The Heat Inactivation of Vaccinia Virus

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SUMMARY: Heat inactivation curves of vaccinia virus between 50° and 60° indicate that the virus is heterogeneous in its heat sensitivity. The proportion of heat-resistant particles varies inversely with the temperature of exposure. The inactivation of heat-sensitive virus is temperature dependent and seems to be a first-order reaction, while the heat-resistant fraction is inactivated at a constant slow rate unrelated to temperature over the range 50°-60°.

The work reported here is part of a larger investigation of virus inactivation by physical agents including ultraviolet and ionizing radiations, under conditions that do not destroy antigenicity (McClean, 1945; Collier, McClean & Vallet, 1956). The heat inactivation of vaccinia virus was determined as a preliminary to testing the protective antigenicity of heat inactivated vaccines.

METHODS

Virus. Most of the experiments were done with the Lister Institute strain of vaccinia virus adapted to the chick embryo; two experiments were done with the WR mouse neuro-adapted strain of virus. Infected chorioallantoic membranes were extracted in dilute McIlvaine buffer by grinding with sterile powdered neutral glass. Extracts were centrifuged at 1000 g for 15 min. to clear them of debris, titrated and stored at 4°. For inactivation, extracts were diluted to a titre between about 10⁷ and 10⁸ infective units/ml.

Experimental procedure. One ml. samples of virus were heated in small, thin-walled test tubes in a water bath accurate to ±0.2°. Samples were removed from the bath at intervals and immediately cooled in a melting ice bath. They were stored at 4° until infectivity titrations were made: this was generally within a few hours, but on a few occasions there was an interval of 2 or 3 days.

Infectivity titrations were made by pock counts on the chorioallantoic membranes of 12- or 13-day chick embryos. The refinements of membrane handling technique described by Westwood, Phipps & Boulter (1957) were used. In titrations of WR strain 3-day instead of the usual 2-day incubation was used, because after 2 days the pocks were pale and poorly developed.

Diluent. All extractions and dilutions were made in McIlvaine's phosphate + citrate buffer, pH 7.2, 0.004 M-phosphate.

In graphs of the results each point is the arithmetic mean of at least four experiments unless otherwise stated. The results are plotted as log $V_o/V$ against time, where $V_o$ is the original concentration and $V$ the concentration at any time $t$. This enables all the inactivation curves to be given a common
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origin for ease of comparison, and it is also one of the terms of the first-order reaction rate equation: \( \log \frac{V_0}{V} = Kt/2.3 \).

RESULTS

At 60° there is an initial rapid inactivation of most of the virus, and a slower inactivation of a small residual fraction. An experiment was done to see whether this greater resistance to heat was genetically determined. From a membrane inoculated with a sample heated for 60 min. a single pock was taken and extracted in 1 ml. of buffer. The bacteriologically sterile extract

was passaged undiluted on the chorioallantoic membranes of five 12-day embryos and 2 days later the virus was extracted from the infected membranes. Fig. 1 shows the course of inactivation at 60° of the original virus and of the clone derived from the single pock. In four tests the inactivation at 60° of preparations descended from 'heat-resistant' virus did not differ from that of the original virus.

The clone derived from 'heat-resistant' virus was inactivated at 50°, 52.5° and 55°. Over the range 50°–60° inactivation appeared to proceed as a first-order reaction until more than 90% of the infectivity had been destroyed (Fig. 2). The inactivation curves suggest two processes, and are consistent with the view that the second process not only succeeds the first but may start simultaneously with it. The velocity constants of both the fast and the slow inactivation, calculated for each temperature (Table 1), show that the rate of fast inactivation is dependent on temperature, and that of the slow
inactivation of the heat resistant survivors is apparently independent of temperature—at least over the 10° range investigated. Fig. 3, a composite of several experiments at 50°, shows two inflexions, indicating two changes of inactivation rate. This phenomenon was observed in four experiments at 50° and may indeed prove to be real. In this connexion it may be noted that we have obtained similar double inflexions in the curves of inactivation of vaccinia virus by γ rays (unpublished work). This was not looked for at any other temperature.

Table 1. *Velocity constants of fast and slow inactivations of vaccinia virus showing temperature dependence of fast reaction* (*K*<sub>f</sub>) *and lack of temperature dependence of slow reaction* (*K*<sub>s</sub>)

<table>
<thead>
<tr>
<th>Temperature (°C.)</th>
<th><em>K</em>&lt;sub&gt;f&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th><em>K</em>&lt;sub&gt;s&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.14</td>
<td>0.07</td>
</tr>
<tr>
<td>52.5</td>
<td>0.42</td>
<td>0.073</td>
</tr>
<tr>
<td>55</td>
<td>0.87</td>
<td>0.074</td>
</tr>
<tr>
<td>60</td>
<td>1.48</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Table 2. *Activation energies* (*E*) *of heat-sensitive fraction showing decrease of E as the temperature of exposure increases*

<table>
<thead>
<tr>
<th>Temperature range (°C.)</th>
<th><em>E</em> (cal./mole/°C.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50–52.5</td>
<td>91,800</td>
</tr>
<tr>
<td>52.5–55</td>
<td>61,600</td>
</tr>
<tr>
<td>55–60</td>
<td>23,000</td>
</tr>
<tr>
<td>50–60</td>
<td>50,270</td>
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</table>

The energies of activation (*E*) for the various temperature ranges investigated were calculated from the velocity constants of the fast reactions (Table 2). The decrease in activation energy over the temperature range studied suggests that as temperature increases either a smaller input of energy is necessary to induce physicochemical changes in the virus or there is less virus present which is susceptible to heat inactivation.

The results of two experiments with the WR strain of virus at 55° are similar to those with the Lister Institute strain at 55° (Fig. 4). Clearly, strains which behave very differently in infected hosts may have similar susceptibility to heat.

**DISCUSSION**

It has been generally assumed that the inactivation of viruses at different temperatures is exponential (Luria, 1953). Adams (1949) found that the inactivation of bacteriophage by heat was exponential, and increased very rapidly over the 10° range from 63° to 73°. Bronson & Parker (1943) reached similar conclusions about the inactivation of myxoma virus. Fenner (1953) had similar results with two strains of vaccinia virus. He did not, however, continue his experiments long enough to reach zero infectivity; this point was estimated by graphic extrapolation of the line obtained in short-period
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experiments. Bourdillon (1944), working with a partially purified mouse-brain preparation of Columbia SK virus, obtained a family of inactivation curves at 49.5°, 56.5° and 63°. His results suggested that the reaction did not follow first-order kinetics, but are insufficient to allow any firm conclusions to be drawn from them. Lauffer & Carnelly (1945) and Scott & Lauffer (1946) were unable to show that influenza virus haemagglutinin was destroyed as a first-order reaction when heated. They managed to obtain a linear relationship

by plotting the reciprocal of the square root of residual haemagglutinating activity against time. This relationship indicates that influenza haemagglutinin is heterogeneous in its heat sensitivity—a state of affairs readily ascertained by less circuitous methods.

Lauffer, Wheatly & Robinson (1949) inactivated influenza virus at various temperatures in the presence of urea or formaldehyde. In only one case did they continue an experiment for as long as 30 min. They made no comment about heterogeneity of response to heat, but since the lowest titres reached in their experiments were in the region of $10^4$ LD50 doses they may not have reached the level at which heterogeneity would be manifest. Lauffer & Wheatly (1951) re-investigated the problem of the alleged $3/2$ order reaction with influenza haemagglutinin and concluded that the behaviour of their virus was conditioned by the presence, in even the most highly purified preparations, of a mixture of rapidly and slowly reacting haemagglutinin particles. Bachrach, Breese, Callis, Hess & Patty (1957) showed that foot-and-mouth disease virus has a heat inactivation pattern very similar to that found by us for vaccinia.

Our results suggest that vaccinia virus preparations also contain particles
with two or three sensitivities to heat. It is more likely, however, that a
given vaccinia virus population contains a great preponderance of particles
which are rapidly inactivated and a smaller proportion of varying suscepti-
bility to heat. This can be seen in Fig. 2, in which the resistant fraction is
decreased from about 10 % at 50° to less than 0.000001 % at 60°. The values
of E in Table 2 also suggest that as the temperature increases there is a pro-
gressively smaller proportion of heat-resistant particles which have to be
endowed with enough energy to raise them to the activated state in which
changes in molecular structure may be expected to occur.

The suggestion that virus populations are heterogeneous in various respects
has been made for several animal viruses. Smith & Cohen (1956) separated
influenza virus strains into rapidly- and slowly-eluting components, and ob-
tained fractions unable to elute spontaneously from human red cells. The pro-
geny both of rapidly-eluting and of non-eluting fractions proved in each case to
be of mixed elution behaviour, i.e. similar to the original strain. Gard (1957),
in a discussion on the chemical inactivation of viruses by formaldehyde,
concluded that the manifest divergence from first-order kinetics of not only
poliomyelitis virus but also tobacco mosaic virus and probably influenza
virus was evidence of heterogeneity. He put forward two theories: that hetero-
genality is genetically determined, or that it is induced by the exposure to
formaldehyde. He considered that the fact that genetically homogeneous strains
of virus could not be obtained was evidence against the first and in favour of
the second hypothesis. Although our results agree with Gard’s in that resis-
tance to inactivation is not genetically controlled, heterogeneity of various
types is not excluded as a normal attribute of virus populations. Although it
is possible that the coagulating action of formaldehyde renders surviving
virus steadily less susceptible to other chemical actions, it is very difficult
to imagine any way in which exposure to heat could make the exposed virus
particles more resistant to heat.

No explanation can be offered for the fact that the inactivation rate of the
resistant fraction of virus is independent of temperature over the range studied.
It is possible that some light may be shed on this by studies of other methods
of inactivation. We obtained curves of similar shape when vaccinia virus was
exposed to large doses of γ-radiation. Whatever its explanation, there is
little doubt that the pattern of inactivation of this virus, and of others (Bour-
dillon, 1944; Lauffer & Carnelly, 1945; Scott & Lauffer, 1946; Lauffer &
Wheatly, 1951; Woese, 1956; Timm, McLean, Kupsky & Hook, 1956;
Taylor et al. 1957) is a character of many viruses when they are inacti-
vated by agents as different as heat, formaldehyde and ultraviolet radiation.
This means that the margin between inactivation of infectivity and destruc-
tion of antigenicity may often be too small to be of practical value. It also
means that, since small numbers of virus particles may resist inactivation for
long periods, sensitive and stringent tests must be used to detect this minute
proportion of survivors in inactivated virus vaccines. This point has recently
been made by Gard and other workers in the poliomyelitis vaccine field, but it
is a subject of such great practical importance that it cannot be overstressed.
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REFERENCES


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