Thermal Inactivation of Semliki Forest Virus

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SUMMARY

Semliki Forest virus was thermally inactivated between 20° and 50° by two processes, one of which predominated at temperatures below 41° and the other at higher temperatures. At pH 6·5, the rates of inactivation were greater than at pH 7·5 but the nature of the reactions was unchanged. The stability of the virus in phosphate buffer solutions was greatly reduced at lower concentrations of protein in the suspending medium. The rate of inactivation was reduced in the dark. At higher temperatures, a change occurred in the surface properties of the virus that did not, of itself, cause loss of infectivity.

It is suggested that at the higher temperatures the inactivation was a consequence of a structural breakdown of a surface unit in the virus; at lower temperatures a more subtle change in the substructure was responsible for inactivation.

INTRODUCTION

Viruses vary widely in their sensitivities to both temperature and environment. Thus conditions of temperature or pH can be selected at which poliovirus is stable for many days but Semliki Forest virus (SFV) loses its activity in a few minutes.

The complexity of the inactivation reactions is shown by the variation of the kinetics of the processes from the most simple to the extremes in which a long period of stability is followed by rapid loss of activity: herpes simplex virus and cytomegalovirus (Plummer & Lewis, 1965); Guaroa virus (March & Hetrick, 1967) or in which rapid initial inactivation is followed by slow decay: vaccinia virus (Kaplan, 1958); Sindbis virus (Simpson & Hauser, 1968).

The increased stability of SFV in the presence of proteins has been described (Osterreith, 1964) and a similar effect has long been noted for other viruses. This paper presents further results on the thermal inactivation of SFV and attempts to relate loss of infectivity to modification of structure and impairment of specific biological function.

METHODS

Semliki Forest virus. The initial virus was the second suckling mouse passage of the 'Original' strain (American Type Culture Collection, VR67) obtained at the twelfth mouse passage, and designated v14. This was a 20% suspension of infected mouse brain in 0·75% bovine plasma albumin (Armour, Fraction V), M/20 phosphate buffer solution, pH 7·6.

A working stock of virus was prepared from v14 by a single passage in a stable line of mouse embryo cells (v14.L). A confluent monolayer was inoculated with approximately $1.5 \times 10^7$ p.f.u./cm²; after adsorption for 2 hr at 34°, the cell sheet was washed twice with nutrient medium (10% calf serum in Earle's saline containing yeast extract plus tryptic
meat broth) and fresh medium was added. The extracellular virus was harvested after incubation for 17 hr at 34° in an atmosphere of air plus 5% carbon dioxide. All virus samples were stored at −75° until used.

Assay of virus infectivity. All assays were carried out in suspensions of chick embryo cells (CEC) in agar (Bradish, Allner & Maber, 1971). One ml. of virus suspension was added to 4 ml. of cells in Parker’s medium 199 containing 10% calf serum (199,CS); 5 ml. of 1:2% Oxoid no. 1 agar in 199 was then added and the mixture quickly poured into a 9 cm. Petri dish. Plaques were counted after 40 hr incubation at 34° in an atmosphere of air plus 5% carbon dioxide, followed by staining with 0.005% neutral red in physiological saline.

Thermal inactivation. Inactivations were carried out in well-stirred water baths maintained within 0.1° of the required temperature. A volume of 1 ml. of virus sample was added to 9 ml. of pre-heated solution to give the desired mixture constitution. Inactivation was stopped by tenfold dilution in the stabilising medium, 199,CS.

Reagents. Phosphate buffers of final ionic strength 0.1 were prepared from Analar grade reagents, sterilized by autoclaving for 15 min. at 15 lb./in². Bentonite was prepared by the method of Brownhill, Jones & Stacey, (1959). Pancreatic ribonuclease (Bohringer, Mannheim) at 1 µg./ml. reduced the infectivity of SFV-RNA from 10⁴ to < 1 p.f.u./ml. in 10 min. at 37°.

Antisera. Antisera were prepared by intravenous inoculation of nine rabbits with doses of 10⁶ p.f.u. of mouse brain SFV (v14) at intervals of 1 and 9 months; the animals were bled 1 week after the third dose.

Neutralization by antiserum. The ability of antiserum to neutralize virus infectivity was measured by incubating mixtures of virus with antiserum under conditions of excess antibody for 1 hr at 35°. Residual infectivity was determined as above by adding 1 ml. of the virus-antiserum mixture to the assay suspension; this was compared with the infectivity of virus incubated without antiserum under identical conditions.

RESULTS

Treatment of data

The theory applying to thermal inactivation of viruses is derived from two equations; the first

\[ k' = \frac{(kT/h) K}{k'} \]

relates the reaction velocity (k') to Boltzmann's constant (k), Planck's constant (h), the absolute temperature (T) and the equilibrium constant (K). This equation holds if, as is the case in thermal inactivation of viruses, the reaction is solely in one direction. The second equation

\[ \Delta G = \Delta H - T \Delta S = -RT \ln K \]

relates the thermodynamic parameters to the equilibrium constant at constant temperature.

If the entropy change (ΔS) is independent of temperature, combination and development of equations (1) and (2) gives the changes of Gibb's function (ΔG) and enthalpy (ΔH) explicitly in terms of measurable quantities:

\[ \Delta G = 4.58T(10.32 + \log T - \log k'), \]

\[ \Delta H = -4.58(d \log k'/d(1/T)) - RT. \]

Equation (3) shows that, at any given temperature, ΔG is a function of the reaction velocity only and is a measure of the energy required by the system for the reaction to proceed at the rate k'.
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For a first-order reaction the concentration of surviving infective virus, $V_s$, is given by

$$V_s = V_0 e^{-k't} \quad \text{or} \quad k' = -(\ln V_s/V_0)/t,$$

where $V_0$ is the original concentration of infective virus. When a population is inactivated by two or more simultaneous and independent processes then a first-order relationship is obtained (Fig. 2a) in which the observed velocity constant is the sum of those for the individual reactions.

![Graph showing inactivation of SFV](image)

**Fig. 1.** Inactivation of SFV (v14.L) at 22°. Virus diluted 1/10 in medium 199,CS then serially ten-fold in phosphate buffer solution, pH 7.5, ionic strength 0.1. Infectivity assayed after 3 hr in the dark (○); in subdued daylight (●); in subdued daylight with solutions containing $10^{-2}$ M-EDTA (△).

**Loss of capacity to replicate**

The considerable influence of the concentration of the stabilizing medium (199,CS) on the rate of loss of infectivity was shown by reduction of the ability to form plaques after 3 hr exposure at 22° (Fig. 1). At concentrations of stabilizing medium less than one-tenth the original, the virus samples showed a smaller loss of infectivity when kept in the dark. Even in the dark, however, with the medium diluted $10^{-4}$ in phosphate buffer, more than 99% of the original infectivity was lost in 3 hr at 22°. These effects were so important that all subsequent experiments were carried out under conditions of accurately controlled medium concentration; all virus samples were suspended in this medium before dilution in test buffer systems. In comparative experiments the same preparation of medium was used and the solutions were handled under similar conditions of subdued, indirect daylight.

The kinetics of inactivation are shown in Fig. 2. At 35° the rates of the reaction of the virus sample v14, and of two passages derived from it (v14.c from chick embryo cells, and v14.L) were identical; at 50°, not only were the rates of inactivation different, but a gross heterogeneity was evident in the virus derived from chick embryo cells.

The virus sample, v14.L, used for this work was prepared to provide virus from L-cells for comparison with the initial material (v14) from mouse brains and with the single passage of this in primary CEC (v14.c). It was selected as a model in thermal inactivation studies because it was at least 99% homogeneous in this property.
Fig. 2. Kinetics of thermal inactivation of SFV. Infectivity assayed after exposure in subdued daylight at (a) 35° in phosphate buffer solution, pH 7.5, ionic strength 0.1 containing 0.1% 199, CS, and (b) 50° in 199, CS. Original mouse brain material, v14 (▲); after one CEC passage, v14-c (■); after one L-cell passage, v14-l (○).

Fig. 3. Arrhenius plot for thermal inactivation of SFV (v14-l). The reaction velocity constants are derived from graphs such as those shown in Fig. 2. Virus diluted in phosphate buffer solution: pH 7.5 with stabilizing medium 199, CS at overall dilutions (A) 10⁻¹, (B) 10⁻², (C) 10⁻³, and (D) 10⁻⁴; pH 6.5 with stabilizing medium at overall dilutions 10⁻¹ (▲) and 10⁻³ (△). Data at pH 7.5 (B and C) near the intersections have been analysed as described in the text (□ and ○).
Thermal inactivation of SFV

Results for the inactivation of the plaque-forming capacity of v 14.1 are summarized in the Arrhenius plot of Fig. 3 (equation (4)) and cover the temperature range 20° to 50° at pH 6:5 and 7:5; the concentrations of stabilizing medium were selected to give conveniently measurable reaction rates. The existence of two simultaneous inactivation processes is demonstrated; where the two rates are comparable (near the intersections in Fig. 3) the individual k' values have been derived in conjunction with linear extrapolation of data from temperatures at which one reaction predominates. The thermodynamic parameters derived from the data of Fig. 3 and equations (2), (3) and (4) are collated in Table 1.

Table 1. Thermodynamic parameters for the inactivation of SFV (v 14.1) in phosphate buffer, ionic strength 0.1, pH 7.5

<table>
<thead>
<tr>
<th>Concentration of stabilizing medium (% v/v)</th>
<th>Temp. (°)</th>
<th>ΔH in Kcal./mole</th>
<th>ΔG in Kcal./mole</th>
<th>ΔS in Kcal./mole/°</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>20 to 40</td>
<td>26</td>
<td>21.5 to 21.2</td>
<td>15</td>
</tr>
<tr>
<td>0.1</td>
<td>20 to 40</td>
<td>26</td>
<td>22.7 to 22.2</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>26</td>
<td>24.6</td>
<td>4</td>
</tr>
<tr>
<td>0.1</td>
<td>42</td>
<td>242</td>
<td>21.8</td>
<td>700</td>
</tr>
<tr>
<td>0</td>
<td>42 to 47</td>
<td>242</td>
<td>24.2 to 21.3</td>
<td>694</td>
</tr>
<tr>
<td>1</td>
<td>47 to 50</td>
<td>242</td>
<td>23.5 to 21.8</td>
<td>684</td>
</tr>
</tbody>
</table>

Variations in the composition of the stabilizing medium affect the reaction velocity causing small variations of the absolute value of both ΔG and ΔS; the value of ΔH is not influenced by such variations.

Addition of 10⁻² M-EDTA as a chelating agent did not affect the rates of inactivation at 22° (Fig. 1), or that at 50° in 199, CS; thus pronounced effects due to heavy metal ions were precluded.

It has been suggested for poliovirus, that inactivation at high temperatures proceeds as a result of an opening of the structure which allows action of RNases (Dimmock, 1967). The addition of an RNase (1 mg./ml.) effective against SFV-RNA had no effect on the thermal inactivation at 50°. The addition of bentonite, an RNase inhibitor, increased the rate of loss of infectivity at 50°, probably due either to binding with virus or to contamination with divalent cations. It is thus possible that the media already contained sufficient RNase for enzymic inactivation of a thermally expanded structure.

Neutralization by antiserum

Under the conditions of antibody excess used in these experiments, the ratio (ρ) of the residual infectivity (Vᵣ) to the infectivity before neutralization (Vₒ) is independent of the initial concentration of infective virus (Bradish, Farley & Ferrier, 1962). Fig. 4 shows the variation of ρ following thermal inactivation of SFV at 35° and 50°; some results for the inactivation by ultraviolet radiation at 20° are included for comparison. The extent of inactivation before the neutralization test was performed is defined by Vₒ/Vᵣ; the test mixture contained 0.02 µl. antiserum/ml. before it was added to the cell suspension for assay of surviving infectivity. After heating virus at 50° the value of ρ increased and indicated a reduced ability of antiserum to neutralize infectivity; no such change occurred following heating at 35° or exposure to ultraviolet radiation. The differences (Fig. 4) were not due to dissociation of virus-antibody complexes, which can occur with some SFV derived from CEC preparations, since the complexes of antibody with either the original virus, or with the infectivity surviving the heating, were not dissociated after dilution ten- to a hundredfold in the cell suspension.
DISCUSSION

The Arrhenius plot (Fig. 3) indicates that two processes contribute to the loss of infectivity of SFV; the nature of each reaction determines the change of enthalpy involved and this then fixes the slope of each limb of the plot. When the enthalpy changes for the two reactions are as different as those shown, the two reactions proceed at similar rates only in a narrow temperature range. Thus for SFV, one inactivation process predominates except between 38° and 42°. This appears to be a general feature of the thermal inactivation of viruses; the temperature at which the two reaction rates are equal is also in the region 40° to 43° for foot-and-mouth disease virus (Bachrach et al. 1957), poliovirus and rhinoviruses (Dimmock, 1967). The analysis of the results for SFV within the range 38° to 42° suggests that the two reactions are independent, but this is not conclusive.

The thermal inactivation of viruses is frequently attributed to a degradation of the nucleic acid at low temperatures, and to a breakdown of structure at high temperatures. With SFV the difference in the nature of the two reactions is shown by the tenfold increase in the enthalpy change in the higher temperature range; the intrinsic entropy change, determined by extrapolation to zero concentration of stabilizing medium, is nearly fifty times larger in the high temperature inactivation due to a much greater change in the conformation of the virus structure.

At all temperatures tested, the enthalpy change was independent of the concentrations of stabilizing medium, and the similarity of this value below 41° with that for RNA inactivation (Eigner, Boedtker & Michaels, 1961; Ginoza, 1958) and for the breakage of phosphodiester bonds, suggests the involvement of RNA at these temperatures. However, although the enthalpy change indicates specific bond breakages, the Gibb’s energy change is the controlling factor and the increase of this parameter at higher medium concentrations, together with its comparison with the value for the breakdown of free RNA (30 Kcal./mole; Eigner et al. 1961), implicates a reaction rate controlled by the concentration of extraneous protein; such protein may interact with the virus protein and in turn affect the virus RNA. Dimmock
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(1967) demonstrated that the infectivity of free RNA was more stable than that of intact virus particles, and this may indicate a stressed configuration of RNA due to its association with virus protein. The differences in the rates of low temperature inactivation of viruses may therefore depend on interaction between virus protein and RNA, on the number of more susceptible cleavage points in the RNA (cytosine-adenine and uracil-adenine; Singer & Fraenkel-Conrat, 1963) or simply on the stability of a virus protein. Studies on multiplicity reactivation would determine whether inactivation at low temperatures and by u.v. light were both due to similar degradation of virus nucleic acid.

Rates of inactivation at high temperatures depend on several factors; for a given strain of SFV these rates vary with the cell from which the virus was derived and indicate that host cell derived material is directly involved. The differences at higher temperatures that are envelope determined may indicate the variation in the proportion of those cells in a heterogeneous population capable of supporting virus replication and provide a useful phenotypic marker. However, thermal inactivation at these temperatures may indicate genotypic differences if the cell population from which the virus is derived is particularly uniform, as for the mouse L-cells in these experiments. Under these special conditions, variations of thermal properties would be a valid marker for the virus genome.

The genome determined rates of inactivation of SFV at temperatures below 37°C may correlate with the differences of virulence between various strains of SFV (Bradish et al. 1971), should the technique prove sufficiently sensitive.

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REFERENCES


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