabbitpox viruses also lost infectivity when exposed to light, but poliovirus was photoresistant.

INTRODUCTION

The inactivation of viruses by means of γ-rays, X-rays, or ultraviolet light is a phenomenon that has been intensively studied and has found application in a number of fields of virology (Kleczkowski, 1957; Gard & Maaløe, 1959). Visible light, with its relatively low energy per quantum, is generally considered to have little effect on viruses except in the presence of a photosensitizing substance. However, some animal viruses are inactivated by light in the absence of an added photosensitizer. Skinner & Bradish (1954) reported the inactivation of vesicular stomatitis, influenza and vaccinia viruses by daylight or artificial light, and Cutchins & Dayhuff (1962) described a similar phenomenon with measles virus. Recently Zwartouw observed that Semliki Forest lost infectivity when exposed to daylight (personal communication). The present work confirms that observation, and investigates the mechanism of inactivation.

METHODS

Virus. Semliki Forest virus strain 25639 was originally obtained from the Rockefeller Foundation Virus Laboratories. It had received 15 mouse brain and 3 suckling mouse brain passages, and was passed by us not more than 5 times in cultures of chick embryo fibroblasts. Virus was titrated as plaque forming units (p.f.u.) in chick embryo fibroblast monolayers (Hawkes, 1964).

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For virus growth, confluent chick embryo fibroblast cultures in Roux bottles were inoculated with about 10^8 p.f.u. virus in 20 ml. maintenance medium (Earle's saline plus 0.5% (w/v) lactalbumin hydrolysate, 0.1% (w/v) yeast extract, 0.1% (w/v) bovine plasma albumin and 0.1 μg./ml. actinomycin D) and incubated at 37°C for 16 hr. The infected medium, centrifuged at 3000 rev./min. for 10 min. to remove free cells, contained virus at a titre of about 10^9 p.f.u./ml.

Most experiments were performed with infected tissue culture medium diluted 10^-2 or 10^-3 in gelatin saline (0.5% (w/v) gelatin in calcium magnesium saline). When more concentrated virus was required, it was separated from the medium by one or two cycles of alternate low speed (10,000 rev./min. for 15 min.) and high speed (20,000 rev./min. for 60 min.) centrifugation, and finally resuspended in gelatin saline.

Illumination of virus. Virus suspensions, usually 2 ml. in 1.4 cm. diameter test tubes or 1.2 cm. diameter bottles, were exposed to daylight or artificial light. For daylight illumination, the virus was placed on the inner sill of a window facing south (i.e. away from the sun). Daylight was the most convenient source of intense illumination, and if heavily overcast days were avoided its inactivating effect varied over little more than a twofold range. The artificial light source, less intense but more constant than daylight, was a 15 w `daylight' fluorescent striplamp 40 cm. in length (Philips TLD 15 w/55). Virus suspensions were exposed at a distance of 5 cm. from the lamp.

When ultraviolet (u.v.) illumination was used 2 ml. volumes of virus suspension in 5 cm. diameter Petri dishes were exposed, with constant agitation, 20 cm. below a u.v. striplamp (Philips TUV 15, 40 cm. length).

Infectious RNA. Virus suspensions, containing 0.2% bentonite (Fraenkel-Conrat, Singer & Tsugita, 1961), were extracted twice at room temperature by shaking with an equal volume of water-saturated redistilled phenol. The phenol was removed by two extractions with peroxide-free ether, and the ether evaporated with a current of air.

The phenol extracts were diluted in m-NaCl in 0.1M-2-amino-2-hydroxymethylpropanediol-1,3 (tris) buffer pH 8.2. Volumes of 0.1 ml. were inoculated on to chick embryo fibroblast monolayers that had been washed once with 0.4 m-NaCl. The RNA was allowed to adsorb at room temperature for 15 min., agar overlay medium was added, and the cultures were incubated as for virus titrations.

Viral antigen. Virus was concentrated and partially purified by two cycles of low and high speed centrifugation to give a suspension of about 10^11 p.f.u./ml in gelatin saline. It was examined by micro-immunodiffusion (Crowle, 1958) against γ-globulin prepared from the peritoneal fluid of mice immunized with Semliki Forest virus.

RESULTS

Inactivation of virus by light

Samples of virus in gelatin saline were titrated after 2 and 4 hr illumination or storage in the dark (Fig. 1). Control virus was stored at 4°C in the dark. There was no significant fall in the titre of virus kept at room temperature (23°C) in the dark. At 37°C virus infectivity, expressed as common logarithms, fell at the rate of 0.26 log/hr
Exposure of the virus to daylight on the windowsill caused the loss of 2.6 log infectivity/hr, and on the laboratory bench 2 m. from the window infectivity fell at the rate of 0.63 log/hr. Inactivation of virus by daylight at 23° therefore considerably exceeded thermal inactivation at 37°. In other experiments, virus exposed on the windowsill to direct sunlight lost infectivity at the rate of about 10 log/hr; the temperature did not rise above 35°.

When the strip lamp was used as a constant source of illumination, there was an exponential loss of infectivity with time (Fig. 2). There was no obvious lag period and no photoresistant fraction of virus could be detected.

The active wavelengths of light

At first it seemed possible that the virus was inactivated by a small amount of 2600 Å u.v. light that might penetrate both the window and the glass container. However, when two additional sheets of glass, each 6 mm. thick, were placed between the light source and the virus, the rate of inactivation was reduced by only 35%. The active wavelengths must therefore have been greater than 3300 Å, for the glass was found to transmit less than 5% of light below this wavelength. A more precise estimate of the active wavelengths was made by observing the effect of light filters on the rate of inactivation. The filters used were the Wratten gelatin filters 2B, 6 and 8, the Wratten glass filter 18A, and the Jena glass filters BG 12 (3.8 mm. thickness) and UG 5 (2.8 mm. thickness); their transmission spectra were determined by the Shimadzu continuous recording spectrophotometer (Fig. 3). Virus samples were placed in a closed box and illuminated by daylight through openings covered by the various
filters, and inactivation rates were determined (Table 1). Virus inactivation was caused by wavelengths between 3300 and 4700 Å, the peak of activity probably being in the region of 3500–4000 Å.

![Transmission spectra of Wratten and Jena glass filters.](image)

Table 1. Effect of filters on the inactivation of Semliki Forest virus by daylight

<table>
<thead>
<tr>
<th>Filter</th>
<th>UG 5</th>
<th>18 A</th>
<th>BG 12</th>
<th>2 B</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivation rate* (% of value without filter)</td>
<td>46</td>
<td>42</td>
<td>29</td>
<td>20</td>
<td>13</td>
<td>3</td>
</tr>
</tbody>
</table>

* Each figure is the mean value from at least three experiments.

Protection of virus against photoinactivation

Substances capable of protecting virus against light might have some practical significance, and their nature could also suggest the mechanism of inactivation.

Calf serum

The presence of calf serum reduced, but did not abolish, the photoinactivation of Semliki Forest virus. Maximum protection, about 75% reduction of the inactivation rate, was obtained with about 5% calf serum; higher concentrations of serum sometimes reduced virus survival by a direct virucidal action. The protective principle of calf serum was not removed by dialysis and was unaffected by heating at 56° for 1 hr. Heating at 100° for 10 min., which left about 10% of the protein in solution, reduced the protective effect of serum to about 30% of its original value. Tests with individual serum fractions (Nutritional Biochemicals Corporation, Cleveland, Ohio) showed that at the optimal concentration (about 2 mg./ml.) bovine plasma albumin fraction V, human \(\beta\)-lipoprotein fraction III–0 and bovine glycoprotein fraction IV each reduced the rate of virus inactivation by about 60%. Some other proteins had a smaller effect. The protective action of serum was probably due to its mixed protein content.

Cysteine

Virus suspensions containing \(6.5 \times 10^6\) p.f.u./ml. were exposed to the striplamp for 3 hr. The titre of the suspension without cysteine was reduced to \(6.3 \times 10^8\) p.f.u./ml., but in the presence of cysteine 10 mg./ml. the titre remained unchanged at \(7.7 \times 10^6\).
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p.f.u./ml. Lower concentrations of cysteine, around 0·1 mg./ml., increased the rate of inactivation. This was due to the fact that at low (0·1 mg./ml.) but not at high (10 mg./ml.) concentrations, cysteine had a direct virucidal action, the reduction in titre in the dark being sometimes as much as 2 log over a period of 3 hr. The reason for the anomalous effect of cysteine concentration was not determined.

Reduction of oxygen tension

Thunberg tubes containing 2 ml. volumes of virus suspension were evacuated on a water pump, filled with nitrogen and evacuated a second time. Control tubes were not evacuated. The virus suspensions were then exposed to the striplamp for periods up to 3 hr. Light had little or no effect on the virus under ‘anaerobic’ conditions (Table 2). Similar results were obtained when the virus was exposed to daylight.

Table 2. Illumination of Semliki Forest virus under anaerobic conditions

<table>
<thead>
<tr>
<th>Period of illumination (hr)</th>
<th>Virus titre (p.f.u./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>0</td>
<td>2·8 × 10⁶</td>
</tr>
<tr>
<td>1</td>
<td>4·0 × 10⁶</td>
</tr>
<tr>
<td>2</td>
<td>4·1 × 10⁶</td>
</tr>
<tr>
<td>3</td>
<td>4·0 × 10⁶</td>
</tr>
</tbody>
</table>

The effect of light on viral protein

Light might inactivate Semliki Forest virus by damaging either the nucleic acid or the lipoprotein. The effects of light on these viral components were therefore studied separately. The results in each case were compared with those obtained with virus inactivated by u.v. illumination or by heating at 56°. The viral protein was examined by testing its ability to react as an antigen in immunodiffusion tests. Virus was concentrated and partially purified by two cycles of low and high speed centrifugation to give a suspension containing 1·2 × 10¹¹ p.f.u./ml. in gelatin saline. Samples of this suspension were inactivated by exposure to daylight for 7 hr (giving a titre of 5·7 × 10⁶ p.f.u./ml.), illumination with u.v. light for 2 min. (titre 5·1 × 10⁶ p.f.u./ml.), or heating in a 56° waterbath for 1 min. or 2 min. (titres 3·1 × 10⁸ and 4·2 × 10⁸ p.f.u./ml.). Inactivation by light was considerably slower than usual (an average rate of only 0·6 log/hr), probably due to the large amount of protective impurities in the concentrated suspension. Immunodiffusion tests on the control and inactivated virus preparations revealed no alteration of the viral antigens by either u.v. or visible light. The heated virus suspensions on the other hand were considerably affected; the four lines produced by the control virus were weakened or lost and were replaced by one apparently new line.

The effect of light on viral nucleic acid

The integrity of the viral RNA was examined by testing its infectivity. Samples of virus were exposed to daylight for various times up to a maximum of 2½ hr. They were then titrated for surviving virus and for infectious RNA after phenol extraction. The infectivity of the extractable RNA decreased at a rate similar to that of whole virus (Fig. 4). The losses of infectivity over the whole 2½ hr period were 2·7 log of virus and 3·1 log of RNA. It was concluded that daylight probably inactivated Semliki Forest
virus by affecting its nucleic acid. Virus was also inactivated by u.v. light and by heat (Figs. 5, 6). As would be expected, u.v. light destroyed the infectivities of whole virus and of extractable RNA at similar rates, whereas heat affected the extractable RNA much less than the virus.

An alternative test was to study the inactivation of free RNA, i.e. RNA that had

![Graph 1](Fig. 4)

**Fig. 4.** Effect of light on the infectivity of Semliki Forest virus (○) and its contained RNA (■). Initial titres were: virus, $3.6 \times 10^9$ p.f.u./ml.; RNA, $4.0 \times 10^5$ p.f.u./ml.

![Graph 2](Fig. 5)

**Fig. 5.** Effect of u.v. light on the infectivity of Semliki Forest virus (○) and its contained RNA (■). Initial titres were: virus, $1.5 \times 10^9$ p.f.u./ml.; RNA, $2.5 \times 10^5$ p.f.u./ml.

![Graph 3](Fig. 6)

**Fig. 6.** Effect of heating at 56°C on the infectivity of Semliki Forest virus (○) and its contained RNA (■). Initial titres were: virus, $6.8 \times 10^9$ p.f.u./ml.; RNA, $4.2 \times 10^5$ p.f.u./ml.
been previously extracted from virus not exposed to inactivating agents. The effects of heat and u.v. light on free RNA were similar to those on the virus-associated RNA. U.v. light inactivated a virus suspension at 2-6 log/min. and the free RNA obtained from it at 2-1 log/min. Heat had even less effect on free RNA than on virus-associated RNA; treatment at 56° for 5 min. caused the loss of 6-7 log of virus infectivity, whereas the infectivity of free RNA fell by only 0-2 log. The effect of light on free RNA was quite different from that on virus-associated RNA, for free RNA was almost unaffected by either daylight or striplamp illumination: over a period of 3 hr, virus infectivity fell by 5-3 log but infectivity of the free RNA fell by only 0-3 log.

Photosensitization of Semliki Forest virus

The above results suggested that the inactivation of Semliki Forest virus was probably the result of sensitized photo-oxidation of the viral nucleic acid, the sensitizing agent being some substance derived from the cells or culture medium. Several compounds were considered as possible photosensitizers. Phenol red and actinomycin D, both present in the virus growth medium, could be excluded because virus harvested from mouse brain and not exposed to either of these agents was found to be fully light sensitive. Vitamin B₁₂ and cytochrome c were unable to sensitize Semliki Forest virus when added to mature virus or to the virus growth medium. The virus was photosensitized by adding riboflavin or vitamin A, and the effects of these two compounds were therefore studied in detail.

Riboflavin

Virus suspensions were mixed with various concentrations of riboflavin and illuminated by the striplamp for ½, 1 or 2 hr. The rate of virus inactivation was doubled by about 2 μg. riboflavin/ml. and was increased 5 to 10 times by 10 μg./ml. (Fig. 7). Riboflavin also increased the inactivation of extractable infectious RNA to the same extent as that of whole virus. Its photosensitizing effect was reduced by calf serum and abolished by cysteine 10 mg./ml. or anaerobic conditions. In these ways, the photosensitization of Semliki Forest virus sensitized by riboflavin resembled that of unsensitized virus.

The natural photosensitizer must be bound firmly to the virus particles, for neither dilution nor partial purification of virus reduced its sensitivity to light. For example, unpurified virus was inactivated by the striplamp at a rate of 1-4 log/hr; the same virus after two cycles of low and high speed centrifugation was inactivated at 1-5 log/hr. Riboflavin was tested for its ability to photosensitize the virus irreversibly. Virus suspensions were kept in the dark for 3 hr with concentrations of riboflavin up to 10 μg./ml. They were then diluted 10⁻² in gelatin saline and immediately illuminated. There was no difference between the inactivation rates of untreated virus and of virus that had been pretreated with riboflavin. Since it was possible that riboflavin became irreversibly bound to virus only if present in the growth medium, the virus was passed twice through chick embryo fibroblast cultures in the presence of riboflavin 2 μg. or 10 μg./ml. The virus from these cultures was found to be no more photosensitive than virus grown in cells without added riboflavin.

Riboflavin occurs in tissues mainly as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Bessey, Lowry & Love, 1949). FMN was as effective as riboflavin, on a molar basis, in photosensitizing Semliki Forest virus. FAD was less
active; a concentration of 25 μg./ml. (= riboflavin 12 μg./ml.) was required to double the usual rate of inactivation. But neither FMN nor FAD caused irreversible photosensitization when incubated with mature virus in the dark or when added (at 20 μg./ml.) to the tissue culture medium during virus growth.

![Graph](image)

**Fig. 7.** Effect of riboflavin on the photoinactivation of Semliki Forest virus. Riboflavin concentrations: ●, Nil; ○, 0.08 μg./ml.; ■, 0.4 μg./ml.; □, 2 μg./ml.; ★, 10 μg./ml.

### Vitamin A

Vitamin A acetate was used. It was dissolved in ethanol at 10 mg./ml. and diluted in gelatin saline to produce a very fine suspension. At a concentration of 10 μg./ml. vitamin A acetate increased the rate of inactivation of Semliki Forest virus by daylight about fivefold; higher concentrations had no additional effect. As with riboflavin, the infectivity of the extractable RNA was reduced at the same rate as that of the virus, and the action of vitamin A was prevented by cysteine or anaerobic conditions. Virus could not be irreversibly photosensitized merely by mixing with vitamin A. But when vitamin A acetate 10 μg./ml. (the maximum tolerated concentration) was added to cell cultures before and during virus growth, the virus progeny showed about a 50% increase in photosensitivity. It therefore seemed possible that vitamin A was the natural photosensitizer. The next requirement was to determine whether the wavelengths responsible for inactivation of virus sensitized by vitamin A were similar to those which inactivated normal virus. The effects of filters on the inactivation by daylight of normal virus and virus sensitized by vitamin A acetate (10 μg./ml.) or riboflavin (10 μg./ml.) were tested (Table 3). The results with filters 18A and 2B suggested that virus sensitized with vitamin A was inactivated by shorter wavelengths than was unsensitized virus. This was what might have been anticipated, for the absorption peak of vitamin A acetate is at 3260 A (Cama, Collins & Morton, 1951). Vitamin A was therefore unlikely to have been the natural photosensitizer. Inactivation in the presence of riboflavin certainly required light of longer wavelengths. Riboflavin has absorption
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maxima at 2200, 2650, 3650 and 4450 Å (Kuhn, György & Wagner-Jauregg, 1933) and light absorbed in the 4450 Å region was probably responsible for the riboflavin-sensitized photoinactivation.

Table 3. Effect of filters on the inactivation of Semliki Forest virus by daylight in the presence or absence of sensitizing agents

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Sensitizer</th>
<th>UG 5</th>
<th>18 A</th>
<th>BG 12</th>
<th>2 B</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>—</td>
<td>47</td>
<td>—</td>
<td>20</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Vitamin A</td>
<td>—</td>
<td>74</td>
<td>—</td>
<td>5</td>
<td>31</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Riboflavin</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>43</td>
<td>26</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>54</td>
<td>40</td>
<td>31</td>
<td>19</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Vitamin A</td>
<td>68</td>
<td>73</td>
<td>46</td>
<td>6</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>54</td>
<td>41</td>
<td>30</td>
<td>21</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Riboflavin</td>
<td>18</td>
<td>0</td>
<td>32</td>
<td>60</td>
<td>26</td>
<td>11</td>
</tr>
</tbody>
</table>

* Expressed as a percentage of the value (calculated as log/hr) without a filter.

Table 4. Photoinactivation of some animal viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>Inactivation rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sindbis</td>
<td>Chick embryo fibroblast culture</td>
<td>70</td>
</tr>
<tr>
<td>Murray Valley encephalitis</td>
<td>Chick embryo fibroblast culture</td>
<td>70</td>
</tr>
<tr>
<td>Influenza type A (WSN)</td>
<td>Allantoic fluid</td>
<td>30</td>
</tr>
<tr>
<td>Rabbitpox (Utrecht)</td>
<td>Chick embryo fibroblast culture</td>
<td>10</td>
</tr>
<tr>
<td>Poliovirus type 1 (strain U)</td>
<td>U cell culture (human amnion cell line) derived from vs41 by Cooper, 1963)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Inactivation rates are expressed as percentages of that of Semliki Forest virus measured at the same time.

Photosensitivity of other viruses

A few other viruses were tested for their sensitivity to light. Each was diluted $10^{-2}$ in gelatin saline to avoid possible protection by constituents of the growth medium and exposed to striplamp illumination at the same time as Semliki Forest virus (Table 4). Sindbis and Murray Valley encephalitis viruses were inactivated at rates comparable with that of Semliki Forest virus, influenza virus rather more slowly, and rabbitpox virus very slowly. Poliovirus showed no detectable inactivation over a period of 4 hr.

DISCUSSION

One purpose of this investigation was to determine whether the photosensitivity of Semliki Forest virus could introduce errors into virus titrations. Appreciable inactivation of virus did occur if suspensions were exposed for more than a few minutes to intense daylight. The main safeguard against this is an awareness of the possibility of photoinactivation so that simple precautions can be taken against it. Our procedure is to handle the virus as little as possible in unshielded daylight, and to store it in darkness when not in use. In addition, 2% (v/v) calf serum is included as a protective agent in the gelatin saline used for virus dilutions.

The second aim was to obtain information on the mechanism of photoinactivation. The observation that the infectivity of the virus-associated RNA decreased at the same rate as that of whole virus indicates that light acts mainly against the viral
nucleic acid, though some damage to the lipoprotein cannot be excluded. However, the inactivation is not the same as that caused by 2600 Å u.v. light, for visible light did not affect the infectivity of free viral RNA. This difference between the photosensitivity of virus-associated and free RNA is similar to the findings of Wilson & Cooper (1965) with poliovirus made photosensitive by growth in neutral red. It suggests that the photoinactivation of Semliki Forest virus is the result of sensitization by some light-absorbent substance which is removed with viral protein in the preparation of free RNA. The ability of cysteine or anaerobic conditions to protect Semliki Forest virus against light supports this idea, for the dye-sensitized photoinactivation of both proteins and nucleic acids is an oxidative process (McLaren & Shugar, 1964).

Virus inactivation is probably, therefore, the result of photo-oxidation of the sensitized viral nucleic acid. The postulated photosensitizing agent could be a naturally occurring compound which has an absorption maximum in the near-u.v. region of 3500 to 4000 Å and is firmly bound to the virus so as to sensitize it irreversibly. It might be either an essential component of the virus or merely extraneous material that happened to be adsorbed or incorporated. Riboflavin and vitamin A photosensitized Semliki Forest virus when added to a virus suspension. Both acted against the viral nucleic acid, and their effects were prevented by anaerobic conditions; but probably neither is the natural photosensitizer, the identity of which remains unknown.

The phenomenon of photosensitivity is not confined to Semliki Forest virus and, if only for practical reasons, deserves more general recognition. The incomplete data available on a number of animal viruses (Skinner & Bradish, 1954; Cutchins & Dayhuff, 1962; present work) suggest that there may be a correlation between virus group and photosensitivity; arboviruses and myxoviruses appear to be photosensitive, picornaviruses photoresistant, and poxviruses perhaps form an intermediate group.

REFERENCES


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