FACTORS DETERMINING THE SITE OF SYNTHESIS OF POLIOVIRUS PROTEINS: THE EARLY ATTACHMENT OF VIRUS PARTICLES TO ENDOPLASMIC MEMBRANES

M. L. FENWICK AND MARGARET J. WALL
Sir William Dunn School of Pathology, University of Oxford, England

SUMMARY
Cytoplasmic extracts of HeLa cells made 1 to 2 h after infection with radioactive poliovirus contained 150-s virus particles and 130-s, perhaps partially disrupted, particles. The latter were resistant to RNase but sensitive to dodecylsulphate. Both particles were associated with fast sedimenting material from which they could be released by deoxycholate, but not by EDTA. In isopycnic gradients of sucrose in D2O the labelled particles formed a sharp band coincident with membrane-bound ribosomes at a density of 1.23 g cm⁻³. It is suggested that attachment of virus particles to endoplasmic reticulum may be an early stage in the infectious cycle, determining the site of subsequent steps.

The inhibition of cellular protein synthesis that develops during infection affects membrane-bound as well as free polysomes and therefore does not determine the membrane-association of viral protein synthesis.

INTRODUCTION
During the first 3 h after infection with poliovirus the rate of protein synthesis in the host cell and the degree of assembly of its ribosomes into polysomes decline markedly (Penman, Scherrer, Becker & Darnell, 1963; Leibowiz & Penman, 1971). Within 4 h, new polysomes are formed to make viral proteins (Penman, Becker & Darnell, 1964) and these polysomes are attached to membranes of the endoplasmic reticulum (Caliguirri & Tamm, 1970; Roumainzef, Maizel & Summers, 1971). In normal growing HeLa cells about 15% of the ribosomes are attached to membranes (Attardi, Cravito & Attardi, 1969; Rosbach & Penman, 1971; Roumainzef et al. 1971).

It is not known why viral protein is synthesized on bound and not on free polysomes, nor how the synthesis of host protein is inhibited while viral protein is being made. We have done experiments to test the following hypotheses: (a) that a property of the infecting virus particle, namely an affinity of the virion for the endoplasmic reticulum, determines the site of functioning of its RNA, and (b) that the inhibitory condition in infected cells is effective specifically against free polysomes, leaving only the membrane-bound system available for the synthesis of viral protein. The results are consistent with the first hypothesis but not with the second.
METHODS

Cells

HeLa cells were grown in suspension (Harris & Watts, 1962).

Virus

Poliovirus type 1, strain ts+ (Cooper, Johnson & Garwes, 1966) was grown in suspended HeLa cells (10^7/ml) infected with 0.1 plaque-forming units (p.f.u.) per cell and incubated for 15 h at 37 °C. Cell lysis was completed by adding sodium deoxycholate (DOC) to 0.2%. Debris was removed by centrifuging for 10 min at 10,000 rev/min (Beckman rotor 30). Virus was sedimented in 2 h at 30,000 rev/min and resuspended in one tenth of the original volume of phosphate-buffered saline containing Ca^2+ and Mg^2+ (PBS, Oxoid Ltd.). After final clarification (10 min at 10,000 rev/min) it contained about 5 x 10^6 p.f.u./ml (Cooper, 1961) and was stored at -70 °C.

3H-virus

5 x 10^7 cells resuspended in 5 ml of growth medium without serum were incubated with 50 p.f.u. of poliovirus per cell for 20 min at 20 °C and then transferred to 45 ml of medium at 37 °C with 5% pig serum and actinomycin D (2 μg/ml, Merck, Sharp & Dohme, Inc.). After 1 h at 37 °C uridine-5-[^3H] (20 μCi/ml, 27 Ci/mmol, Radiochemical Centre, Amersham) was added. At 5.5 h the cells were resuspended in 1.8 ml 'MP' buffer (0.004 M magnesium acetate, 0.04 M sodium phosphate, pH 7.2) and lysed with 0.2 ml of 2% DOC. After centrifuging for 2 h at 25,000 rev/min to remove nuclei, the supernatant was divided between two 30-ml gradients of 30-60% w/v sucrose in 0.1 M NaCl, 0.01 M Tris, pH 7.2, and centrifuged for 2 h at 25,000 rev/min in a Beckman rotor SW 25-1 at 10 °C. Fractions were collected and a drop taken from each for scintillation counting. The fractions containing the peak of radioactivity were pooled and stored at 4 °C. A typical preparation contained 10^6 counts/min/ml and 8 x 10^6 p.f.u./ml. The counts formed a single sharp band at about 150 s when centrifuged in a second sucrose gradient.

Preparation of infected cell lysates

Cells (10^7) were resuspended in 1 ml of medium lacking serum, incubated with virus (5 x 10^5 p.f.u. of unlabelled virus or 8 x 10^7 p.f.u. of 3H-virus) for 30 min at 20 °C and then (0 h after infection) transferred to 9 ml of medium with serum at 37 °C, gassed with 5% CO₂ in air. At the appropriate time they were chilled, centrifuged and resuspended in 1 ml of MP. After 10 min at 0 °C they were broken by 12 strokes of a Dounce glass homogenizer and centrifuged for 5 min at 2000 rev/min to remove nuclei and debris. The supernatant cytoplasmic extract is referred to as 'lysate'.

Polysomes

In experiments involving pulse-labelling with 14C-amino acids, all of the membrane-bound and most of the free polysomes (groups of 4 or more ribosomes) were sedimented by centrifuging 1 ml of lysate for 10 min at 20,000 rev/min in the Beckman rotor SW 50-1. The sediment was resuspended in 0.3 ml of MP, and 0.1 ml analysed by isopycnic centrifugation.

Protein synthesis

10^7 cells were resuspended in 1 ml of PBS and placed in a bath at 37 °C. After 2 min 14C-amino acids (0.04 ml of 62 μCi/ml, algal hydrolysate, Radiochemical Centre) were added and the mixture chilled in ice 2 min later.
Attachment of poliovirus to membranes

Kinetic sucrose gradients

0.2 ml of lysate was applied to a 5-ml exponential gradient of 30-8% (w/v) sucrose in the appropriate buffer (Fenwick, 1968), and centrifuged in a Beckman rotor SW 50-1 before fractionating and monitoring absorbance at 254 nm with an Isco fractionator. Fractions were collected on glass fibre filters for measurement of acid-precipitable radioactivity (Fenwick, 1971).

Isopycnic gradients

Exponential gradients were constructed using the following solutions: (a) 8 ml 50% (w/w) sucrose in deuterium oxide (BDH Chemicals Ltd.) + 2 ml 0.2 M sodium phosphate, pH 7.2 + 0.04 ml 1 M magnesium acetate, and (b) 8 ml D2O + 2 ml 0.2 M sodium phosphate + 0.04 ml 1 M magnesium acetate. The mixing chamber contained 3 ml of solution (a) and the top chamber 2 ml of solution (b). The first 2 ml of the gradient was collected in a sawn-off centrifuge tube. A sample of lysate (0.2 ml) or resuspended polysomes (0.1 ml) was layered on top and centrifuged at 10°C in the rotor SW 50-1 for 3 h at 50,000 rev/min. It was fractionated in the same way as the kinetic gradients. The densities of a few fractions were measured by weighing in a calibrated micropipette.

Rough endoplasmic reticulum formed a sharp band in these gradients at a density of about 1.23 g cm⁻³. The precise position of the band varied between 1.22 and 1.24 g cm⁻³ with different lysates, perhaps depending on the extent of loading of the membrane with ribosomes. The reticulum band can be located by monitoring absorbance at 254 nm, due to the RNA of its attached ribosomes (Fig. 1), or by measuring radioactivity if the cells are previously labelled with radioactive uridine, amino acids, choline or fucose. The other peaks of absorbance in Fig. 1, which are due to ribosomal subunits and single (74-s) ribosomes, have not reached density equilibrium. If centrifugation is prolonged they move to the bottom of the tube. Fragments of plasma membrane or ribosome-free endoplasmic reticulum, with a buoyant density of about 1.08 g cm⁻³ (Mosser, Caliguir, Scheid & Tamm, 1972), would remain at the top of these gradients.

RESULTS

Attachment of infecting virus to membranes

HeLa cells were infected with ³H-poliovirus at 20°C and infection allowed to proceed for 90 min at 37°C. A lysate was prepared and analysed by centrifuging in an isopycnic gradient of sucrose in D2O. A sharp peak of radioactivity coincided with the optically dense band of rough reticulum (Fig. 1A). Only about 10% was recovered from the bottom of the tube where free virus would be expected. The pattern was not altered if pancreatic ribonuclease (10 μg/ml, 5 min at 20°C) was added to the lysate before centrifugation. To a second sample of the same lysate further ³H-labelled virus was added (Fig. 1B). By subtracting the counts in Fig. 1A from those in Fig. 1B it was estimated that about 22% of the added virus became attached to the rough membrane, and most of the remainder sedimented to the bottom of the tube.

When 0.2% DOC was added to a lysate like that of Fig. 1A before centrifugation, no peak of absorbance equilibrated in the membrane position (the ribosomal subunits and monomers remained) and all the radioactivity was in the sediment, i.e. greater than 74 s). In order to characterize this membrane-bound radioactive material more clearly a lysate was treated with 0.2% DOC to dissolve membranes and EDTA to prevent Mg-mediated aggregation and centrifuged through a kinetic sucrose
Fig. 1. Association of infecting poliovirus with membrane-bound ribosomes. A lysate was prepared 90 min after infection with $^3$H-virus and a sample (a) centrifuged in an isopycnic gradient of sucrose in D$_2$O. Peaks of absorbance (---): M, membrane-bound ribosomes; S, small ribosomal subunits; L, large ribosomal subunits; R, single (74-s) ribosomes. The disconnected section of absorbance tracing is recorded on a one-fifth scale. ○, $^3$H; −, density. sed. = cpm recovered from the bottom of the tube. Further $^3$H-virus was added to an identical sample (b) before centrifugation.

The slower-sedimenting RNase-resistant labelled material released from membranes was sensitive to sodium dodecysulphate (SDS). A control sample of infected lysate was treated with 1% DOC and sedimented in a sucrose gradient in 0.1 M NaCl, 0.01 M Tris (Fig. 3A), to display the 150-s and 130-s labelled material. Treatment with 1% SDS (Fig. 3B) left a distinct peak of intact virus, which is not degraded by
Attachment of poliovirus to membranes

Fig. 2. Detachment of ³H-virus from membranes. A sample (A) of a lysate like that of Fig. 1A was treated with 0.02 M EDTA and 0.2% DOC and centrifuged for 25 min at 50,000 rev/min at 10 °C in a kinetic sucrose gradient in 0.1 M NaCl, 0.005 M EDTA. A second sample (B) was treated with EDTA but not with DOC. ----, absorbance; O—O, ³H. S, L = small and large ribosomal subunits, respectively.

Fig. 3. Effect of SDS on membrane-bound virus. Samples of lysate prepared 2 h after infection with ³H-virus were treated with 1% DOC (A) or 1% SDS (B) and centrifuged for 21 min at 45,000 rev/min at 20 °C in kinetic gradients in 0.1 M NaCl, 0.01 M Tris.

this treatment, while all of the slower sedimenting label was released as free viral (35-s) RNA. Thus 90 min after infection the viral RNA that had penetrated the cell was associated with the ribosome-bearing membrane of the endoplasmic reticulum. About one third was still in intact virus particles and two thirds in more slowly sedimenting particles from which it could be released by SDS. The latter may perhaps be partially disrupted virions.

Lysates were prepared at intervals after infection and analysed as in Fig. 3B, after treatment with SDS. The results (Table 1) showed a progressive increase in the proportion of viral RNA released by SDS.

The attachment of virus and of viral RNA to rough reticulum in vitro was further investigated by adding the labelled materials to uninfected lysates. After isopycnic centrifugation about 45% of the intact virus was recovered in the membrane band, and 40% at or near the bottom of the tube. Of the viral RNA about 26% banded with the membranes and 55% sedimented with coefficients of more than 45 s (i.e. it
Table 1. **SDS-sensitivity of intracellular virus**

<table>
<thead>
<tr>
<th>Time after infection, min</th>
<th>Total cpm</th>
<th>% of counts in 35-s peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35-s + 150-s</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>230</td>
<td>42</td>
</tr>
<tr>
<td>60</td>
<td>270</td>
<td>62</td>
</tr>
<tr>
<td>90</td>
<td>310</td>
<td>72</td>
</tr>
<tr>
<td>120</td>
<td>350</td>
<td>73</td>
</tr>
</tbody>
</table>

Fig. 4. Association of virus with membranes *in vitro*. ³H-virus was added to samples of uninfected cell lysate and analysed in kinetic sucrose gradients centrifuged for 20 min at 50,000 rev/min at 10 °C. A, untreated sample, gradient in MP. B, sample treated with 0.2% DOC, gradient in MP. C, sample treated with 0.02 M EDTA, gradient in 0.1 M NaCl, 0.005 M EDTA.

was either aggregated or attached to some fast-sedimenting material). Thus there is some tendency for both virus and viral RNA to stick to rough reticulum *in vitro*. However, unlike the complex formed after infection (Fig. 2B), that formed with virus *in vitro* was completely dissociated by EDTA, releasing free virus (Fig. 4C). In a control gradient containing Mg²⁺ most of the virus reached the bottom of the tube (Fig. 4A). This was presumably due partly to its association with membranes and partly to other forms of Mg-mediated aggregation since treatment with 0.2% DOC slightly reduced the average sedimentation rate, but did not release 150-s virus particles (Fig. 4B).

**Inhibition of membrane-bound protein synthesis after infection**

In an experiment to determine whether membrane-associated protein synthesis was inhibited in infected cells, a culture was incubated for 15 h with 0.1 µCi/ml of
Table 2. Effect of infection on membrane-bound protein synthesis

<table>
<thead>
<tr>
<th>Time after infection, h</th>
<th>Total [3H]fucose in membrane band, % of value at 1 h (= 295 cpm)</th>
<th>Ratio [14C]/[3H], % of value at 1 h (= 4-o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3-0</td>
<td>92</td>
<td>33</td>
</tr>
<tr>
<td>3-5</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>4-0</td>
<td>92</td>
<td>48</td>
</tr>
<tr>
<td>4-5</td>
<td>100</td>
<td>37</td>
</tr>
<tr>
<td>5-0</td>
<td>79</td>
<td>34</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of infection on membrane-bound protein synthesis. Ribosomal RNA was labelled by incubating cells with [3H]uridine before infection. Protein was pulse-labelled for 2 min with 14C-amino acids 3 h after infection (h) or mock-infection, omitting virus (x). Polysomes were prepared and centrifuged in isopycnic gradients. O—O, 3H; •—•, 14C.
[\textsuperscript{3}H]-l-fucose to label membrane polysaccharides (Kaufmann & Ginsburg, 1968). It was then infected with unlabelled poliovirus and incubated in non-radioactive medium. At various times samples of cells were resuspended in PBS and labelled for 2 min with \[^{14}\text{C}\text{-amino-acids. The cells were homogenized and polysomes were sedimented, resuspended and applied to isopycnic sucrose/D}_2\text{O gradients. The amount of }^{3}\text{H-labelled membrane banding at 1.23 g cm}^{-3}\text{ did not change significantly up to 4.5 h after infection (Table 2) but the rate of protein synthesis, measured by the ratio of }^{[14}\text{C}]\text{ to }^{[3}\text{H}], \text{ fell to a minimum of 33% of the value at 1 h by 3 h after infection and then rose slightly between 3.5 and 4.5 h, suggesting that a substantial proportion of the product from 3 h onwards was virus-specific protein. If this is so, then the extent of inhibition of host synthesis was more than the observed overall depression of 67%. It is clear that the membrane-associated synthesis of cellular protein is not immune to virus-induced inhibition.}

Fig. 5 shows the results of a similar experiment performed 3 h after infection. In this instance the cells were incubated for 14 h with \[^{3}\text{H}\text{-uridine (0.5 }\mu\text{Ci/ml} before infection in order to label ribosomal RNA. The }^{[14}\text{C}]/^{[3}\text{H}] \text{ ratio in the membrane-bound peak was 38% of that in the uninfected control gradient. The }^{[3}\text{H]} \text{ and }^{[14}\text{C}] \text{ radioactivities in the free polysomes recovered from the sediment were respectively 15 and 6% of the control levels. Thus the functioning of free polysomes was more inhibited after infection than that of bound polysomes, but, as mentioned above, the residual membrane-bound synthesis includes an unknown proportion of viral protein synthesis.}

It has been demonstrated that synthesis of host protein is inhibited after infection even if multiplication of viral RNA is prevented by adding guanidine (Penman & Summers, 1965; Leibowitz & Penman, 1971). If much of the protein being synthesized at 3 h is virus-specific, the rate should be further lowered by guanidine. Therefore we measured membrane-bound protein synthesis in infected cells incubated for 3 h in the presence of 3 mM guanidine. The inhibition was indeed greater (about 80%) than in the absence of guanidine. However, there was always some inhibition (about 50%) of amino acid incorporation in uninfected guanidine-treated control cultures. Consequently it was not possible to attribute all of the 80% decline to the presence of virus.

\textbf{DISCUSSION}

\textbf{Fate of infecting virus}

Poliovirus-specific protein is made on membrane-bound polysomes (Caliguiri & Tamm, 1970) and we have confirmed (unpublished experiments) that much of the newly made viral RNA in cells infected for 4-5 h equilibrates in our sucrose/D\textsubscript{2}O gradients with the bound ribosomes at a density of 1.23 g cm\textsuperscript{-3}. This RNA can be released from the polysomes by treatment with EDTA. It then sediments in sucrose gradients (as does viral RNA added to a lysate) as a nucleoprotein particle at about 80 S (Huang & Baltimore, 1970) which dissociates into free viral RNA and protein in the presence of 1% DOC (M. L. Fenwick, unpublished observation).
Attachment of poliovirus to membranes

The experiments described here show that many of the virus particles that had adsorbed to cells and were found in the cytoplasmic fraction after breaking the cells were also attached to ribosome-bearing membranes. By 2 h after infection three-quarters of the original virus particles had been converted to an SDS-sensitive but still RNase-resistant form sedimenting at about 130 s, but no free viral RNA or 80-s nucleoprotein was detected. Virus attached to fragments of plasma membrane or to ribosome-free endoplasmic reticulum would be expected to remain at the top of our sucrose/D₂O gradients, at about 1.08 g cm⁻³ (Mosser et al. 1972). Mosser et al. (1972) also found lysosomes distributed broadly over isopycnic sucrose gradients. Association of some virus particles with these organelles is not excluded by our experiments but is unlikely to account for the accumulation of radioactivity at a density of 1.23 g cm⁻³.

The interpretation of these experiments is complicated by the fact that in order to recover sufficient radioactivity for accurate measurement cells were infected with an average of 2 to 4 adsorbed p.f.u. Assuming a ratio of at least 100 virus-like particles per p.f.u. (see Discussion in Fenwick & Cooper, 1962) probably several hundred particles enter the cell. The fate of the majority of these, which is studied in such experiments, cannot be presumed to indicate the path taken by that particle which actually initiates the infectious cycle. It is conceivable, on the contrary, that affinity for membranes deflects most of the intracellular virus particles from the true course of infection. However, the observation that viral protein synthesis occurs later in association with the same type of membrane is at least consistent with the hypothesis that the attachment of the infecting particle determines the site of the subsequent processes of virus production. If this is so, it appears from these experiments that the efficiency of uncoating of the membrane-attached particles is low, since no free viral RNA was observed. Conversion to the 130-s form may be an early stage in the uncoating process: although we have not excluded the possibility that the 130-s particles carry cellular protein, they differ in sedimentation rate and sensitivity to DOC from the complex formed in vitro between viral RNA and cellular protein.

Some virus became attached to membrane in vitro, probably linked by Mg²⁺ ions since it was dissociated by EDTA. Such an attachment may also precede the formation of the stronger EDTA-resistant bond in vivo. We have observed, in unpublished experiments, that incubation of poliovirus with a crude membrane-containing fraction of cell lysate also results in the appearance of a 130-s SDS-sensitive form of virus, but no free viral RNA. The conversion, which occurred at 37 °C but not at 0 °C, was prevented by 1% DOC and, with doubly-labelled virus, did not involve detectable loss of viral protein.

Inhibition of host protein synthesis

There have been reports of functional differences between free and membrane-bound polysomes. To assemble amino acids in vitro the 2 classes required different specific RNA fractions as well as specific factors extractable with 0.8 M KCl (Uenoyama & Ono, 1972). The activity of bound polysomes was stimulated more
than that of free polysomes by poly-U in vitro (Shaffritz & Isselbacher, 1972) and inhibited more by cycloheximide in vivo (Glazer & Sartorelli, 1972). We considered the possibility that some such difference might cause the bound and the free systems to respond differently to infection with poliovirus with the result that viral protein synthesis (which is membrane-associated) could continue while free synthesis was inhibited.

We found, however, that both systems were inhibited in infected cells. Thus the choice of site for the synthesis of viral protein is not determined simply by the elimination of one of the alternatives. The mechanism of the inhibition of host protein synthesis presumably depends on the recognition of some difference between cellular and viral messages.

REFERENCES

Attachment of poliovirus to membranes


(Received 19 January 1973)