Thermal Inactivation of Untreated and Gamma-irradiated A2/Aichi/2/68 Influenza Virus

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SUMMARY

The infectivity and the haemagglutinin activity of A2/Aichi/2/68 influenza virus were unchanged during a 2-year storage in allantoic fluid at −80 °C. Over the temperature range from −20 to +37 °C, exponential slopes could be drawn by means of the regression analysis, the velocity constants showing very low values. Conversely, at +56 °C inactivation took place in a two-component fashion, each following first-order kinetics. Haemagglutinin and neuraminidase activities were not impaired by exposure to [6°Co]-γ-rays (3 × 10⁶ rad), which completely removed infectious particles. The specific rate constants for inactivation of haemagglutinin and neuraminidase activities of untreated and irradiated virus were overlapping when samples were stored for 2 years in the frozen state (−80 and −20 °C), whereas a significantly increasing rate of inactivation was recorded for γ-irradiated samples following storage at temperature above 4 °C. Nevertheless, the energy of activation required for thermal inactivation was very low and the entropy of activation showed negative values for both the untreated and the irradiated virus preparations.

Previous work (De Flora & Badolati, 1970) has shown that [6°Co]-γ-rays exert a selective action on some functions of A2/Hong Kong virus (A2/Genova/8060/69 strain). In fact, the doses required to achieve complete removal of infectious particles (2 × 10⁶ rad) do not affect its haemagglutinin (HA) titre nor alter its ultrastructural appearance. HA ability is abolished following exposure to 15 × 10⁶ rad and high doses of γ-rays are also needed to alter the virus morphology.

Our investigation has been now extended by comparatively exploring, at various temperatures, the inactivation kinetics of untreated and of γ-irradiated A2/Aichi/2/68 prototype strain of A2/Hong Kong influenza virus. Vials of virus, grown in allantoic fluid, were exposed to 3 × 10⁶ rad in a Vickers Vickrad laboratory irradiation unit and then kept at −80, −20, +4, +20 and +37 °C, together with samples of untreated virus. At various time intervals, samples were checked for infectivity and for HA activity and, at the end of a 2-year storage, also for neuraminidase activity, which was determined according to Webster & Laver (1967). The inactivation patterns of infectivity, HA and neuraminidase properties were comparatively checked also at +56 °C.

No decrease of infectivity could be detected at −80 °C after 2 years (Fig. 1, dotted lines), whereas the half-life of infective particles was about 100 days at −20 °C, 15 days at +4 °C, 5 days and 14 h at +20 °C and 7 h at +37 °C, respectively.

Over this range of temperature, straight lines, derived by means of regression analysis, could be interpolated between symbols plotted on a semilogarithmic graph, indicating that the survival ratio was a decreasing exponential function of time:

\[ N/N_0 = e^{-kt}, \]
Fig. 1. Rate of inactivation of haemagglutinin activity of untreated (○—○) and γ-irradiated (3 × 10⁶ rad) (●—●) A2/Aichi/2/68 virus stored in allantoic fluid at temperatures ranging from −80 to +37 °C. The dotted lines refer to the rate of inactivation of infectivity.

where $N_0$ was the initial activity (8.5 EID₅₀/ml) and $N$ was the activity after storage at a given temperature for time $t$. The velocity constants ($k$) for the inactivation of infectivity, calculated according to the equation

$$k = \frac{(\ln N_0/N)}{t},$$

are listed in Table 1.

The velocity constants for the inactivation of infectivity at +4, +20 and +37 °C, drawn on an Arrhenius plot as a function of the reciprocal of absolute temperature, showed a linear relationship. Hence, the thermodynamic parameters of inactivation were calculated according to Eyring’s theory (Eyring, 1935) of absolute reaction rates by simultaneously solving at two different temperatures the equation

$$k = K T / h \times e^{-\Delta H / R T} \times e^{\Delta S / R},$$

where $K$ is the Boltzmann’s constant, $T$ is the absolute temperature, $h$ is the Planck’s constant, $R$ is the gas constant, $\Delta H$ and $\Delta S$ are the energy and the entropy of activation of the process, respectively.

The results of these calculations showed that inactivation of A2/Aichi/2/68 virus infectivity over the temperature region from +4 to +37 °C is associated with a low $\Delta H$ value (18.6 kcal/
Table 1. Velocity constants ($k \times 10^{-6}/s$) for the inactivation of infectivity, haemagglutinin and neuraminidase properties of untreated and $\gamma$-irradiated ($3 \times 10^6$ rad) A2/Aichi/2/68 virus stored in allantoic fluid

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>Untreated virus</th>
<th></th>
<th>Irradiated virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infectivity</td>
<td>Haemagglutinin</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>$-80$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$-20$</td>
<td>0.003</td>
<td>0.111</td>
<td>0.003</td>
</tr>
<tr>
<td>+4</td>
<td>0.004</td>
<td>0.542</td>
<td>0.013</td>
</tr>
<tr>
<td>+20</td>
<td>0.006</td>
<td>3.198</td>
<td>0.021</td>
</tr>
<tr>
<td>+37</td>
<td>0.009</td>
<td>21.656</td>
<td>0.032</td>
</tr>
<tr>
<td>Primary rate</td>
<td>8666</td>
<td>483</td>
<td>1835</td>
</tr>
<tr>
<td>Secondary rate</td>
<td>4833</td>
<td>28</td>
<td>181</td>
</tr>
</tbody>
</table>

mol) and with a negative $\Delta S$ value ($-19.9$ cal/mol/deg). This is rather typical for inactivation of RNA viruses (Woese, 1960). Similar values were calculated for instance by Pollard (1953) for inactivation at low temperatures of TMV ($\Delta S = 17$ kcal/mol and $\Delta H = -21$ cal/mol/deg), by Ginoza (1958) for inactivation of TMV-RNA ($\Delta H = 19$ kcal/mol and $\Delta S = -19$ cal/mol/deg) and by Black (1959) for inactivation of measles virus ($\Delta H = 18$ kcal/mol).

The decay of HA activity at temperatures ranging from $-80$ to $+37^\circ$C followed first-order kinetics for both the untreated and the irradiated virus samples and proceeded at very low rates if compared to inactivation of infectivity (Fig. 1), thus indicating that thermal inactivation of infectivity is firstly due to chain breakage of RNA.

In particular, HA activity was unmodified after a 2-year storage at $-80^\circ$C. At $-20^\circ$C a very slight decrease was detectable for both the untreated and the $\gamma$-irradiated samples, without any significant difference ($P > 0.05$) between the corresponding regression coefficients ($t = 0.080$).

Conversely, at temperatures above $+4^\circ$C the irradiated samples displayed a greater sensitivity to thermal inactivation, which was more and more marked by increasing the temperature. The comparison of regression coefficients showed significant differences ($P < 0.01$) at $+4$, $+20$ and $+37^\circ$C ($t = 8.685$, $10.708$ and $26.313$, respectively).

The velocity constants for HA inactivation in the range from $+4$ to $+37^\circ$C fit on an Arrhenius plot two parallel straight lines, whose levels were significantly different. This means that the HA inactivation of untreated and of $\gamma$-irradiated A2/Aichi/2/68 virus proceeded on two parallel levels characterized by different thermodynamic parameters.

The energy of activation for the decay of HA activity showed, however, very moderate values for both the untreated and the $\gamma$-irradiated virus preparations ($\Delta H = 3.3$ and $4.0$ kcal/mol, respectively) and the entropy of activation had negative values ($\Delta S = -84.7$ and $-79.7$ cal/mol/deg, respectively). Since the thermal inactivation of protein molecules is generally associated with high values of $\Delta H$ and large positive values of $\Delta S$ (Woese, 1960), the considerable resistance of A2/Aichi/2/68 virus HA to chain breaking at low temperatures could be ascribed to the glycoprotein nature of spike subunits of influenza viruses (Compans et al. 1970; Schultze, 1970; Skehel & Schild, 1971).

After a 2-year storage, no statistically significant difference ($P > 0.05$) could be revealed between the neuraminidase activities of untreated and of irradiated samples at $-80$ or at $-20^\circ$C ($t = 0.919$ and $0.478$, respectively). Conversely, neuraminidase activity, like HA activity, was significantly higher ($P < 0.01$) for untreated than for $\gamma$-irradiated virus at $+4$, $+20$ and $+37^\circ$C ($t = 7.015$, $5.555$ and $9.956$, respectively). Neuraminidase of irradiated virus was completely inactivated within 2 years at $+37^\circ$C.
Fig. 2. Ninety per cent destruction at +56 °C of untreated (---, infectivity; ○--○, haemagglutinin and △--△, neuraminidase activities) and of γ-irradiated (●—●, haemagglutinin and, △—▲, neuraminidase activities) A2/Aichi/2/68 influenza virus in allantoic fluid.

At +56 °C the loss of infectivity was very rapid (Fig. 2). The reaction followed first-order kinetics for 22 min, when only 0.001 % of infectious particles was still viable, and then a second slower step was detectable. Similarly, the inactivation of HA and of neuraminidase took place in two stages, each following first-order kinetics. As determined by extrapolating the slow inactivation slope to the ordinate, each stage was found to account for approximately 50 % of the virus populations for both the untreated and the irradiated samples. As shown in Table 1, also at +56 °C, the velocity constants for the inactivation of neuraminidase and HA properties were higher for γ-irradiated than for untreated virus.

A number of factors could have been involved in the two-step patterns observed as +56 °C, such as inhomogeneity of the virus population, different inactivation mechanisms (chain breakage and denaturation) or formation of virus aggregates. It could be also assumed that the denaturation of the two different types of molecules, which have been associated with both the HA and the neuraminidase activities (Webster, 1970; Laver, 1971; Skehel & Schild, 1971), occurs at different inactivation rates, whereas phenomena of multiplicity reactivation could have been involved in the course of infectivity inactivation.

It is noteworthy that the HA displayed an identical sensitivity to thermal inactivation at +56 °C when virus was diluted in freshly collected, uninfected allantoic fluid or in γ-irradiated, uninfected allantoic fluid stored at +37 °C for 2 years. This indicated that no difference could occur in the protective action of medium on virus activity. It must then be assumed that exposure to γ-rays (3 × 10⁶ rad) results in a subtle damage of molecules responsible for HA and neuraminidase activities of A2/Aichi/2/68 virus, which is, however, undetectable by means of usual techniques just after irradiation or also after prolonged storage in the frozen state, but is revealed under non-optimal conditions of storage.

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REFERENCES


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