Newcastle Disease Virus: Virus Particle and RNA Synthesis in Different Host Cells and at Different Temperatures*

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SUMMARY
When incubated in vitro at 40 °C, chorioallantoic membranes produce 80 to 200 p.f.u./cell of Newcastle disease virus (NDV), whereas chick embryo cells and mouse L cells produce only 2 to 10 and 0.1 p.f.u./cell, respectively. Under such conditions NDV ‘minus’ strand RNA accumulates in chick embryo cells (as well as in mouse L cells) but not in chorioallantoic membranes.

Lowering the incubation temperature from 40 to 36 °C resulted in delays in virus release by 5 h from chick embryo cells, but by 1 h from chorioallantoic membranes. The maximum rate of virus RNA synthesis was observed at times when the release of progeny virus had been terminated.

When protein synthesis is inhibited by early addition of cycloheximide, some NDV ‘minus’ strand RNA is found in the chick embryo cells but not in the chorioallantoic membranes. When the inhibitor is added later (2 h after virus infection), no virus RNA is labelled in the chick embryo cells, although some ‘plus’ strand RNAs are found labelled in the chorioallantoic membranes.

It is suggested that the observed difference in the permissiveness of various cell types for NDV depends on the amount of some cellular component involved in NDV ‘plus’ strand RNA synthesis.

INTRODUCTION
Cells infected with Newcastle disease virus (NDV) accumulate several species of RNA (so called ‘minus’ strand RNAs) which are complementary to the RNA extracted from the virus particles (‘plus’ strand RNA) (Kingsbury, 1966a, b, 1967; Bratt & Robinson, 1967). ‘Minus’ strand RNA was also found in cells infected by virus even when its infectivity was destroyed by u.v. irradiation prior to use (Huppert, Hillova & Gresland, 1969; Clavell & Bratt, 1971). Further, in these NDV-infected cells, the amount of virus-specific double-stranded RNA is small and it has a rapid rate of turnover (Zhdanov & Kingsbury, 1969; Huppert, Gresland & Hilo, 1970; Bratt & Robinson, 1971). A functional role of messenger has been attributed to those ‘minus’ strand RNAs found associated with cellular ribosomes (Bratt & Robinson, 1967, Blair & Robinson, 1968). However, the cause of ‘minus’ strand accumulation in the virus infected cells is not known.

Experiments, in which the accumulation of ‘minus’ strand RNA were observed, were done exclusively with chicken embryo cells (CEC) incubated either at 37 °C or at 40°C.

* Dedicated to the memory of Nicole Granboulan.
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The choice of this condition was probably guided by the fact that a supposed homogeneous population of chicken cells (considered to be essentially fibroblasts) is easily obtained in culture, that the normal body temperature of the chicken is about 40 °C and that eggs are hatched at 36 °C. It should be born in mind that CEC, under the above conditions, are poor NDV producers. Further, at temperatures higher than 37 °C, the in vitro RNA polymerase activity associated with NDV particles is markedly reduced (Huang, Baltimore & Bratt, 1971).

This paper provides information on the relationship between NDV particle production and RNA synthesis at the two temperatures (36 and 40 °C) using different types of cells.

**METHODS**

**Virus**

*Newcastle disease virus (Kansas Loevenhorst strain)* was propagated in 11-day-old embryonated eggs of lymphomatosis free chicken (Brown Leghorn-J. Cart's strain) bred in our Institute. The allantoic fluid was harvested 48 h after inoculation of 10^4 p.f.u./egg. The fluid was stored at −70 °C. Usually it contained 3 × 10^8 p.f.u./ml of NDV as titrated on CEC. In some instances the virus was further purified by ammonium sulphate precipitation and repeated sucrose gradient centrifugings by a modified procedure of Haslam, Cheyne & White (1969).

Essentially identical results were obtained with crude and purified virus.

**Cell cultures and infection**

*Chicken embryo cell (CEC).* Eleven-day-old chicken embryos from the lymphomatosis free chicken were trypsinized, seeded in large glass Roux bottles in Eagle's medium supplemented with 10% calf serum and 10% tryptose phosphate broth (TPB). Cultures were incubated in a 7% CO_2 atmosphere and used for experiments 24 h after planting when each bottle contained about 10^8 cells.

For infection the medium was removed, and enough virus was added to give 10 p.f.u./cell in a total vol. of 5 ml of medium per bottle. The cells were kept for 1 h at 20 °C, washed 5 times with phosphate-buffered saline (PBS) and refed with 80 ml of warm (40 °C) medium containing 2% calf serum and 2% TPB. For plaque assays, 4 × 10^6 CEC in 50 mm ‘Nunclon’ plastic dishes were infected with serial dilutions of virus and covered with medium containing 0.9% agar. After 3 days the dishes were fixed with formaldehyde, the agar was removed and the cells stained with methylene blue.

For assays of haemagglutinin (H.A.) samples of the virus preparations, diluted by two-fold steps, were mixed with an equal vol. of a suspension containing 7 × 10^5 washed chicken erythrocytes per ml. The last dilution showing haemagglutination after 15 h at 4 °C, was considered to contain 1 H.A.U.

*Chorioallantoic membranes (CAMs).* CAMs from 11-day-old eggs were rinsed with PBS, pooled, immersed in medium containing NDV (6 ml and 10^6 p.f.u.) and stored 30 min at 4 °C to allow virus adsorption. The CAMs were then washed 6 times with PBS, cut into small fragments and redistributed into flasks with 100 ml of Eagle's medium supplemented with 2% tryptose phosphate broth. Each flask contained the equivalent of 1 CAM, or according to Cairns & Fazekas de St Groth (1957) about 5 × 10^7 cells. The suspensions were incubated with constant stirring (60 rev/min) at 36 or 40 °C. At indicated intervals samples of the medium were assayed for p.f.u. on CEC.
Mouse L cells were propagated as monolayers in Eagle's medium containing 10% TPB and 10% calf serum with twice weekly passages. Infection with NDV was effected as described for the CEC and the produced virus assayed on chicken cells.

Materials. RNases (pancreatic and T1) and DNase (RNase free) were purchased from Worthington Biochemical Corporation. Actinomycin D was the gift of Merck, Sharp and Dohme. Cycloheximide (Acti-dione) was provided by the Upjohn Co. Eagle's medium (Dulbecco BHK modified 1959) was purchased from Eurobio (Paris). Tryptose phosphate broth (TPB) was a Difco product. All chemical reagents were Merck's analytical grade.

Radioactive materials. [5-3H]-Uridine (20 Ci/mmol) and [14C]-labelled amino acids (algae hydrolysate) were obtained from C.E.A. (Saclay–France).

Labelling of cells and extraction of RNA. For labelling, the infected cells after washing, were refed with medium containing 4 µg/ml of actinomycin D. At indicated times 10 µCi/ml of [3H]-uridine was added. RNA was extracted essentially according to the procedure of Bratt & Robinson (1967) by lysing the cells in the bottle with a solute of NTE (NaCl 0.14 M, tris 0.01 M, pH 8.4, EDTA 10−3 M), β-mercaptoethanol 1% (v/v) and sodium dodecyl sulphate 1%. The lysate (in 20 ml of final vol.) was twice extracted with NTE saturated phenol and the aqueous phase, precipitated with ethanol. The precipitate was dissolved in NTE, pH 7.4, incubated with 20 µg/ml of DNase, MgCl2 10−3 M, extracted with phenol and reprecipitated with ethanol. The amount of RNA was determined either by u.v. extinction or by orcinol reaction. RNA from purified virus particles was prepared in a similar way, except that the virus suspension was first incubated with pronase for 3 h at 37 °C (Huppert et al. 1966).

For gradient centrifuging the RNA was layered on 5 to 20% sucrose in NTE at pH 7.4. Two types of centrifugings were used: for analytical purposes 80 min at 64,000 rev/min (Spinco rotor SW 65); for preparative purposes, 17 h at 24,000 rev/min (SW 25). With the second type of centrifuging the 57S component sedimented to the bottom but a good separation of the 28S and 18S region was achieved. Fractions were collected from the bottom of the tube and assayed for E260 and for acid-insoluble radioactivity.

Annealing experiments. Labelled RNA in selected fractions from sucrose gradients were pooled, precipitated with ethanol and redissolved in 2 x SSC (1 x SSC = 0.15 M-NaCl, 0.015 M-Na-citrate). Samples were mixed with 0.25 µg of 57S RNA extracted from purified NDV particles and the vol. made up to 0.1 ml. The mixture was heated for 5 min at 100 °C, incubated for 1 h at 85 °C and cooled gradually (5 h) to 20 °C. The sample vol. was increased to 1 ml with SSC containing 10−3 M-EDTA and each sample was divided into two parts. One part was immediately precipitated in the presence of carrier RNA with cold trichloracetic acid (final concentration 5%). The other part was incubated with RNases (pancreatic 10 µg; T1 5 units) for 30 min at 37 °C and then precipitated. Samples were placed on Whatman GFC filters, washed, dried and counted with a toluene based PPO, POPOP mixture on a Beckman scintillator.

The difference in counts between the RNase treated and untreated part of the same sample was used to calculate the percentage of RNase resistance.

Controls consisted of RNA heated and rapidly cooled (indicating the residual RNase resistant counts, considered as background); of unheated RNA (in order to estimate the amount of double-stranded RNA originally present in the fraction) and of RNA annealed without the addition of ‘plus’ strand RNA (in order to estimate the amount of ‘plus’ strand synthesized by the infected cells).
Saturation experiments (Huppert, Gresland & Hillova, 1970) have shown that 0.1 µg of the 'plus' strand RNA from virus particles was sufficient to anneal 80 to 100% of the labelled RNA extracted from 1 to 2 x 10^8 infected cells.

RESULTS

NDV infection of chicken embryo cells at 40 and 36 °C

Cultures of chicken embryo cells (CEC) were infected with NDV in the presence of actinomycin D and incubated either at 40 °C or at 36 °C. At 40 °C, the first infectious progeny virus particles appeared in the medium 3 to 4 h after infection and production levelled off after another period of about 3 h. In contrast, at 36 °C, virus release occurred at 10 to 11 h but reached its plateau also after 3 h (Fig. 1 A). In general virus yield was in the order of 2 to 10 p.f.u./cell, however, it was consistently higher at the lower temperature; between 20 and 80% in nine experiments with an average of 40%. Assays for haemagglutinin have shown, for virus produced at both temperatures, a ratio in the order of 1 H.A.U. for 5 to 10 x 10^4 p.f.u. The precision of the H.A. assay was not sufficient to confirm the difference (in the 40% range) found by the p.f.u. estimation. This was also the case with estimates of the number of virus particles obtained by electron microscopy or by incorporation of [14C]-labelled amino acids.

Sucrose gradient analysis of RNA extracted from infected cells showed several labelled components (Fig. 2 A). The fast component (57S) was similar to the RNA from NDV particles (‘plus’ strand RNA); labelled RNA sedimenting around 28S and 18S were complementary to the virus RNA as shown by annealing experiments; these represent the
Fig. 2. Sucrose gradient analysis of RNA from NDV infected chicken cells. Centrifuging in a Beckman SW 65 rotor for 80 min at 63,000 rev/min at 4 °C. The arrows (28S and 18S) indicate the position of the E\textsubscript{480} peaks of the preparation. The 57S position was determined in a separate run using RNA extracted from NDV particles. A, CEC infected for 5 h and then labelled for 2 h. B, CEMs infected for 1.5 h and then labelled for 2 h. C, CEMs infected for 3 h and pulse labelled for 30 min. ••••, RNA from infected cells; ○○○○, RNA from uninfected, actinomycin D treated controls.
### Table 1. Annealing of RNA from various cells infected with NDV

<table>
<thead>
<tr>
<th></th>
<th>Percentage of acid insoluble counts after digestion with RNase</th>
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<tbody>
<tr>
<td></td>
<td>Before annealing</td>
<td>After annealing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ct/min/sample</td>
<td>Without ‘plus’ strand RNA</td>
<td>With NDV ‘plus’ strand RNA</td>
</tr>
<tr>
<td>CAMs incubated at 36 °C*</td>
<td>3400</td>
<td>4.7</td>
<td>3.8</td>
</tr>
<tr>
<td>CAMs incubated at 40 °C*</td>
<td>4900</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>CEC incubated at 36 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30S peak†</td>
<td>2800</td>
<td>18.4</td>
<td>20.0</td>
</tr>
<tr>
<td>18S peak†</td>
<td>6000</td>
<td>3.3</td>
<td>12.7</td>
</tr>
<tr>
<td>CEC incubated at 40 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30S peak†</td>
<td>3200</td>
<td>19.3</td>
<td>23.0</td>
</tr>
<tr>
<td>18S peak†</td>
<td>5000</td>
<td>4.1</td>
<td>11.7</td>
</tr>
<tr>
<td>Mouse L cells incubated at 40 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30S peak†</td>
<td>6000</td>
<td>8.2</td>
<td>15</td>
</tr>
<tr>
<td>18S peak†</td>
<td>17000</td>
<td>1.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Fractions no. 4 to 18 (40 to 10S) of sucrose gradients similar to that in Fig. 2B were pooled and after concentrating by ethanol precipitation used for annealing.

† The peak fractions (1 tube) of sucrose gradients centrifuged under conditions sedimenting the 57S ‘plus’ strand to the bottom of the tube were used for annealing.

Annealing as described under Methods. The indicated values are averages of 2 to 4 determinations.
NDV virus particle and RNA synthesis

Table 2. Effect of temperature on synthesis of NDV RNA

<table>
<thead>
<tr>
<th>Time of incubation at different temperatures</th>
<th>ct/min/μg of RNA</th>
<th>% of maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 h at 40 °C</td>
<td>1000</td>
<td>100.0</td>
</tr>
<tr>
<td>6.5 h at 36 °C</td>
<td>129</td>
<td>12.9</td>
</tr>
<tr>
<td>5.5 h at 40 °C + 1 h at 36 °C</td>
<td>373</td>
<td>37.3</td>
</tr>
<tr>
<td>5.5 h at 36 °C + 1 h at 40 °C</td>
<td>276</td>
<td>27.6</td>
</tr>
<tr>
<td>7.5 h at 36 °C</td>
<td>382</td>
<td>38.2</td>
</tr>
<tr>
<td>5.5 h at 36 °C + 2 h at 40 °C</td>
<td>644</td>
<td>64.4</td>
</tr>
<tr>
<td>6 h at 36 °C</td>
<td>1060</td>
<td>100.0</td>
</tr>
<tr>
<td>5 h at 36 °C</td>
<td>130</td>
<td>12.6</td>
</tr>
<tr>
<td>6 h at 36 °C + 2 h at 40 °C</td>
<td>360</td>
<td>33.8</td>
</tr>
<tr>
<td>3 h at 36 °C + 3 h at 40 °C</td>
<td>490</td>
<td>46.0</td>
</tr>
<tr>
<td>2 h at 36 °C + 4 h at 40 °C</td>
<td>550</td>
<td>52.1</td>
</tr>
<tr>
<td>1 h at 36 °C + 5 h at 40 °C</td>
<td>665</td>
<td>62.5</td>
</tr>
<tr>
<td>6.5 h at 40 °C</td>
<td>798</td>
<td>100.0</td>
</tr>
<tr>
<td>6.5 h at 36 °C</td>
<td>186</td>
<td>23.2</td>
</tr>
<tr>
<td>1 h at 40 °C + 5.5 h at 36 °C</td>
<td>209</td>
<td>26.2</td>
</tr>
<tr>
<td>2 h at 40 °C + 4.5 h at 36 °C</td>
<td>129</td>
<td>16.3</td>
</tr>
<tr>
<td>3 h at 40 °C + 3.5 h at 36 °C</td>
<td>182</td>
<td>22.8</td>
</tr>
<tr>
<td>4 h at 40 °C + 2.5 h at 36 °C</td>
<td>161</td>
<td>20.3</td>
</tr>
</tbody>
</table>

Chicken embryo cells were infected as described in Methods. Actinomycin D was added and cultures were incubated for indicated time at one and then at the other temperature. [3H]-uridine was added 1 h before the end of incubation. RNA was extracted and specific radioactivity determined. The maximum specific radioactivity varied from one experiment to another therefore only results from the same experiment were compared. For each point at least two separate cultures were used.

‘minus’ strand RNA (Table 1). (The experiments in Table 1 were designed to study the polarity of RNA and not the amount, therefore a number of counts convenient for annealing was used regardless of the specific radioactivity).

RNA from non-infected CEC treated with actinomycin D contained only small amount of label (less than 1% of that from infected cells). It sedimented slowly and could not be annealed with NDV RNA, though self annealing rendered it 40 to 50% RNase resistant. Therefore, the specific radioactivity of RNA from infected actinomycin D-treated cells was considered a good estimate of the amount of virus RNA synthesized. The amount of NDV ‘minus’ strand RNA found in infected CEC incubated at 36 °C was 12 to 30% of that found in cells incubated at 40 °C, when estimated by the specific radioactivity (see, for examples, Table 2), the ‘polarity’ being verified by annealing.

The rate of synthesis of virus RNA was determined by pulse labelling the infected, actinomycin D-treated cells (Fig. 3). The maximum rate at 36 °C (observed at 1 h after infection) was only 30% of that at 40 °C (observed at 6 h after infection).

At both temperatures the maximum rate of virus RNA synthesis was observed at times when the release of infectious virus particles had been already terminated (compare Figs. 1A and 3).

When the infected CEC were first incubated at 40 °C, then transferred to 36 °C and labelled for 1 h an immediate drop of incorporation of [3H]-uridine was observed. The reverse shift from 36 to 40 °C provoked an increase of the labelling; however, the values observed remained always lower than those in cells incubated permanently at 40 °C (Table 2). Cells held at 40 °C (allowing time for ‘minus’ strand accumulation) and then shifted down to 36 °C did not produce more infectious virions than those held all the time at 36 °C (Table 3) indicating that the increased rate of RNA synthesis does not influence virus synthesis.
Fig. 3. Rate of NDV RNA synthesis in chick embryo cells at 36 °C and 40 °C. At indicated times [5'-3H]-uridine (20 μCi/ml) was added for 15 min and RNA was extracted. The total amount of RNA in each sample was determined by the orcinol reaction and the specific radioactivity calculated. ○—○, cells incubated at 36 °C; ●—●, cells incubated at 40 °C. (Three separate experiments indicated by different symbols (*, □) are superimposed.)

**NDV infection of chorioallantoic membranes**

Cultivated chicken embryo cells are rather poor NDV producers as compared to embryonated eggs or to chicken lung cells. Since lung cells do not proliferate well in culture, CAMs were used. In CAMs infected as described under 'Methods' and incubated at 40 °C, virus release started 3 h after infection and was terminated about 1 h later (Fig. 1B). Lowering the temperature to 36 °C delayed the onset of virus release only by 1 h (compared to 5 h delay observed with infected CEC). The CAMs incubated at 36 °C continued to release virus at decreasing rate until the 7th hour after infection. However, the most striking difference was found in the final yield of infectious virus: the infected CAM produced 80 to 200 p.f.u./cell whereas the fibroblasts yielded 2 to 10 p.f.u./cell. Also in infected CAMs, the yield was higher at 40 °C than at 36 °C. The addition of 5 μg/ml of actinomycin D did not modify virus production. The NDV produced by the CAM had a H.A. to p.f.u. ratio (1 H.A.U. for 5 to 10×10^4 p.f.u.) similar to that of the virus coming from the CEC. This indicates that the CAMs produced more virus particles (infectious and non-infectious) than the CEC.
NDV virus particle and RNA synthesis

Table 3. Effect in shift of temperature on production of infectious virus

<table>
<thead>
<tr>
<th>Incubation of cells</th>
<th>P.f.u./ml of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Control) 0 time</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>Incubated 24 h at 36 °C</td>
<td>$1.6 \times 10^7$</td>
</tr>
<tr>
<td>Incubated 24 h at 40 °C</td>
<td>$1.3 \times 10^7$</td>
</tr>
<tr>
<td>Incubated 2 h at 40 °C+22 h at 36 °C</td>
<td>$1.8 \times 10^7$</td>
</tr>
<tr>
<td>Incubated 4 h at 40 °C+20 h at 36 °C</td>
<td>$1.6 \times 10^7$</td>
</tr>
<tr>
<td>Incubated 6 h at 40 °C+18 h at 36 °C</td>
<td>$1.6 \times 10^7$</td>
</tr>
</tbody>
</table>

Chicken fibroblasts ($2 \times 10^7$ per flask) were infected as described under Methods, and incubated with 10 ml of medium during indicated times. Then samples of medium were assayed for plaque forming virus. Values for each time are averages of two flasks and four plates.

To study virus RNA synthesis, infected minced CAMs were incubated in the presence of actinomycin D and labelled with [³H]-uridine (the label was present during the following time intervals: 1 to 5 h, 2.5 to 7.5 h and 6 to 7.5 h in case of suspensions incubated at 36 °C and 1 to 5 h, 1.5 to 4 h and 6 to 7.5 h in case of suspensions incubated at 40 °C). RNA was extracted as usual except that the cells were disrupted by a Dounce homogenizer.

After sucrose gradient centrifuging part of the labelled RNA sedimented at 57S; the rest as less than 5S (Fig. 2B). Peaks of NDV ‘minus’ strand RNA could not be detected either in the preparations from CAMs incubated at 36 °C or from those incubated at 40 °C with the labelling times used. $E_{260}$ showed the presence of well separated and abundant 28S and 18S ribosomal RNA. This indicates that the extraction of RNA was efficient and that no important degradation had occurred. The RNA which sedimented at 57S corresponded to NDV ‘plus’ strand RNA; this was verified by annealing with unlabelled ‘minus’ strand RNA (result not shown), which indicates that labelling of virus RNA was efficient (Fig. 2B). Annealing experiments using pooled fractions from the central part of the sucrose gradients and NDV ‘plus’ strand RNA showed that RNase resistance increased by 5 to 10% over that found in self-annealed controls (Table 1), an observation which clearly indicated the presence of some ‘minus’ strand RNA. Another experiment was performed under conditions permitting a better visualization of an RNA with a fast turnover: actinomycin D-treated CAMs infected for 3 h and non-infected controls were pulse-labelled for 30 min; sucrose gradients of the extracted RNA showed peaks of acid-insoluble radioactivity at fractions corresponding to 57S, 25 to 30S, 18S and those less than 5S (Fig. 2C). However, it should be noted that the RNA from infected CAMs was labelled only 2.4 times more than that from non-infected controls (24900 ct/min versus 11 100 ct/min) and that its radioactivity corresponded to 5% of that found under similar conditions in infected CEC (500000 ct/min).

NDV infection of mouse L cells

The finding that NDV ‘minus’ strand RNA accumulates in CEC producing a small amount of infectious virus particles but not in CAMs producing a large amount of virus led us to study NDV RNA synthesis in another cell line, mouse L cells. These cells are known to respond to NDV infection in a non-productive way. The amount of newly synthesized virus particles varies slightly with the NDV strain used, but is generally less than 1 p.f.u./cell. Nevertheless, infection induces lethal metabolic changes and also interferon production (Wilcox, 1959; Paucker, Skurska & Henle, 1962; Reda, Rott & Schafer, 1964; Thacore & Youngner, 1969; Esminger & Tamm, 1970).

Mouse L cells in monolayer cultures were infected with NDV and labelled with [³H]-uridine in the presence of actinomycin D as described above for the CEC. We have found
an average yield of 0.1 p.f.u./cell of progeny virus particles produced after 18 h at 37 °C, in good agreement with the quoted data. Sucrose gradient analysis of RNA extracted from infected cells showed that it contained all of the virus specific ‘minus’ strand components but that no 57S RNA could be detected (Fig. 4). The ‘minus’ strand RNA was identified by annealing with RNA from NDV virus particles, the RNase resistance of the annealed fractions being close to 100% (Table 1).

Effects of inhibition of protein synthesis on NDV RNA synthesis in CEC and CAMs

Cycloheximide, a potent inhibitor of protein synthesis, suppresses completely Sendai virus and NDV production in CEC, but does not block the synthesis of ‘minus’ strand RNA (Clavell & Bratt, 1971; Robinson, 1971). We performed comparative experiments with CEC and CAMs to see if the inhibition of protein synthesis and the consequent blockage in utilization of RNA would lead to an increase in the relative amount of ‘minus’ strand detected in infected CAMs.

CEC and CAMs were infected with NDV at 40 °C in the presence of actinomycin D and of 5 μg/ml of cycloheximide (preliminary experiments showed this concentration of cycloheximide reduced the incorporation of [3H]-labelled amino acids into proteins by 90%, and that concentrations up to 100 μg/ml did not augment this degree of inhibition to any extent). After 1 h the CEC were labelled with [3H]-uridine for 2 h and RNA was extracted. The CAMs were labelled from the beginning of infection.

The RNA from cycloheximide-treated CEC contained typical ‘minus’ strands though in
NDV virus particle and RNA synthesis

![Figure 5](image)

Fig. 5. Sucrose gradient analysis of RNA from NDV infected chicken embryo cells and chorio-allantoic membranes labelled in the presence or absence of cycloheximide. A, Cells infected in the presence of cycloheximide and labelled between 1 and 3 h after infection (CEC) or between 0 and 2 h (CAMs). B, Cycloheximide added 2 h after infection and cells labelled between 2 and 4 h after infection. Centrifugings as in Fig. 2. ○—○, RNA from CAMs; ●—●, RNA from CEC. +—+, RNA from control CEC infected in the absence of cycloheximide.

smaller amount than the untreated controls. The distribution of radioactivity in RNA from CAMs was similar in cycloheximide treated and untreated cells, without visible peaks which could correspond to 'minus' strand (Fig. 5 A).

In another experiment cells were infected first, then after 2 h cycloheximide and [3H]-uridine were added and after 2 h more (a time when in the untreated control according to Fig. 3 the rate of virus RNA synthesis is close to maximum) RNA was extracted. Under such condition RNA from cycloheximide-treated CEC contained still less label than in the previous experiment. In the CAMs (both treated and untreated with cycloheximide) again no 'minus' but, already, some 57S RNA was detected (Fig. 5B).

Another possibility to dissociate NDV 'plus' and 'minus' strand RNA synthesis was suggested by the finding that u.v. irradiated virus which does not multiply still induces 'minus' strand synthesis in the CEC (Huppert et al. 1969; Clavell & Bratt, 1971). However, after infecting the CAMs with inactivated NDV we have not been able to detect 'minus' strand RNA (results not shown).

**DISCUSSION**

The results of the present study show a inverse relationship between the production of infectious NDV particles and the accumulation of 'minus' strand RNA. When three types of cells were compared it was found that in the CAMs, which yielded the highest p.f.u. (80 to 200/cell), the presence of the 'minus' strand could not be demonstrated except by short pulse labelling; the CEC, which yielded only 2 to 10 p.f.u./cell, contained about 50-
fold more of this RNA species. Mouse L cells which in general agreement with published results (Wilcox, 1959; Pauker et al., 1962; Reda et al., 1964; Thacore & Youngner, 1969; Esminger & Tamm, 1970), produced the lowest p.f.u. (0·1/cell), contained ‘minus’ strand RNA in quantities even greater than those in the CEC. The observed values were: CAMs and CEC uninfected: 11 ct/min/μg of RNA; infected CAMs: 30 ct/min/μg; infected CEC: 500 to 1000 ct/min/μg; infected L cells: 3000 ct/min/μg. This reverse relationship is further illustrated by the observation with CEC (Table 2) that raising the temperature from 36 to 40 °C resulted in a decrease in the production of virus with a concomitant increase in the synthesis of virus RNA in the cell.

Bratt & Robinson (1967) and Blair & Robinson (1968) suggested a ‘messenger’ function for the intracellular ‘minus’ strand RNA in the case of paramyxoviruses, first because RNA extracted from virus particles is non-infective and secondly because in the cell the ‘minus’ strands are associated with the polyribosomes. This suggestion is in accord with the recent finding that polyadenylate sequences, characteristic of messenger RNAs (Darnell, Wall & Tushinski, 1971; Edmonds, Vaughn & Nakazato, 1971; Lee, Mendecki & Brawerman, 1971), are present in the intracellular ‘minus’ strand but not in the ‘plus’ strand RNA in the case of Sendai virus (Pridgen & Kingsbury, 1972). Our observation that in infected CAMs the ‘minus’ strand RNA has a rapid turnover, could be in favour of such a conclusion. However, the ‘minus’ strand RNA, in addition to its contribution in protein synthesis, must have a function as a template for RNA synthesis, as is evident from a review of the sequence of events known or supposed to occur in NDV replication.

The virus particle-associated replicase (Huang et al., 1971) transcribes virus RNA in the cell into a complementary RNA, the ‘minus’ strand RNA. The latter, after the addition of poly A to it (Marshall & Gillespie, 1972), directs the synthesis of virus proteins (including replicase) as well as serving as template for the synthesis of progeny ‘plus’ strands. A part of the new ‘plus’ strands is transcribed again into ‘minus’ strands and another part is associated with proteins to form virus particles. While the first transcriptional event is not sensitive to inhibitors of protein synthesis (Clavell & Bratt, 1971; Robinson, 1971), all consecutive events, leading to the production of virus particles (including ‘plus’ strand RNA synthesis) are dependent on new protein synthesis.

The factor responsible for the low yield of NDV in several cell types is not clear, but interferon is excluded as causative agent at least in mouse L cells (Youngner & Scott, 1968; Lancz & Johnson, 1966) and also probably in chick cells, since in the latter only previously inactivated NDV is capable of inducing interferon (Ho & Breining, 1965; Youngner et al. 1966). But, whatever the cause, the accumulation of the ‘minus’ strand RNA in those cells after NDV infection, reflects a block in the synthesis of some virus component. A blockage either in coat protein synthesis or in virus maturation is unlikely, since hindering virus release should induce also the accumulation of ‘plus’ strand RNA corresponding to the virus genome which remained in the cell and not only that of the ‘minus’ strand corresponding to ‘messengers’. The accumulated ‘minus’ strand RNA seems not involved in virus particle production. The highest rate of synthesis of this RNA is observed when progeny virus particles are already released (compare Figs. 1 and 3). This is also supported by the results of the temperature shift experiment with CEC. Also when CEC were allowed to accumulate ‘minus’ strand RNA at 40 °C and were then shifted to 36 °C (a condition which is favourable for more virus particle production) no increase in the amount of p.f.u. was observed (Table 3).

We suggest the impairment of NDV production to be at the level of the synthesis of new ‘plus’ strand RNA. This synthesis would need a cellular enzyme (or a cellular component
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for a virus coded enzyme) the availability of which varies in different cell types and is the factor limiting virus particle production.

NDV production varies widely depending on the cell type used. Therefore, the permissiveness of a cell for the production of the virus is not an all-or-none phenomenon but covers different degrees. Our hypothesis concerning the cellular component necessary for the transcription of the 'minus' strand RNA to its complementary 'plus' counterpart, if correct, would provide an explanation for these variations.

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REFERENCES


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