Stability and Precursor Relationships of Virus RNA

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SUMMARY
Pulse-chase experiments with [3H]-uridine were performed with glucosamine-treated chick cells infected by RNA viruses. It was established that in cells infected by fowl plague virus both virus RNA and the RNA with the complementary base sequence were metabolically stable. Newcastle disease virus-specific RNA was also metabolically stable in chick fibroblasts. In cells infected by Semliki Forest virus, radioactivity was not lost from the virus-induced 26 S RNA, while the activity of the partially RNase-resistant 20 S RNA could be chased to RNase-sensitive RNA of high mol. wt.

INTRODUCTION
In order to measure the stability of virus RNA or to establish its precursor relationships, pulse-chase experiments with radioactive RNA precursors are essential. In animal cells the nucleoside triphosphate pools are too large for their influence to be diluted out by non-labelled nucleosides after the labelling period. In a new method which overcomes this difficulty (Scholtissek, 1971) the UTP pool is reduced drastically by incubating the cells with glucosamine prior to pulse labelling with uridine. Alternatively, the UTP pool is drained by the amino sugar after incorporation of [3H]-uridine. This method has been used to measure the in vivo stability of the RNA of Newcastle disease, fowl plague, and Semliki Forest viruses.

It has been shown recently that glucosamine interferes with the production of infectious fowl plague and Semliki Forest virus without interfering with the synthesis of virus RNA. The amino sugar has no significant effect on the multiplication of Newcastle disease virus (Kaluza, Scholtissek & Rott, 1972). In view of these differences we compared the stability and precursor relationships of the virus RNA of these three viruses, which show characteristic differences in their cellular synthesis of virus RNA and its complementary RNA (Kingsbury, 1966; Bratt & Robinson, 1967; Scholtissek & Rott, 1970).

METHODS

Virus strains. The virus strains used were: Newcastle disease virus, strain ITALIEN (paramyxovirus); fowl plague virus, strain ROSTOCK (orthomyxovirus); Semliki Forest virus, strain OSTERRIETH (arbovirus).

Cell culture and virus titration. All experiments were performed in primary chick embryo fibroblasts (2 × 10^7 cells/dish), covered with 6 ml of minimal medium (Earle's medium containing twice the amount of glucose plus 2 mM-glutamine) in Petri dishes of 9 cm diameter. Cells were used 46 h after seeding. The multiplicity of infection was from 10 to 50 p.f.u./cell. Virus multiplication was followed either by plaque assay for Semliki Forest
virus or by haemagglutination for Newcastle disease virus and fowl plague virus (Rott & Scholtissek, 1968).

**Pulse-chase experiments.** Two methods were used in pulse-chase experiments (Scholtissek, 1971). In the first method, cells were incubated with glucosamine at 37 °C for 90 min before the pulse of [3H]-uridine at 4 °C. At this temperature uridine was taken up and phosphorylated to UTP, which was not metabolized further (Scholtissek, 1967). The cells were washed twice and covered again with minimal medium. The incorporation of [3H]-UTP into RNA and UDP-sugar derivatives was started by warming the cells to 37 °C. After 10 to 30 min the remainder of the labelled UTP was diluted by 100 µg/ml of non-labelled uridine (= chase). In the second method, [3H]-UTP or [3H]-uridine was incorporated into RNA in the presence of glucosamine. After about 30 min at 37 °C residual radioactivity of the UTP-pool was diluted out by 100 µg/ml of non-labelled uridine, as given in the Fig. or Table legends.

**Determination of virus RNA.** The labelled RNA of fowl plague virus was determined in the presence of newly synthesized cellular RNA after phenol extraction by specific hybridization with a surplus of either non-labelled virus RNA or its complementary RNA. Cells were labelled with [3H]-uridine starting 2.5 h after infection. At the times indicated total RNA was extracted by phenol plus SDS and dissolved in 5 mM-Tris-HCl, pH 8.1, containing 1 mM-EDTA. Samples were taken for determination of radioactivity in total RNA, in virus RNA, or in complementary RNA (Scholtissek & Rott, 1970). The RNA of Newcastle disease or Semliki Forest virus was determined by adding either actinomycin D at 2 µg/ml 90 min before the pulse was started or at 0.2 µg/ml immediately after infection, respectively. In a few cases, Newcastle disease virus RNA was also characterized by specific hybridization with a surplus of non-labelled virus RNA (Kingsbury, 1966).

**Sucrose-gradient analysis of virus RNA.** This was as described by Scherrer & Darnell (1962). The radioactivity of samples precipitated with trichloroacetic acid in the presence of carrier protein was determined as before (Scholtissek, 1971).

**Reagents.** [3H]-uridine (29 Ci/m-mol) was purchased from the Radiochemical Centre, Amersham, England. Actinomycin D was a gift from Merck, Sharpe and Dohme (New York, U.S.A.). D-glucosamine-HCl was obtained from Sigma (St Louis, Missouri, U.S.A.).

**RESULTS**

**Rate of incorporation of [3H]-uridine into virus and cellular RNA**

When cells were incubated with glucosamine for 90 min at 37 °C and then labelled at 4 °C with [3H]-uridine, [3H]-UTP was incorporated into RNA for no longer than about 30 min after warming the pre-labelled cells to 37 °C. After this time, most of the radioactivity disappeared from the UTP-pool and was found in RNA and in UDP-N-acetylglucosamine (Scholtissek, 1971). This method for pulse-chase experiments with virus RNA can be applied only when the labelling of virus RNA starts without significant lag. As shown in Fig. 1, this holds for fowl plague and Semliki Forest virus, but for Newcastle disease virus a lag of about 20 min was indicated by extrapolation to the abscissa of the linear part of the curves of Fig. 1b. The pulse-chase experiments with cells infected by Newcastle disease virus were performed only at 37 °C. Under these conditions the incorporation into cellular RNA did not cease within 40 min of the addition of the [3H]-uridine (Scholtissek, 1971). In cells pre-labelled at 4 °C, most of the radioactive UTP would have been used up for the synthesis of UDP-N-acetylglucosamine before its incorporation into Newcastle disease virus RNA had started. Indeed, in the former method very little radioactivity was found in NDV-RNA.
Precursor-product relationships of virus RNA

Fig. 1. Kinetics of the labelling of virus RNA with [3H]-uridine.
A, each point represents the mean of RNA analyses on five cultures. Each culture was labelled with 50 μCi [3H]-uridine starting 2-5 h after infection with fowl plague virus. At the times indicated the RNA was isolated. ●—●, total RNA x 10^-4; ○—○, virus RNA x 10^-4; Δ—Δ, complementary RNA x 10^-4.
B, each point represents the mean of RNA analyses on four cultures. Each culture was labelled with 50 μCi [3H]-uridine in the presence of actinomycin D starting 6-5 h after infection with Newcastle disease virus. RNA was extracted as described in A. ●—●, total RNA x 10^-4; ○—○, radioactivity in RNA x 10^-4, after hybridization with excess Newcastle disease virus RNA and treatment with RNase.
C, each point represents one culture in which 0.2 μg/ml actinomycin D was added immediately after infection with Semliki Forest virus. The pulse with 2.5 μCi [3H]-uridine was started 4.5 h after infection. ●—●, total RNA x 10^-4.

Fig. 2 shows the results of a pulse-chase experiment with cells infected by Semliki Forest virus after pre-incubation with glucosamine and pre-labelling at 4 °C with [3H]-uridine. Although the chase with non-labelled uridine was immediate and efficient, without addition of non-labelled uridine the incorporation of radioactive UTP ceased almost completely after 30 min of incubation at 37 °C. After larger chase some virus RNA was lost from the infected cells. It is not known whether this is due to breakdown or to package into immature particles which are released. Fig. 2 also demonstrates that the RNA of Semliki Forest virus is synthesized slowly even at 4 °C: 2500 ct/min were recorded before the pulse at 37 °C. This effect is in contrast to the synthesis of cellular RNA (Scholtissek, 1967) and is under investigation.

Stability of RNA’s of fowl plague and Newcastle disease viruses

The virus RNA and the complementary RNA of fowl plague virus show no significant breakdown to non-hybridizable material during incubation for more than 2 h (Table 1). There is, however, considerable loss of radioactivity from cellular RNA during the chase. Sucrose-gradient analysis of virus RNA cannot be applied to this system, since actinomycin D cannot be used to inhibit cellular RNA during synthesis of influenza virus RNA (Rott & Scholtissek, 1964; Scholtissek & Rott, 1970).

In NDV-infected cells the prevailing synthesis is of RNA with the base sequence complementary to that of virus RNA (Kingsbury, 1966) of which most is of a sedimentation
Fig. 2. Pulse-chase experiment with RNA of cells infected with Semliki Forest virus. Immediately after infection with Semliki Forest virus, cells received 0.2 μg/ml actinomycin D and 15 mM-glucosamine. At 4.5 h after infection the cells were cooled to 4 °C and incubated with 2.5 μCi [PH]-uridine per culture for 1 h. At 15 min (arrow) after the pulse was started at 37 °C, some cultures received 100 μg/ml non-labelled uridine (chase). ●—●, without uridine chase; ○—○, chase with non-labelled uridine.

Table 1. Stability of fowl-plague RNA under chase conditions

<table>
<thead>
<tr>
<th>RNA component</th>
<th>Time of chase (min)</th>
<th>Ct/min x 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>316</td>
<td>250</td>
</tr>
<tr>
<td>Virus</td>
<td>44</td>
<td>42</td>
</tr>
<tr>
<td>Complementary</td>
<td>31</td>
<td>27</td>
</tr>
</tbody>
</table>

After infection for 2.5 h, each culture was incubated for 1 h at 4 °C with 25 μCi [PH]-uridine. After removal of surplus [PH]-uridine the cultures were incubated with 10 mM-glucosamine at 37 °C for 30 min (pulse). Thereafter, 100 μg/ml of non-labelled uridine was added (chase). Each value is the mean for the RNA component analysed on five separate cultures. Replicate analyses showed a maximum range of 5%.

coefficient of 18 S (Bratt & Robinson, 1967). As shown in Fig. 3, the sucrose gradient pattern of RNA isolated from Newcastle disease virus-infected cells does not change significantly under chase conditions. In this experiment 2 μg/ml of actinomycin D and 10 mM-glucosamine were added to each culture 5 h after infection. The pulse with 25 μCi/culture of [PH]-uridine was started 90 min later. The RNA from six cultures was layered on to each sucrose gradient.
Precursor-product relationships of virus RNA

Precursor-product relationships of Semliki Forest virus RNA's

Pulse experiments with [3H]-uridine demonstrated that in Semliki Forest-infected cells a partially RNase-resistant 20 S RNA appears first and was followed by RNA of 26 S and 42 S (Friedman, Levy & Carter, 1966; Cartwright & Burke, 1970). Thus it was possible that these components were in precursor-product relationship.

In Fig. 4 the results of an experiment are presented in which cells received 0.2 µg/ml of actinomycin D and 10 mM-glucosamine immediately after infection with Semliki Forest virus. At 4.5 h after infection the cells were cooled to 4 °C and incubated with 5 µCi [3H]-uridine/culture for 1 h. The pulse was started by warming the cultures to 37 °C after removal of the surplus of labelled uridine. The radioactivity was chased 10 min later by an excess of non-labelled uridine. The 20 S RNA which appeared first was partially RNase-resistant and a 26 S component was recognized at about the same time. Although the label of the 20 S RNA was chased easily to single-stranded RNA of high mol. wt., the radioactivity of the 26 S RNA did not change significantly. The 42 S virus RNA appeared during the chase with other single-stranded RNA molecules of high mol. wt. but intermediate size (about...
Very similar results were obtained when [3H]-uridine was incorporated at 37 °C into virus RNA before the chase was performed with glucosamine plus non-labelled uridine, although the chase was now less efficient. Under these conditions breakdown to 15 S and 12 S RNA fragments was observed (Fig. 5), which was in agreement with in vivo results for Sindbis virus (Stollar, Shenk & Stollar, 1972).

DISCUSSION

The half-life times of messenger RNA's vary widely. Since both virus RNA and the RNA with the complementary base sequence can function as messenger, it was of interest to study their catabolism. This was aided by the development (Scholtissek, 1970) of a reliable method for the analysis of RNA metabolism in chick fibroblasts by pulse-chase experiments without the application of actinomycin D. When such studies were extended to the analysis of RNA in fowl plague-infected cells the surprising result was that both the newly synthesized virus RNA and the complementary RNA were rather stable. In cells infected with Newcastle disease virus most of the newly synthesized virus-specific RNA had the base sequence complementary to that of virus RNA (Kingsbury, 1966; Bratt & Robinson, 1967).
and here again this RNA was almost completely stable during chase. No precursor–product relationships of any kind were established between the several RNA components.

A different pattern was obtained when these methods were applied to cells infected by Semliki Forest virus. While the amount of the 26 S RNA of unknown function did not change during the chase, partially double-stranded 20 S RNA disappeared and label accumulated in high mol. wt. RNA. Thus the 20 S RNA may be a true replicative intermediate for the production of virus RNA, as suggested already by pulse experiments (Friedman et al. 1966; Cartwright & Burke, 1970).

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REFERENCES


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