Biochemical Studies on Influenza Virus Multiplication at Reduced Temperatures

By R. ROTT AND C. SCHOLTISSEK

Institut für Virologie, Justus Liebig-Universität, Giessen, Germany

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SUMMARY

At 25° all components of fowl plague virus were synthesized in chick embryo fibroblasts. The rate of formation was about a quarter of that at 37°, but the final yield was the same. Infectious virus, however, was not formed at 25°. If infected cells were preincubated at 25° for 20 hr and thereafter kept at 37°, a normal yield of infectious progeny was found. With longer preincubation at 25° a decreasing yield of infectious virus was found. This effect may have been due to the synthesis at 25° of a viral component responsible for the cytopathic effect which acts only at the higher temperature. Preincubation of infected cells at 37° for different times and further incubation at 25° revealed a virus-specific precursor synthesized early in the infectious cycle which was rate limiting at 25°.

INTRODUCTION

During the multiplication of influenza viruses at 37° the formation of the various complex viral subunits occurs at different times and at different sites in the host cell. Little is known about the synthesis of the macromolecules of the different subunits. With fluorescent antibodies, for example, the viral inner component, the RNP-antigen, has been demonstrated in the cell nucleus, while part of the envelope, the haemagglutinin, is confined to the cytoplasm. The assembly of the viral subunits into infectious particles is assumed to occur at the cell membrane (for review see Rott & Scholtissek, 1967).

It might be expected that after incubation of influenza virus in infected cells at reduced temperatures one or several steps in virus reproduction would be slowed down or inhibited completely. We hoped that by this method intermediate steps and possibly existing precursors could be studied.

METHODS

Virus. The ROSTOCK strain of fowl plague virus was used in all experiments. The virus was propagated in chick embryos.

Growth experiments were made by infecting chick embryo fibroblasts at an added multiplicity of about 10. After an adsorption period of 30 min. at 37° the cells were washed and incubated at the temperatures mentioned.

Where not otherwise stated, the biological activities were determined in extracts of infected cells which were broken up by three times freezing and thawing with 2 ml. buffered saline.
Infectivity was determined by a plaque test (Schäfer, Zimmermann & Schuster 1959).

Haemagglutination tests were performed by a standard method in plastic plates using a 1% suspension of chicken erythrocytes (Davenport, Rott & Schäfer, 1960).

Complement-fixation tests were done by a modification of the micromethod of Fulton & Dumbell (1949) described by Hennessen (1955). The complement-fixing activity of viral antigen was compared using convalescent serum from mice infected with influenza A2/FM1 and normal mouse serum. Q is the ratio of the amount of complement causing 50% haemolysis in the reaction mixture with antiserum to that with normal serum.

Neuraminidase activity was determined according to Drzeniek (1967) using bovine sialolactose as substrate. The reaction mixture was incubated for 15 min. at 37° in 0.1 M-phosphate buffer, pH 6.5. The enzymic activity was expressed in μg. sialic acid released per ml. virus material.

Experiments using radioactive isotopes. The same materials and methods were used as already described by Scholtissek, Becht & Drzeniek (1967).

RESULTS

Virus multiplication at 25°

RNP-antigen, haemagglutinin and neuraminidase of fowl plague virus were synthesized at 25° (Fig. 1). Compared to normal conditions (37°) about a fourfold incubation period was necessary until the corresponding activities could be demonstrated.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Temp. (°C.)</th>
<th>Infected or not</th>
<th>Counts/min. in TCA-precipitate</th>
<th>Counts/min. in TCA-soluble fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]uridine</td>
<td>37</td>
<td>No</td>
<td>20,400</td>
<td>9,750</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>28,900</td>
<td>15,300</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>No</td>
<td>11,810</td>
<td>12,300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>12,650</td>
<td>16,700</td>
</tr>
<tr>
<td>[14C]leucine</td>
<td>37</td>
<td>No</td>
<td>26,350</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>24,520</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>No</td>
<td>11,980</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>11,620</td>
<td>—</td>
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</table>

After infection half of the cultures were incubated at 25°, the other half at 37°. Two hr after infection either 0.3 μC [14C]uridine or 0.1 μC [14C]leucine were added and the cultures were processed 2 hr later. One tenth of the neutralized TCA-extract was dried and its radioactivity counted disregarding self-absorption.

When 25° was chosen as incubation temperature, the intervals between the appearance of the RNP-antigen and the haemagglutinin were longer than at 37°. At 25° haemagglutinating and neuraminidase activities seemed to appear at the same time as at 37°. Infectious particles, however, were not formed at 25° even when cells were incubated for 48 hr after infection with fowl plague virus. There was no difference whether the
Influenza virus multiplication at reduced temperatures

infected cells were incubated in minimal medium or growth medium. At 25° virus-specific activities were found, at any time after infection, only inside the cells and were not released into the medium.

Fig. 1. Synthesis of fowl plague virus and its subunits at 25° in chick fibroblasts. O, Cell-associated p.f.u.; ●, cell-free p.f.u.; □, cell-associated complement-fixing antigen; △, cell-associated neuraminidase activity; ▲, cell-associated haemagglutinin.

Fig. 2. Influence of transfer from 25° to 37° at various periods on fowl plague virus development. Cells infected with fowl plague virus were incubated at 25° and at the times indicated by the arrows transferred to 37°. Eight hr later infectivity was determined.

Cells kept at 25° retained their full capacity to produce virus particles. This became obvious when non-infected cells were incubated at 25° for 40 hr and transferred to 37° after infection. After 8 hr at 37° the normal yield of virus progeny was obtained. On the other hand no increase in infectivity was observed when the infected cells were kept
at 25° for 40 hr and then incubated at 37° for 8 hr. By shortening the incubation time at 25° of infected cells an increasing number of infectious particles was found when the temperature was changed to 37°. A normal yield of infectious virus was obtained when the infected cells were kept at 25° up to 20 hr after infection (Fig. 2).

In order to elucidate the reason for the retarded synthesis of the viral subunits and the lack of formation of infectious virus particles at 25° the synthesis of macro-

Table 2. Incorporation of [14C]uridine into RNA and into the TCA-soluble extract of infected and uninfected cells

<table>
<thead>
<tr>
<th>Period of incubation at 25° (hr)</th>
<th>Period of incubation at 37° (hr)</th>
<th>Period of pulse (hr)</th>
<th>Infected or not</th>
<th>Counts/min. in RNA</th>
<th>Counts/min. in TCA-extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 to 3</td>
<td>2 to 3</td>
<td>No</td>
<td>45,250</td>
<td>43,300</td>
</tr>
<tr>
<td>0 to 24</td>
<td>None</td>
<td>23 to 24</td>
<td>No</td>
<td>57,500</td>
<td>49,000</td>
</tr>
<tr>
<td>0 to 23</td>
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<td>23 to 24</td>
<td>No</td>
<td>4,080</td>
<td>8,170</td>
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<td>0 to 23</td>
<td>23 to 24</td>
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<td>Yes</td>
<td>6,200</td>
<td>11,100</td>
</tr>
<tr>
<td>0 to 48</td>
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<td>47 to 48</td>
<td>No</td>
<td>19,770</td>
<td>17,050</td>
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<tr>
<td>0 to 47</td>
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<td>21,800</td>
</tr>
<tr>
<td>0 to 47</td>
<td>47 to 50</td>
<td>49 to 50</td>
<td>No</td>
<td>30,550</td>
<td>24,800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>29,800</td>
<td>23,050</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6,210</td>
<td></td>
</tr>
</tbody>
</table>

1 μc [14C]uridine per culture was added as indicated in the Table. One hr later the cultures were processed. For further details see Table 1.

Table 3. Incorporation of [14C]uridine into RNA, UDP-X (X = glucose or N-acetylglucosamine), and UTP at 37° or 25°, respectively, in uninfected cells

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>RNA</th>
<th>UDP-X</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>20,400</td>
<td>28,300</td>
<td>25,400</td>
</tr>
<tr>
<td>25</td>
<td>11,810</td>
<td>16,800</td>
<td>56,200</td>
</tr>
</tbody>
</table>

The same experiment as in Table 1. All data were corrected for self-absorption. There was no significant radioactivity found in uridine, UMP, and UDP.

molecules and their precursors in infected and non-infected cells was examined by radio-isotopic techniques. The rates of RNA and protein synthesis were reduced at 25° by a factor of about 2 as could be demonstrated by the immediate addition of the isotopes after changing the temperature (Table 1). If the preincubation time at 25° was longer than 20 hr, less [14C]uridine was incorporated into the RNA (Table 2). The label accumulated at 25° as [14C]UTP which is relatively slowly incorporated into RNA and into the carbohydrate precursors UDP-glucose and UDP-N-acetylglucosamine (UDP-X) (Table 3). The unexpected high rate of phosphorylation of [14C]uridine at 25° is due to the fact that the temperature optimum of uridine kinase is 30° (Scholtissek, 1968). RNA labelling reduced at 25° returned to a sizeable rate when
non-infected cells were transferred to 37°. When infected cells, however, were pre-incubated for 47 hr at 25° and then transferred to 37°, RNA metabolism broke down rapidly, owing to decreased synthesis of energy-rich phosphate precursors (Table 2).

A cytopathic effect, as defined by the breakdown of RNA metabolism in infected cells (Scholtissek et al. 1967), could not be observed when infected cells were kept at a constant temperature of 25° for 48 hr (Table 2). If the temperature was raised after this period to 37° the cytopathic effect occurred very soon. This may at least partly explain why no infectious virus was formed under these conditions.

![Graph showing influence of transfer from 37° to 25° on the early phase of fowl plague virus development](image)

Fig. 3. Influence of transfer from 37° to 25° on the early phase of fowl plague virus development. Infected cells were kept at 37° and transferred to 25°, 1 (■), 2 (□), 3 (●) and 4 (○) hr after infection, respectively. △—△, normal development of haemagglutinin at 37°. Haemagglutinin was assayed after infection at the times indicated on the abscissa.

**Effect of temperature pulses on virus multiplication**

When incubation at 25° was only briefly interrupted (37° for 30 min.) at different times after infection, the short lasting higher temperature had no effect on the formation of infectious fowl plague virus.

Since the formation of viral subunits was considerably retarded at 25°, infected cells were kept, starting from the time of infection, for different times at 37° before incubation at 25°. The shorter the time of preincubation at 37° the slower were the initial rates of production of haemagglutinin which levelled off at different yields of the viral subunit (Fig. 3). The same pattern of production was also found for the neuraminidase and the RNP-antigen.

Delays in penetration of virus into the cell or in synthesis of early protein(s) cannot explain the results for three reasons. First, 2 hr after infection the synthesis of a stable early protein necessary for the synthesis of viral RNA is completed in the majority.
of the cells (Scholtissek & Rott, 1961). At this time the retardation at 25° was still very obvious. Secondly, when penetration occurred at 37° in cells treated with actidione, an inhibitor for protein synthesis, treated and untreated cells behaved in the same way (Fig. 4). It is already known that in the presence of inhibitors of protein synthesis the virus penetrates normally (Zimmermann & Schäfer, 1960). Thirdly, in immuno-fluorescence experiments with antibodies specific for the surface antigen of fowl plague virus 3 to 4 hr after infection practically all cells showed fluorescence in the cytoplasm, irrespective of whether they had been kept at 37° all the time or only for 2 hr and then further incubated at 25°. As expected, the intensity of fluorescence was greater in cells continuously incubated at 37°.

These findings clearly demonstrate that the results shown in Fig. 3 cannot be explained by any delays in penetration or the formation of early protein(s).
**DISCUSSION**

Our first object was to separate the different steps of fowl plague virus multiplication and to study them in greater detail. We achieved this by reducing the incubation temperature to 25°. At this temperature all viral subunits are formed at a slower rate but with a normal yield. No increase in infectious virus, however, was observed which might be due to an inhibition of the maturation process at the cell membrane.

Our second object was to find possible virus-specific precursors for the various viral subunits. The results in Fig. 3 demonstrate that the rate of synthesis and the yield of all viral subunits at the break of the curve at 25° depends on the length of time of preincubation at 37°. Since delays in penetration or in the formation of early protein(s) can be excluded as being responsible for these results, it must be assumed that the formation of a precursor(s) necessary for the synthesis of all three viral subunits is particularly sensitive to lower temperatures. By changing the temperature from 37° to 25° the amount of the precursor present at this time determines the rate of synthesis of the viral subunits. The breaks in the curves at different levels suggest that the precursor formed might be used up by incorporation into a viral subunit or by breakdown, and that there is no further production of this substance at the former rate after the reduction of temperature.

The nature of this precursor is unknown. Since the synthesis of all viral subunits depends on its presence, its formation must occur very early in the replication cycle. The early protein(s) can be excluded, since its synthesis is finished 2 hr after infection and its activity is stable during the infectious cycle (Scholtissek & Rott, 1961). The viral RNA or its replicative form is a reasonable candidate for this precursor.

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**REFERENCES**


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