Interferon Production by Semliki Forest Virus Inactivated with Hydroxylamine

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SUMMARY

The inactivation of Semliki Forest virus by hydroxylamine was a first-order reaction. The ability of the partially inactivated virus to induce the production of infective virus, virus haemagglutinin, virus-induced RNA synthesis, virus-induced RNA polymerase and interferon were all inactivated with first-order kinetics. It is concluded that functional nucleic acid is essential for interferon production in this system.

INTRODUCTION

Many different viruses induce interferon production by a process resembling derepression. Indirect evidence (summarized by Burke, 1966) suggests that the virus nucleic acid is the essential stimulus for interferon production, but the evidence is not unequivocal. This paper reports investigations of the effect of hydroxylamine inactivation of Semliki Forest virus on its interferon-inducing capacity, and on its ability to induce a number of other virus-directed processes. It is concluded that functional viral nucleic acid is essential for interferon production.

Hydroxylamine inactivates the infectivity of a number of animal viruses through reaction with the viral nucleic acid. Franklin & Wecker (1959) showed that the infectivity of mouse encephalomyelitis virus was inactivated as rapidly as the infectivity of viral RNA, and at approximately the same rate as the infectious RNA extracted from the untreated virus. Hydroxylamine had no effect on the serological and enzymic properties or the haemagglutinin of a number of viruses (Franklin & Wecker, 1959; Schäfer & Rott, 1962), showing that viral proteins are little affected under the conditions used. Hydroxylamine also inactivated certain bacterial viruses, probably as a consequence of attack on thiol-ester bonds (Kozloff, Lute & Henderson, 1957). However, the activation energy of this process is 19 kcal./mole whereas the activation energy for the inactivation of western equine encephalomyelitis virus is only 4 kcal./mole (Franklin & Wecker, 1959). The effect of hydroxylamine is therefore much faster on viral nucleic acid than on viral protein.

The mode of action of hydroxylamine has been extensively investigated because of its mutagenic action. Hydroxylamine reacts with the cytosine residues of polynucleotides to form 5,6-dihydro-6-hydroxylamino cytosine, which then pairs with adenine, and it also reacts with uracil residues, with the elimination of an isoxazolone and consequent loss of template function (Schuster, 1961; Phillips et al. 1965). Inactivation by hydroxylamine does not, however, cause breakdown of RNA since the sugar phos-
phosphate bond is not attacked, and it has no effect on the molecular weight of the RNA of polio virus (Holland et al. 1960).

A brief account of some of the present results has already been published (Skehel & Burke, 1967).

METHODS

Materials. Hydroxylamine was obtained from Hopkins and Williams Ltd. Phosphoenolpyruvate and pyruvate kinase were obtained from C. F. Boehringer u. Soehne, Mannheim, German Federal Republic. Actinomycin was a gift from Merck, Sharpe and Dohme, New Jersey, U.S.A. Sodium dodecysulphate (specially pure) was obtained from British Drug Houses Ltd, Poole, Dorset. Ether and phenol were freshly redistilled before use. [14C]uridine (specific activity 42.3 mc/m-mole) and [3H]uridine (specific activity 31 c/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks. [3H]UTP (specific activity 2-24 c/m-mole) was obtained from Schwarz Bioresearch Ltd, New Jersey, U.S.A. The nucleoside triphosphates were obtained from Sigma Ltd, London.

Media, cells and viruses. These were as described previously (Walters, Burke & Skehel, 1967). Semliki Forest virus was partially purified from mouse brains by differential centrifugation or was more extensively purified from mouse brains or tissue culture fluids by a slight modification of Cheng's (1961) method. Virus haemagglutinin was measured by the method of Clarke & Casals (1958), using goose red cells and plastic trays, at pH 5.8. One haemagglutinating unit is defined as the least quantity of virus giving partial haemagglutination.

Inactivation of virus by hydroxylamine. Purified virus (about 2 x 10⁹ p.f.u./ml.) in 0.1 M-tris in 0.15 M-NaCl pH 7.0 was mixed with an equal volume of hydroxylamine in Earle's saline +0.2 % bovine plasma albumin pH 7.0. Inactivation proceeded at room temperature (23 °) and samples were removed at intervals, diluted four- to ten-fold with maintenance medium and dialysed against a large volume of Earle's saline at 2 ° for 24 hr with two changes.

Interferon production and assay. Interferon was produced by infection of cells for 1 hr at 37°, followed by incubation at 42° as described by Burke, Skehel & Low (1967). Fluids were harvested 12 hr after infection. Interferon was assayed as described by Burke & Buchan (1965), the interferon titre being defined as the reciprocal of the dilution necessary to give a 50 % depression of the control plaque count (PDD 50). An internal standard, included in all assays, gave a PDD 50 of 2.552 log. units with a standard deviation of 0.052 log. units (11 determinations). All titres were the logarithmic mean of at least two titrations.

Incorporation of radioactive precursors. This was carried out as previously described (Skehel et al. 1967).

Extraction and sedimentation of viral RNA. Viral RNA was extracted from virus suspensions by vigorously shaking with water-saturated phenol and sodium dodecysulphate (final 0.5 %). The aqueous phase was removed by centrifuging, washed with phenol and the residual phenol removed by ether extraction.

The extracted RNA (0.2 ml.) was then centrifuged for 2 hr at 39,000 rev./min. on a 5 % to 20 % sucrose gradient in 0.1 M-acetate buffer, pH 5.0 at 5° in the SW 39 rotor of the Spinco L 2 centrifuge. Fractions (0.2 ml.) were collected by puncturing the tube and 0.1 ml. of bovine plasma albumin (10 mg./ml.) and 0.3 ml. of cold 10 % tri-
chloracetic acid were added. The precipitated RNA fractions were collected on glass-fibre filters (Whatman GF/C) washed with cold 5% trichloracetic acid, ethanol and ether, dried and then suspended in a toluene scintillation mixture (40 g. diphenyloxazole, 0.05 g. 1,4-bis[4-methyl-5'-phenyl-oxazolyl] benzene/1. of toluene).

Assay of infectious RNA. This was carried out as described by Méc et al. (1967).

Virus-induced RNA synthesis. Cells were infected with hydroxylamine-inactivated virus, and then incubated with maintenance medium containing 0.5 μg./ml. of actinomycin. The cells were pulsed with [3H]uridine (2.5 μc/culture) for 30 min. at 4 hr after infection, when nearly all the RNA synthesis is virus-directed (Burke et al. 1967).

Preparation and assay of virus-induced RNA polymerase. The procedures of Martin & Sonnabend (1967) were followed. Chick cell monolayer cultures (approximately 120 × 10^6 cells per culture) were exposed to infection with Semliki Forest virus (5 p.f.u./cell) for 1 hr at 37°C. Six hr after infection the cultures were washed twice with ice-cold saline, and then with hypotonic buffer (10^{-3} M-tris+HCl pH 8.3 containing 10^{-3} M-MgCl₂ and 10^{-3} M-mercaptoethanol). All following procedures were performed at approximately 0°C. The cells were then scraped from the Petri dishes and homogenized in a Dounce homogenizer until all cells were broken but nuclei were seen to be intact (35 strokes). Nuclei and cell debris were removed from the suspension by centrifuging at 1000 rev./min. for 5 min. and the supernatant fluid was then centrifuged at 20,000 rev./min. for 10 min. Both resulting pellets were resuspended in assay buffer (10^{-3} M-tris+HCl pH 8.5 containing 2 × 10^{-3} M-MgCl₂ and 10^{-3} M-mercaptoethanol) to a final concentration of approximately 5 mg./ml. of protein.

The incubation medium used for the assay of polymerase activity contained in a volume of 0.35 ml.: tris (pH 8.5), 35 μmole; MgCl₂, 0.8 μmole; β-mercaptoethanol, 7.0 μmole; phosphoenolpyruvate, 5.0 μmole; pyruvate kinase, 10 μg.; actinomycin D, 1 μg.; [H]UTP, 0.5 μc; 1.0 μm/mole; ATP, CTP and GTP, 50 μm/mole; and approximately 250 μg. enzyme protein. After incubation for 30 min. reactions were stopped by adding 0.1 ml. ice-cold bovine serum albumin (10 mg./ml.) rapidly followed by 1 ml. ice-cold 5% trichloracetic acid. The resulting precipitate was washed twice by centrifuging in cold 5% trichloracetic acid and finally collected on glass-fibre discs, washed with 5% trichloracetic acid, ethanol and ether, and then dried in vials before the addition of 10 ml. toluene scintillator.

Under these conditions polymerase activity was predominantly present in the 20,000 rev./min. sediment. It increased linearly with time of incubation up to 25 min., and with protein concentration up to 0.5 mg./assay. It was not present in fractions of uninfected cells, was completely dependent on the presence of all four nucleoside triphosphates, and had similar pH, mercaptoethanol and Mg^{2+} concentration requirements for optimal activity as those reported by Martin & Sonnabend (1967).

When the product of the polymerase reaction was identified (Fig. 1) the reaction volume and reactants were doubled and the amount of [H]UTP increased to 4.0 μc; 0.05 ml. of a 14C labelled ribosomal RNA extract was added to the mixture after 30 min. incubation and the product was then isolated using an identical procedure to that described by Martin & Sonnabend (1967).

The more slowly sedimenting component of the reaction product was constantly observed, and appeared to increase on freezing and thawing of the extracted product. The major component of the reaction mixture had a similar sedimentation coefficient and resistance to ribonuclease to that reported by Martin & Sonnabend (1967).
RESULTS

Inactivation of Semliki Forest virus

The inactivation of the infectivity of partially purified virus by hydroxylamine was a first-order reaction (Fig. 2). Virus haemagglutinin was inactivated much more slowly than infectivity (Table 1), showing that the effect of hydroxylamine was primarily on viral nucleic acid. This was confirmed by showing that the infectivity of Semliki Forest virus nucleic acid was rapidly inactivated by incubation of the nucleic acid with 0.1 M-hydroxylamine at room temperature (Table 2). This loss of infectivity was not accompanied by any breakdown of viral nucleic acid since the rate of sedimentation of virus nucleic acid labelled with $[^{14}C]$uridine was unaffected following incubation with 0.2 M-hydroxylamine for 20 min. at room temperature. The rate of inactivation varied slightly with the batch of virus, inactivation proceeding more rapidly with more ex-

![Fig. 1. Sucrose gradient analysis of Semliki Forest virus polymerase reaction product. Fractions were assayed for total and ribonuclease-resistant activity after treatment with 1 µg./ml. of enzyme. $[^{14}C]$radioactivity of ribosomal marker before (○—○) and after (○—○) ribonuclease treatment; $[^{3}H]$radioactivity of polymerase product before (△—△) and after (△—△) ribonuclease treatment.]

<table>
<thead>
<tr>
<th>Table 1. Effect of 0.2 M-hydroxylamine on the infectivity and haemagglutinin titre of Semliki Forest virus purified from tissue culture fluids</th>
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</thead>
<tbody>
<tr>
<td><strong>Time (hr)</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>$\frac{1}{2}$</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>7</td>
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</table>
Interferon production by inactivated virus

tensively purified material (Fig. 2). Multiplicity reactivation was not observed. The ability of the partially inactivated virus to induce the virus-coded properties described below was independent of the source of virus or the rate of inactivation.

Schuster (1961) found that cytosine residues of tobacco mosaic virus were attacked more rapidly than uracil residues at pH 6·15 whereas the reverse was true at pH 9·15. Semliki Forest virus infectivity was destroyed by dialysis at pH 6·0, but the virus was inactivated more rapidly at pH 7·0 than at pH 9·0, both reactions being first order.

Table 2. The effect of 0·1 M-hydroxylamine on the infectivity of Semliki Forest virus nucleic acid

<table>
<thead>
<tr>
<th>Time of inactivation (min.)</th>
<th>Infectivity (log. p.f.u./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3·36</td>
</tr>
<tr>
<td>5</td>
<td>2·78</td>
</tr>
<tr>
<td>10</td>
<td>2·40</td>
</tr>
<tr>
<td>15</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Control incubated for 40 min.</td>
<td>3·08</td>
</tr>
<tr>
<td>1 µg./ml. ribonuclease for 15 min.</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

Fig. 2. Inactivation of partially purified Semliki Forest virus by 0·2 M-hydroxylamine (□—□) and of more extensively purified Semliki Forest virus by 0·2 M-(■—■), 0·1 M-(○—○) and 0·05 M-hydroxylamine (●—●).

Properties of inactivated virus

Samples of partially purified virus which had been inactivated for various times (Fig. 2) were used to infect fresh tissue cultures, and the yields of various virus-induced processes measured. Several other batches of virus gave similar results.

Virus infectivity and haemagglutinin production. The yield of infectivity and haemagglutinin was measured 8 hr after infection. There was little effect of hydroxylamine until the effective multiplicity of infection fell below 1 p.f.u./cell (the multiplicity of exposure of virus inactivated for 2 hr was 6 while that of virus inactivated for 3 hr was 2). Both virus-induced properties were then inactivated with first-order kinetics.
(Fig. 3a). Extrapolation of this portion of the curve to the ordinate gives an effective multiplicity of infection of untreated virus of 16, compared with the known multiplicity of exposure of 30.

Table 3. The effect of 0.2 M-hydroxylamine on the ability of Semliki Forest virus to induce RNA-dependent RNA polymerase activity

<table>
<thead>
<tr>
<th>Time of inactivation (hr)</th>
<th>Polymerase activity (counts/min./mg. protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ ATP, GTP, CTP</td>
</tr>
<tr>
<td>0</td>
<td>1920</td>
</tr>
<tr>
<td>½</td>
<td>1680</td>
</tr>
<tr>
<td>1</td>
<td>1456</td>
</tr>
<tr>
<td>2</td>
<td>1368</td>
</tr>
<tr>
<td>3</td>
<td>1940</td>
</tr>
<tr>
<td>4</td>
<td>2560</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of hydroxylamine inactivation of Semliki Forest virus on the yield of (a) virus infectivity (●●, control yield 1.76 × 10⁹ p.f.u./culture) and virus haemagglutinin (■■, control yield 640 haemagglutinating units per 0.5 ml.); (b) Virus-induced RNA synthesis (○○, control specific activity 224 counts/min./μg. RNA) and virus-induced RNA polymerase (▲▲, control yield 1655 counts/min./mg. protein); (c) virus-induced interferon formation (▲▲, control yield 241 PDD 50).

Virus-induced RNA synthesis and RNA polymerase. Both virus-induced RNA synthesis and RNA-dependent polymerase were inactivated at the same rate as virus yield (Fig. 3b). During polymerase assays it was observed that the incorporation of radioactivity into the acid-insoluble fraction, in the absence of the three unlabelled nucleoside triphosphates, increased with the period of hydroxylamine inactivation (Table 3). This effect is being further examined.

Virus-induced interferon production. When the hydroxylamine-inactivated virus samples were used to induce interferon production, the capacity to induce interferon was inactivated at the same rate as that of the other virus-induced properties (Fig. 3c).
DISCUSSION

All the virus-induced properties that were measured in this study were inactivated at the same rate by hydroxylamine. In this respect, Semliki Forest virus shows similar behaviour to Newcastle disease virus but differs from that of fowl plague virus (Scholtissek & Rott, 1964). Since hydroxylamine is known to react selectively with nucleic acid, and since the production of infectious virus particles, haemagglutinin, viral RNA and viral polymerase all depend upon the presence of functional viral RNA molecules, it follows that interferon production also requires functional viral RNA for its initiation. Little is known about the mechanism by which viral RNA causes derepression of the host genome, although recent studies suggest that a very limited amount of viral RNA synthesis may be essential (Skehel & Burke, in preparation).

Since this work was completed, Lampson et al. (1967) have shown that a double-stranded RNA extracted from the mycelium of *Penicillium funiculosum* induced production of an interferon in the rabbit.

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REFERENCES


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