DNA Synthesis in Mengovirus-infected Cells: Mechanism of Inhibition

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SUMMARY
We have studied several aspects of the inhibition of DNA synthesis in Mengovirus-infected cells. In mouse L-929 cells at 5 h after infection at a multiplicity of 200 p.f.u./cell, there was a decrease in DNA synthesis. However, the rate of DNA replication fork movement, measured by equilibrium sedimentation of DNA sequentially labelled with BrdUrd and ^3H-thymidine, was not significantly reduced in infected cells despite an 84% inhibition of DNA synthesis. Enzymic assay of the total cellular dTTP pool size showed it to be increased 37% in infected cells compared to controls. This was not accounted for by enhanced entry of thymidine nucleotides into the pool from either exogenous or endogenous sources, since the buoyant density of DNA pulse-labelled with ^3H-BrdUrd was the same in infected and uninfected cells and there was no evidence for breakdown of DNA in infected cells. There was also a moderate decrease in the uptake of exogenous thymidine into the cell, shown by a reduction in the Vmax for transport with little change in the Ka. By 6 h after infection, the dTTP pool size was slightly smaller in infected cells than in the controls and there was a marked inhibition in the rate of replication fork movement. These results show that Mengovirus infection blocks the entry of exogenous thymidine into the cell, shown by a reduction in the Vmax for transport with little change in the Ka. By 6 h after infection, replication fork movement is also retarded and there is a more generalized derangement in DNA synthesis.

INTRODUCTION
Mengovirus infection causes an inhibition of the incorporation of ^3H-thymidine into cellular DNA (Franklin & Baltimore, 1962; Ensminger & Tamm, 1970a) that starts within 2 h and is virtually completed by 6 h. This most probably represents an actual decrease in synthesis rather than a reduction in specific activity of labelled DNA although the amount of DNA synthesized early in infection has never been measured. At late times (6 h), the amount of DNA synthesized in infected cells is decreased (Ensminger & Tamm, 1970b) and there is a reduction in the rate of DNA replication fork movement (Hand, 1976a).

Early in infection (5 h and before), despite the marked decrease in thymidine incorporation, the proportion of infected cells synthesizing DNA is normal (Hand & Tamm, 1971) and the rate of replication fork movement is not reduced (Hand & Tamm, 1972). Since there is no block to entry of cells into the S phase of the cell cycle and no retardation of replication fork movement, it has been suggested that the primary DNA synthetic event blocked by Mengovirus is the multifocal initiation of new DNA chain synthesis on individual replication units within chromosomal DNA (Ensminger & Tamm, 1970a; Hand & Tamm, 1972).
We began the present experiments to test the hypothesis that Mengovirus infection blocks DNA initiation. First, we wanted to obtain evidence that infection actually inhibits DNA synthesis at 5 h and earlier. Previous studies had shown only that $^3$H-thymidine incorporation into acid-precipitable material was decreased in infected cells. We wished also to confirm that infection does not retard replication fork movement until later times and to determine if there were other alterations of thymidine nucleotide metabolism in infected cells that could decrease the incorporation of thymidine into DNA. Our data indicate that Mengovirus infection directly inhibits DNA synthesis by blocking the entry of dTTP into DNA. Up to 5 h after infection this block is manifested by an inhibition of initiation of new chain synthesis. At later times, rate of replication fork movement is also retarded.

**METHODS**

**Cells and virus.** Published methods were used for the growth and maintenance of the L-929 cell line in monolayers (Hand, 1975) and for the production of stocks of the small plaque variant of Mengovirus and their assay (Ensminger & Tamm, 1970a). With this variant, the onset of cell rounding in L-929 cells takes place 6 to 7 h after infection and is completed by 9 h.

**Infection of cells with Mengovirus.** Monolayers of cells in logarithmic growth were infected at a multiplicity of infection of 200 p.f.u./cell. Absorption was allowed to take place for 1 h in one-tenth the usual amount of medium. Control cells were mock-infected. Various aspects of cellular DNA synthesis were examined from 4 to 7 h after infection.

**Extraction of DNA.** The method has been described elsewhere (Hand, 1976b). It is a modification of the Marmur phenol-chloroform extraction as published by Britten, Graham & Neufield (1974). After extraction, the DNA was sheared to fragments of 4 μm by passage four times through a 25-gauge needle.

**Equilibrium sedimentation of denatured DNA.** This was performed as described elsewhere (Hand, 1976b). A sample of extracted DNA containing about 5000 cts/min of $^{14}$C-thymidine-labelled normal density marker DNA was made up to 5 ml in a solution containing 0.002 M-NaCl, 0.002 M-EDTA, 0.1% Sarkosyl and 5.6 g CsCl. This was centrifuged to equilibrium in a fixed angle rotor (S40.2, Beckman Instruments) for 36 to 60 h. Fractions of the gradient were collected by gravity and analysed for density and radioactivity.

**Assay of the total cellular dTTP pool.** We used *Escherichia coli* DNA polymerase (EC. 2.7.7.7.) to measure limiting dTTP concentrations in acid-extracts of cells (Solter & Handschumacher, 1969; Lindberg & Skoog, 1970) as described elsewhere (Hand, 1976b). The acid-soluble pool was extracted with 0.5 M-perchloric acid for 30 min at 4 °C. The extract was neutralized and the perchlorate precipitated with KOH. The dTTP concentration in samples of the neutralized extract was measured. Cell number was determined in replicate cultures by measuring the protein concentration of the monolayers by the method of Lowry et al. (1951).

**Measurement of the uptake of thymidine into cells.** Replicate monolayers of cells were labelled for 2 min with $^3$H-thymidine (5 μCi/ml) at concentrations from $1 \times 10^{-7}$ to $6 \times 10^{-7}$ M. At the conclusion of the pulse, the cells were washed four times with phosphate-buffered saline. The cells were then treated with 5% (w/v) trichloroacetic acid at 4 °C for 10 min and the precipitates were suspended in the trichloroacetic acid solution. Samples of the suspensions were spotted on glass fibre filters (GF/A) for liquid scintillation counting. The radioactivity in the suspension was used to calculate total incorporation of thymidine into the cell, taking into account the specific activity of the thymidine. Results from four replicate monolayers were averaged for each point.
DNA synthesis and replication fork movement in Mengovirus-infected cells

The technique for measurement of rate of fork movement developed by Painter & Schaefer (1969) was adapted. It measures by equilibrium sedimentation the density of sheared DNA that has been sequentially labelled with $^3$H-thymidine and BrdUrd. With this type of labelling protocol, a proportion of the nascent DNA will have end-to-end association of tritium- and density-substituted DNA. The faster the rate of replication fork movement, the more likely that a sheared segment of DNA from within a replication unit will be substituted with $^3$H-thymidine alone. With slower rates of fork movements, segments will be more likely to be substituted with both BrdUrd and $^3$H-thymidine. Thus, the proportion of $^3$H-labelled DNA with heavy density increases as the rate decreases.

This type of experiment also permits an estimate of the amount of DNA synthesis. The bulk DNA is uniformly labelled with $^{14}$C-thymidine before the experiment and the $^3$H-thymidine pulse takes place in the presence of FdUrd, which blocks the synthesis of endogenous thymidine nucleotides. The ratio of $^3$H:$^{14}$C in the DNA is therefore proportional to the amount of DNA made during the $^3$H-thymidine pulse. For these experiments, cells were labelled overnight with $^{14}$C-thymidine to provide normal density marker DNA. They were infected and then labelled with BrdUrd for 2 h starting 1.75 h after infection. This was followed by a pulse with $^3$H-thymidine for 30 min with the midpoint at 5 h after infection. This was a reversal of the sequence of the density and radioactive labelling described by Painter & Schaefer (1969). The radioactive pulse must take place at the time point at which one wishes to measure the rate of fork movement. If the density pulse came after the radioactive pulse, it would have been at a time when the infected cells were rounding and beginning to lyse. The sequence of labelling is otherwise immaterial; the amount of heavy DNA is still inversely proportional to the rate of replication fork movement. At the completion of the labelling period the DNA was extracted and centrifuged to equilibrium in alkaline caesium chloride.

Gradients from a representative experiment in which replication fork movement was examined 5 h after infection are shown in Fig. 1. The infected cells incorporated less $^3$H-thymidine into DNA than controls as measured by the ratio of $^3$H to $^{14}$C d/min in nascent and template DNA (compare Fig. 1a with 1b). This shows that there is less DNA made in infected cells. When the gradients are normalized to estimate the DNA of heavy density (Painter & Schaefer, 1969) it can be seen that the proportion is about the same in infected and control cells (compare Fig. 1c with 1d). This shows that the rate of fork movement is unchanged in infected cells despite the marked decrease in DNA synthesis.

DNA replication fork movement was also examined in cells 6 h after infection. The protocol was the same as for the previous experiment, except that the BrdUrd labelling period was started at 3.75 h after infection so the midpoint of the $^3$H-thymidine pulse was at 6 h. In infected cells, there is again a marked decrease in the amount of $^3$H-thymidine incorporated into DNA (Fig. 2a, b) reflecting the decrease in DNA synthesis. However, in contrast to the situation at 5 h, there is a much higher proportion of DNA of heavy density in the infected cells than in controls (Fig. 2c, d). This shows that DNA replication fork movement is inhibited in the infected cells.

The results of several experiments at different times after infection are presented in Table I. The ratio of $^3$H to $^{14}$C incorporation has been used as an estimate of overall DNA synthesis while the reciprocal of the fraction (F) of DNA of heavy density has been used to estimate the relative rates of replication fork progression. The value of F varies inversely with the
rate of fork movement (Painter & Schaefer, 1969). The data show clearly that Mengovirus causes an inhibition of DNA synthesis of more than 80% by 4 h after infection, but that there is no reduction in the rate of replication fork movement until 6 h after infection.

**dTTP pool in Mengovirus-infected cells**

The concentration of exogenous nucleotide in the dTTP pool and the size of the pool was measured for two reasons: (1) to determine if pool dilution was responsible for the discrepancy between thymidine incorporation and the rate of fork movement observed 5 h after infection and (2) to determine whether pool depletion might cause the inhibition of fork movement seen 6 h after infection.

To find out if pool dilution was occurring in infected cells, the density and radioactivity of DNA labelled during a short pulse with ³H-BrdUrd in the absence of FdUrd was determined. A decrease in the concentration of this exogenous density analogue of dTTP in the pool should be revealed as a decrease in both radioactivity and density of the nascent DNA.
DNA synthesis in Mengovirus infection

Fig. 2. Buoyant density of DNA sequentially labelled with BrdUrd and 3H-thymidine to measure replication fork movement in cells 6 h after Mengovirus infection. Cells were labelled with 14C-thymidine, BrdUrd, FdUrd and 3H-thymidine as in the experiment in Fig. 1 except that the density pulse began 3.75 h, and the 3H-thymidine pulse 5.75 h after infection so that the midpoint of the 3H pulse was at 6 h. •—•, 3H, d/min; ○—○, 14C, d/min; ▲—▲, density. (a) Uninfected cells. (b) Mengovirus-infected cells. (c) Gradient from (a) with 3H disintegrations normalized. (d) Gradient from (b) with 3H disintegrations normalized.

Table 1. DNA synthesis and replication fork movement in Mengovirus-infected cells

<table>
<thead>
<tr>
<th>Time after infection</th>
<th>Number of experiments</th>
<th>3H:14C</th>
<th>% of control</th>
<th>F†</th>
<th>1/F</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>1</td>
<td>4.31</td>
<td>—</td>
<td>0.145</td>
<td>6.90</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.73</td>
<td>16</td>
<td>0.158</td>
<td>6.33</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Mengo-infected</td>
<td>0.59</td>
<td>13</td>
<td>0.134</td>
<td>7.49</td>
<td>78</td>
</tr>
<tr>
<td>5 h</td>
<td>3</td>
<td>4.45</td>
<td>—</td>
<td>0.104</td>
<td>9.62</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.59</td>
<td>13</td>
<td>0.134</td>
<td>7.49</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Mengo-infected</td>
<td>0.22</td>
<td>5</td>
<td>0.917</td>
<td>1.09</td>
<td>17</td>
</tr>
<tr>
<td>6 h</td>
<td>2</td>
<td>4.11</td>
<td>—</td>
<td>0.160</td>
<td>6.25</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.22</td>
<td>5</td>
<td>0.917</td>
<td>1.09</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Mengo-infected</td>
<td>0.22</td>
<td>5</td>
<td>0.917</td>
<td>1.09</td>
<td>17</td>
</tr>
</tbody>
</table>

* DNA synthesis is estimated from the ratio of 3H to 14C incorporated into DNA that had been uniformly labelled with 14C-thymidine before infection and pulse-labelled with 3H-thymidine in the presence of FdUrd at the indicated time after infection. The exact labelling protocol is detailed in the legend to Fig. 1.

† F is equal to the fraction of DNA of heavy density from cells subjected to the labelling protocol in the legend to Fig. 1. The reciprocal of F is directly proportional to the rate of replication fork movement (Painter & Schaefer, 1969). The reciprocal is used here as a relative estimate of the rate of fork movement.
Fig. 3. Buoyant density of DNA pulse labelled with \(^3\)H-BrdUrd from cells at 5 h after infection with Mengovirus. For 16 h before infection, cells were labelled with \(^1\)C-thymidine (0.0025 \(\mu\)Ci/ml). The radioactive medium was removed, and the cells were infected with Mengovirus. At 4.75 h after infection, the cells were labelled with \(^3\)H-BrdUrd \((5 \times 10^{-6} \text{ M}, 10 \mu\text{Ci/ml})\) for 30 min. The DNA was extracted and sedimented to equilibrium in alkaline cesium chloride. 

- O---O, \(^3\)H,d/min;
- 14C,d/min;
- ▲—▲, density. (a) Uninfected cells. (b) Mengovirus-infected cells.

Fig. 4. Uptake of exogenous thymidine by Mengovirus-infected cells. Cells were labelled with \(^8\)I-thymidine \((5 \mu\text{Ci/ml})\) at the indicated concentrations for 2 min at 5 h after infection. 

- O---O, Uninfected cells; O---O, Mengovirus-infected cells. (a) Total uptake into cells. The curves were fitted by regression analysis after linear transformation. (b) Double-reciprocal plot of the data in (a) \(V, \text{ } ^3\)H-thymidine incorporation; \(S, \text{ thymidine concentration.}\)

Table 2. \textit{dTTP pool in Mengovirus-infected cells}

<table>
<thead>
<tr>
<th>Experiment</th>
<th>\textit{dTTP pool*}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mol/g protein (\times 10^{-12})</td>
</tr>
<tr>
<td>5 h post-infection</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>73</td>
</tr>
<tr>
<td>Mengo</td>
<td>100</td>
</tr>
<tr>
<td>6 h post-infection</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>76</td>
</tr>
<tr>
<td>Mengo</td>
<td>63</td>
</tr>
</tbody>
</table>

* \textit{E. coli} DNA polymerase was used to measure limiting dTTP concentrations in acid extracts of cells. The reaction was followed by the incorporation of \(^3\)H-dATP into acid-precipitable material. The amount of protein was used as an estimate of cell number. Under the conditions of culture used, 1 mg of protein is equal to 3 × 10^9 cells.

DNA was labelled with \(^3\)H-BrdUrd at \(5 \times 10^{-6} \text{ M}\) for 30 min with the midpoint of the pulse 5 h after infection. Under these conditions, thymidine nucleotide in nascent DNA was 40% substituted by BrdUrd nucleotide. The DNA was extracted and sedimented to equilibrium in alkaline CsCl. The gradients from this experiment (Fig. 3) show that in infected cells
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compared to controls, less $^3$H was incorporated into DNA, since there was a decrease in the amount of $^3$H in nascent DNA compared to $^4$C in pre-labelled DNA. The density of the nascent DNA was the same in infected and control cells; therefore there is no change in the concentration of exogenous nucleotide in the dTTP pool in infected cells. Similar results were obtained when $^3$H-BrdUrd-labelled DNA was examined at 6 h, in that the density of nascent DNA was the same in infected and control cells despite the marked decrease in $^3$H incorporation in infected cells (data not shown).

The size of the total dTTP pool in infected cells was measured next. Since the nuclear dTTP pool is approximately four times the size of the cytoplasmic dTTP pool (Skoog & Bjursell, 1974), significant changes in the nuclear pool will be reflected as changes in the total pool. Acid extracts of cells were assayed using E. coli DNA polymerase to measure limiting amounts of dTTP (Solter & Handschumacher, 1969; Lindberg & Skoog, 1970). The dTTP pool was 37% larger in infected cells than in controls at 5 h (Table 2). The size decreased subsequently so that at 6 h it was 17% smaller than in the controls. The data show clearly that there is no marked decrease in pool size at either time, and this cannot account for the inhibition of DNA synthesis in infected cells. There is an increase in pool size at 5 h after infection, but since the concentration of exogenous nucleotide is unchanged, it is unlikely to represent pool dilution.

Uptake of exogenous thymidine by infected cells

Another possible cause of decreased incorporation of $^3$H-thymidine into DNA in infected cells is decreased uptake. Transport of nucleosides across the cell membrane may be measured by standard kinetic techniques (reviewed by Hauschka, 1973). The uptake of $^3$H-thymidine into total cellular material at different concentrations of thymidine in the external medium was measured. At 5 h after infection, the uptake was decreased at all concentrations tested (Fig. 4a). A double reciprocal plot of the data (Fig. 4b) shows that the inhibition of thymidine transport in Mengovirus-infected cells was non-competitive. The $V_{max}$ was decreased almost 40% while there was little change in the $K_m$ (Table 3). The $V_{max}$ in the control cells was somewhat lower than that obtained by us in the same cell line in previous experiments (Hand, 1976b), but this may have been due to the shorter pulse times in the present experiments (2 min compared with 5 min in the previous experiments). Similar results were obtained in cells 6 h after infection (data not shown). The findings are compatible with the interpretation that Mengovirus infection causes a decrease in the number of functional thymidine transport sites on the cell membrane.

Stability of template DNA in infected cells

Another potential source of dilution of exogenous label in infected cells is thymidine derived from the breakdown of template DNA. Ensminger & Tamm (1970a) showed that the size of template DNA as measured by sedimentation in neutral sucrose gradients was the same in control and Mengovirus-infected cells. In the present study, the re-utilization of nucleotides from template DNA for synthesis of new DNA was investigated. Data from two experiments are given in which the fate of isotope label that had been incorporated into cellular DNA before infection is presented.

Cells were labelled overnight with $^3$H-BrdUrd and then infected. The DNA was extracted and its density determined by equilibrium sedimentation in alkali. In this type of experiment, breakdown and re-utilization should be detected as $^3$H incorporated into DNA of normal density, since the re-utilized $^3$H-BrdUrd is not likely to be present in sufficient concentration in nascent chains to cause a density shift. Control cells showed no $^3$H counts
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**Table 3. Thymidine transport in Mengovirus-infected cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>$V_{\text{max}}^*$ (mol/10^6 cells/min × 10^{-12})</th>
<th>$K_m$ (M × 10^{-6})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.98</td>
<td>0.23</td>
</tr>
<tr>
<td>Mengovirus-infected</td>
<td>0.61</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* The values were calculated from the data in Fig. 4. Straight lines were fitted to the data in the double reciprocal plot by regression analysis. The $V_{\text{max}}$ values were determined from the reciprocals of the y-intercepts and the $K_m$ values from the negative reciprocals of the x-intercepts.

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**DISCUSSION**

The pertinent findings in this report relate to the decrease in thymidine incorporation into cellular DNA at 5 h after infection of L cells with Mengovirus. They show that there is an actual inhibition in DNA synthesis at this time. This is accompanied by a moderate increase...
DNA synthesis in Mengovirus infection

in the size of the thymidine triphosphate pool. This increase is not accounted for by increased uptake of exogenous thymidine, increased synthesis of endogenous thymidine nucleotides, or re-utilization of thymidine nucleotides from breakdown of template DNA. Therefore, it is most probably caused by a block to the entry into DNA of dTTP. A block at this point could have two effects on the replication of DNA: it could reduce initiation or retard replication fork progression. We have shown now with the use of an equilibrium sedimentation technique that replication fork movement is not significantly reduced at 4 to 5 h after infection. This confirms our earlier findings on fork movement in infected cells obtained with DNA fibre autoradiography (Hand & Tamm, 1972). The available evidence strongly supports the supposition that Mengovirus inhibits the initiation of DNA synthesis.

Mengovirus infection also causes a decrease in cellular protein synthesis (Franklin & Baltimore, 1962). Inhibition of protein synthesis by a variety of chemical agents results in a decrease in the rate of replication fork movement (Weintraub & Holtzer, 1972; Gautschi & Kern, 1973; Hand & Tamm, 1973). In addition, it causes a total derangement in the pattern of DNA replication rather than selectively inhibiting one aspect of replication such as initiation (Hand, 1975). Thus, while the late effects of Mengovirus infection on DNA synthesis may be ascribed to inhibition of protein synthesis (Hand, 1976b), the early effects must be attributed to a separate, more specific mechanism.

Separate mechanisms for the cut-off of cellular protein synthesis and cellular DNA synthesis may also exist in poliovirus-infected cells. Temperature-sensitive virus mutants defective in the inhibition of cellular DNA synthesis have been described that are genetically distinct from mutants defective in the repression of protein synthesis (Garwes, Wright & Cooper, 1975).

There is also precedent for two distinct mechanisms in other cytocidal RNA virus infections. Vesicular stomatitis virus infection of BHK 21 cells causes a decrease in DNA synthesis that occurs before the decrease in cellular protein synthesis (Baxt & Bablanian, 1976). In reovirus-infected L cells, the inhibition of DNA synthesis also takes place before the onset of the inhibition of protein synthesis (Ensminger & Tamm, 1969). Since vesicular stomatitis virus (Wertz & Younger, 1970) and reovirus (Zweerink & Joklik, 1970) eventually inhibit cellular protein synthesis, it may be surmised that this would contribute to the inhibition of DNA synthesis at late times in infection.

We have also presented data showing that Mengovirus causes a decrease in the uptake of thymidine across the cell membrane. The inhibition is non-competitive and this is compatible with the interpretation that there are fewer functional receptors for thymidine transport in infected cells. A similar effect on transport has been described in Newcastle disease virus-infected cells (Hand, 1976b). Inhibition of protein synthesis by cycloheximide or puromycin also causes non-competitive inhibition of thymidine transport (Plagemann & Erbe, 1972, 1974; Plagemann et al. 1974, 1975). The most reasonable explanation for the decrease in transport in infected cells is that fewer receptor proteins are synthesized. Alternatively, sites already on the membrane might be rendered non-functional by infection. In either view, the decreased transport of thymidine most probably is unrelated to the virus-induced inhibition of DNA synthesis.

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