The Role of Arginine in the Replication of Herpes Simplex Virus

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SUMMARY

Arginine is essential for the replication of herpes simplex virus. Omission of arginine from the culture medium did not affect the synthesis of viral DNA but prevented the formation of virions. Addition of arginine immediately stimulated protein synthesis which was followed by virus formation.

INTRODUCTION

The need for arginine in the replication of adenovirus was described by Rouse, Bonifas & Schlesinger (1963), who demonstrated the inhibition of virus synthesis in the absence and its restoration on the addition of arginine. Tankersley (1964) showed that arginine was essential for the replication of herpes simplex virus, as did Roizman, Borman & Rousta (1965) and Sharon (1966), but the mechanism was not explained. The present communication deals with the role of arginine in the intranuclear replication of herpes simplex virus and its effect on the synthesis of viral DNA, viral coat proteins and the formation of herpes virions.

METHODS

Virus, cells and medium

The HF strain of herpes simplex virus, plaque-purified, was propagated in the BSC1 line of monkey kidney cells. The cells were grown in milk-dilution bottles using Eagle's medium (Eagle, 1959) supplemented with 10% (v/v) calf serum. Infected cultures which were used as virus stock preparations contained 5% calf serum and were stored at 4 °. The virus was released from the cells before use by ultrasonic vibration for 1 min. in a MSE ultrasonic disintegrator operating at 134. Monolayer cultures were infected at a multiplicity of 10 p.f.u./cell. The virus, suspended in 5 ml. Eagle's medium without serum, was adsorbed to the cells (2 x 10^6 cells per milk-dilution bottle) for 3 hr. At the end of the adsorption period the virus was removed and 10 ml. fresh Eagle's medium was added. Infectivity titrations of the virus stocks were done by the plaque assay described by Levitt & Becker (1967). The amount of leucine in Eagle's medium was reduced to one-hundredth the normal concentration (final concentration 0.008 mM). As required, arginine was either omitted from the medium or added at concentrations ranging from 0.0042 to 4.2 mM. Puromycin dihydrochloride (obtained from N.B. Co., U.S.A.), at a concentration of 10 μg./ml., was used to inhibit protein synthesis.

Radioactive isotopes

The DNA in the infected and control cultures was labelled either with [2-14C]-thymidine or [3H]thymidine (specific activity 35 mc/m-mole and 2.7 mc/m-mole,
respectively). Proteins were labelled with either $[^{14}C]$leucine or $[^{3}H]$leucine (specific activity 165 and 200 mc/m-mole, respectively) and also with $[^{14}C]$arginine (specific activity 150 mc/m-mole). The isotopes were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

**Fractionation of cells**

Infected and control cells labelled with radioisotopes were withdrawn at intervals after infection; the medium was removed and the cells were kept at 4°C. The cells were scraped with a glass rod into 2 to 4 ml. cold hypotonic buffer (RSB) and disrupted by means of twenty strokes in a Dounce glass homogenizer (Levitt & Becker, 1967). By this procedure less than 2% of the radioactive DNA in the nuclei was released into the cytoplasmic fraction. The nuclei were separated from the cytoplasm by centrifugation at 800 g for 2 min., resuspended in hypotonic buffer and disrupted by ultrasonic treatment for 1 min. Samples of 0.1 ml. were removed from the different cell fractions, treated with 20% trichloracetic acid, collected on Millipore filters and counted in a Packard liquid scintillation counter. The amount of DNase-resistant radioactive DNA was determined by treatment with 100 μg. DNase per 0.1 ml. sample, before the addition of trichloracetic acid.

**Sucrose density gradient centrifugation**

Mature herpes virions were isolated from the nuclear fractions by zone centrifugation in sucrose gradients (15 to 30%, w/w). The gradients were centrifuged at 15,000 rev./min. for 50 min. in the SW 25.1 rotor of the Beckman model L-2 ultracentrifuge as described by Levitt & Becker (1967). Fractions were collected and incubated for 30 min. at 37°C with DNase (2 × crystallized; Worthington Co., U.S.A.), 35 μg. per fraction in the presence of $10^{-4}$M-Mg$^{2+}$, before treatment with trichloracetic acid.

**RESULTS**

*The effect of arginine on the formation of herpes simplex virus*

At least 0.105 mM arginine was required to obtain maximum virus yield (Table 1). This was also demonstrated by using the DNase resistance of mature herpes virions to measure virus synthesis (Levitt & Becker, 1967). BSC1 cultures were infected with herpes simplex virus, labelled with tritiated thymidine and incubated in Eagle's medium lacking arginine. Different concentrations of arginine were added to some of the cultures 3 hr and to the rest 13 hr after infection. The cultures were harvested 19 hr after infection and the amount of coated viral DNA determined. The amount of radioactive DNA resistant to DNase increased in the infected cells as the concentration of arginine was increased, indicating that the process of coating of viral DNA occurred only in the presence of arginine (Fig. 1).

An attempt was made to determine whether incomplete herpes virions were synthesized in the nuclei of the infected cells in the absence of arginine. Accordingly, the cells were infected with herpes simplex virus and incubated in Eagle's medium lacking arginine (Fig. 2A) or containing 0.105 mM arginine (Fig. 2B, C). The cultures were labelled with $[^{3}H]$thymidine and $[^{14}C]$leucine (Fig. 2A, B), or with $[^{3}H]$thymidine and $[^{14}C]$arginine (Fig. 2C). Eighteen hr after infection the cultures were harvested and the cell nuclei separated and analysed on sucrose gradients. In the absence of arginine
Arginine and replication of herpes virus

(Fig. 2 A) no virus band was evident and radioactive proteins were found only in the soluble fraction at the top of the sucrose gradient. In the presence of arginine however, mature herpes virions labelled in the coat proteins with either $^{14}$C-leucine or $^{14}$C-arginine appeared in the virus band which was characterized by the presence of radioactive DNA resistant to DNase. In addition, two peaks containing radioactive proteins were evident in the region of the gradient between the virus band and the soluble fraction at the top. These two peaks were shown by electron microscopy to contain viral capsids and capsomeres (Olshevsky, Levitt & Becker, 1967), indicating that arginine was required for the formation both of mature virions and of the viral coat proteins. The two upper protein bands were not evident in the cytoplasmic fraction of infected cells.

![Graph showing the effect of different concentrations of arginine on the extent of virus formation 19 hr after infection.](image)

Fig. 1. The effect of different concentrations of arginine on the extent of virus formation 19 hr after infection. • — , arginine added 3 hr after infection; ○ — , arginine added 13 hr after infection.

Table 1. Dependence of virus yield on the concentration of arginine

<table>
<thead>
<tr>
<th>Arginine concentration (mM)</th>
<th>Virus yield (p.f.u.) after 18 hr incubation</th>
</tr>
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<tbody>
<tr>
<td>Complete medium (3 hr after infection)</td>
<td>Expt 1</td>
</tr>
<tr>
<td>0</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>0.042</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>0.105</td>
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<td>0.420</td>
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<td>4.200</td>
<td>$1.5 \times 10^8$</td>
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<td></td>
<td>$1.7 \times 10^8$</td>
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</table>
The effect of arginine on the synthesis and coating of viral DNA

Infected cells were labelled with [3H]thymidine 3 hr after infection and incubated in Eagle's medium with or without arginine. Samples were withdrawn at different times and the amounts of radioactive DNA before and after DNase treatment were determined. Uninfected controls were similarly treated. More DNA was synthesized in infected than in uninfected cells and the synthesis of DNA was not affected by the absence of arginine. The coating of viral DNA was studied by measuring the increase

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Fig. 2.** Effect of arginine depletion on the synthesis of herpes simplex virus; zone centrifugation in sucrose density gradients. A, without arginine; B, C, with 0.105 mM arginine. A, B, C labelled with [3H]thymidine; A, B with [14C]leucine; C with [14C]arginine. ○—○, [3H]DNA resistant to DNase; ●—●, [14C]proteins.

![Graph D](image4)

**Fig. 3.** The effect of arginine on the synthesis of DNA and herpes virions. ●—●, infected cells plus 0.105 mM arginine; ○—○, infected cells minus arginine; □—□, uninfected control. A, before and B, after treatment with 1 mg./ml. DNase.
in the amount of radioactive DNA resistant to DNase treatment at different times after infection. In the absence of arginine the amount of DNase-resistant DNA was negligible, indicating that although viral DNA was being synthesized, coating of the DNA and formation of herpes virions were prevented (Fig. 3 A, B).

Fig. 4. The effect of arginine depletion on the rate of protein synthesis. A, infected cultures; B, uninfected controls. ●—●, medium containing 0-105 mM arginine; ○——○, medium lacking arginine.

The rate of protein synthesis in the absence of arginine

Cultures were incubated in Eagle's medium with or without arginine and at different times were labelled for 15 min. with [14C]leucine and the radioactivity in the protein was determined. The rate of protein synthesis in infected cells was lower than in uninfected cells (Fig. 4). The lack of arginine in the medium resulted in reduction of the rate of protein synthesis both in infected and in uninfected cells. Further analysis demonstrated the site of arginine incorporation to be localized in the cytoplasm. Treatment with puromycin abolished, to a great extent, the incorporation of [14C]-arginine into cell proteins.

The effect of addition of arginine on the rate of protein synthesis

To determine whether the protein-synthesizing mechanism in the cytoplasm of infected cells was affected by the absence of arginine, cultures infected with herpes virus and incubated for 15 hr in the absence of arginine were pulse-labelled for 5 min. with [14C]leucine at different times before and after the addition of arginine. The rate of protein synthesis increased three- to fourfold after the addition of arginine (Fig. 5). Labelling the cells for 2 min. at 5 min. intervals demonstrated that the increase in the rate of protein synthesis occurred within 2 min. of the addition of arginine.

The effect of arginine on the coating of viral DNA

Cultures infected with herpes simplex virus were labelled with [3H]thymidine and incubated in medium with and without arginine. Twelve hr after infection, arginine
was added to some of the cultures without arginine. Cultures were harvested at different times and the amount of DNase-resistant radioactivity determined. DNase-resistant radioactivity after the addition of arginine 12 hr after infection increased in parallel with the increase in infected cultures kept in complete medium (Fig. 6). This and other similar experiments showed that radioactive DNA resistant to DNase appeared within 2 hr of the addition of arginine. Sucrose-gradient analysis of infected cell nuclei labelled both with [14C]leucine and [3H]thymidine also showed that radioactive proteins and DNA appeared in the band containing mature virions 2 hr after the addition of arginine. This agrees with the finding that under normal circumstances viral proteins are transported to the nucleus and viral DNA is coated within two hr (Olshevsky et al. 1967).

![Graph](image1)

![Graph](image2)

**Fig. 5.** The effect of arginine on the rate of protein synthesis. • •, arginine not added; ○ ○ ○ ○, arginine (0.04 mM) added (arrow).

**Fig. 6.** Reversal of arginine-deficiency effect. • •, complete medium; ○ ○ ○ ○ ○, no arginine; ▼ ▼ ▼ ▼ ▼, arginine added 12 hr after infection (arrow).

**DISCUSSION**

The experiments described in the present paper confirm and extend the initial finding of Tankersley (1964) that arginine is essential for the synthesis of herpes simplex virus. Determination of virus yield both by the plaque assay and by the increase in DNase-resistant radioactive DNA demonstrated that virus synthesis was completely inhibited in the absence of arginine. The extent of virus synthesis was found to be dependent on the amount of arginine present in the medium of the infected cultures. The effect of arginine deprivation was further investigated by studying the synthesis of DNA and proteins in infected cells. DNA synthesis was not affected by the lack of arginine and there was no difference in the increase in the amount of
DNA synthesized in cultures incubated in medium with or without arginine. It was also shown that the decline in the rate of protein synthesis in infected cells continued in the absence of arginine, though the extent of incorporation decreased in both infected and control cultures. These experiments demonstrated that the synthesis of enzymes necessary for the synthesis of viral DNA was not affected in the absence of arginine. Thus, the inhibition of virus formation does not occur at the level of viral DNA.

Utilization of arginine for the synthesis of herpes virions was demonstrated by labelling infected cells with $^{14}{\text{C}}$arginine and analysing the infected nuclei in sucrose gradients. It was found that similarly to $^{14}{\text{C}}$leucine, $^{14}{\text{C}}$arginine is incorporated in the coat proteins of mature herpes virions. This indicates that arginine is essential for the synthesis of the viral coat proteins and is incorporated into them. In the absence of arginine, also the two radioactive protein peaks present in the sucrose gradient above the virus peak do not appear. When arginine was added to deficient medium the synthesis of proteins was resumed immediately. This indicates that the viral messenger RNA was present in the cytoplasm, probably in association with the cytoplasmic ribosomes, but lacked the ability to continue protein synthesis in the absence of arginine. Addition of the essential amino acid may be thought to initiate the synthesis of nascent peptide chains and the viral coat proteins, which are transported from the site of synthesis in the cytoplasm to the nuclei to form mature virions. Bonifas (1967) similarly concluded that arginine is needed for the synthesis of coat proteins of adenoviruses. The possible utilization of arginine in other metabolic processes is being further investigated.

The results of the present experiments therefore indicate that the genetic information required for the early enzymes necessary for the synthesis of DNA polymerase (Keir et al. 1966) as well as the inhibitors of cellular nucleic acid synthesis are not affected in the absence of arginine. Cell damage is evident in the infected cells in the absence of arginine. Arginine is necessary for the expression of the late viral functions concerned with the synthesis of viral coat proteins. Thus in the absence of arginine, the viral DNA molecules remain in the infected nuclei as DNase sensitive DNA. It may be assumed that if the infected cells contain an enzyme capable of degrading arginine, synthesis of herpes virions would similarly be prevented. Such a situation is not known for herpes viruses but the infection of cells with Shope papilloma virus (Shope, 1933) causes the induction of the enzyme arginase which is virus specific (Rogers & Moore, 1963). Under similar conditions Ito & Evans (1961) found only naked viral DNA molecules and not virions in the tumour cells. Recently, it was found that the synthesis of SV40 coat proteins in BSC$_{1}$ cells was inhibited in the absence of arginine but the synthesis of the tumour antigen, which is an early viral function, was not affected (Goldblum, Ravid & Becker, unpublished).

It is of especial interest that Subak-Sharpe et al. (1966) found evidence of arginyl-transfer RNA in herpes infected cells and that this must be specified by the virus genome. Herpes virus resembles adeno- and papovaviruses in its requirement for arginine. All three groups replicate in cell nuclei, and are also characterized by their ability to cause latent infections. The finding that in the absence of arginine, viral DNA synthesis continues undisturbed, whereas the formation of virions is inhibited, might explain the latent behaviour of these intranuclear viruses.
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REFERENCES


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