Requirement of Arginine for the Replication of Herpes Virus

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SUMMARY

Arginine in the medium was essential for the replication of herpes simplex virus in RK 13 cells. Virus adsorption, penetration and eclipse were not affected. When the medium of arginine-deficient infected cultures was exchanged for complete medium, virus growth resumed with the appearance of a near-synchronous infection. Resumption of virus growth was immediate if the exchange was made in the first 7 days, but if it was later a delay resulted which increased as the duration of deprivation increased. A threshold concentration of arginine was found for herpes virus growth. Virus yield was increased when the arginine concentration was raised over a limited range, but further addition of arginine did not continue to improve the yield. Undialysed calf serum could not be substituted successfully for arginine in herpes virus replication, although it supported cell growth and the replication of vaccinia and coxsackie B 3 viruses.

INTRODUCTION

The essential nutritional requirements for the growth of tissue cells in vitro include many amino acids, salts, vitamins and some sugars (Morgan, Morton & Parker, 1950; Eagle, 1955a, b). Depending on the specific omission the degenerative effects become evident within 1 to 14 days (Eagle & Habel, 1956). Many viruses have a generation time of between 10 and 14 hr in cells in tissue culture. It thus becomes possible to determine which of the nutrients, necessary for the cell, are also essential for the propagation of the infecting virus.

Omission of certain amino acids from the growth medium resulted in a marked decrease in the proliferation of herpes simplex virus (Pelmont & Morgan, 1959). A detailed investigation of the amino acid requirements of herpes simplex virus in human cells was made by Tankersley (1964), who found that both virus growth and cytopathic effect ceased when arginine was omitted from the medium. When it was replaced virus growth resumed promptly and extensively.

The present report concerns the replication of herpes virus and the fate of the cell and the virus +cell complex in arginine-deficient medium.

METHODS

Virus. The polykaryocyte forming strain of herpes simplex virus (MP) was obtained from Dr B. Roizman, Johns Hopkins University School of Medicine, Baltimore (Roizman & Roane, 1961, 1963). Virus was propagated in RK 13 cells at 33°, titrated by the plaque method, and stored in 0.5 ml. volumes at −65°.

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Cells. RK 13 cells were obtained from Dr J. McKay, Moredun Research Institute, Edinburgh. This is an uncloned line of rabbit kidney epithelial cells which originated from Glaxo Laboratories Ltd, Greenford, England.

Media. Cells were propagated in Glaxo tissue culture medium 'I99' supplemented by 10% calf serum. The plaque assay overlay medium was made by adding 0.75% methylcellulose to medium 'I99' supplemented by 5% calf serum. The Eagle's medium used in nutritional studies was based on the formula given by Paul (1961). Component solutions were prepared, sterilized by filtration through Millipore filters of porosity 0.22 μ and stored at −30°. Media were made up on the day of use. To prepare a deficient medium, the relevant component was omitted and salt solution added to keep the final volume constant.

Virus assay. Routine virus assay was by plaque titration on cell monolayers (Dulbecco, 1952). Monolayers were prepared by sowing 3.75 × 10⁶ cells in 60 mm. Petri dishes and incubating overnight. Virus dilutions were made in a 1% solution of skimmed milk, the inoculum volume was 0.5 ml. and adsorption was for 3 hr at 37°. After 60 hr at 37° cultures were stained with 0.1% methyl violet in physiological saline and the plaques counted.

Nutritional studies. All tests were made in parallel using test medium and complete Eagle’s medium. Uninfected cell controls were included. Monolayers of cells were prepared in 85 mm. Carrel flasks, 60 mm. Petri dishes or in test tubes.

(a) Inoculation. Hanks's balanced salt solution (BSS) at pH 7.2 was used to wash cell monolayers and as the virus diluting fluid. Cultures were drained, washed twice, inoculated with 0.5 ml. of the virus preparation and adsorption continued either for 2 hr at 37°, or 1 hr at 4°. Residual inoculum fluid was removed and the cultures washed twice. Experimental media were added, incubation continued at 37° and samples withdrawn as required.

(b) Virus yield. Infected cells were lysed by three cycles of freezing and thawing and the virus titrated.

(c) Plaque formation. The effect of nutrition on plaque size was investigated by incorporating methyl-cellulose into the test medium.

(d) Evidence of virus biosynthesis. Cover-slip cultures were fixed at various stages of infection. The occurrence of intracellular viral inclusions was investigated by the slow method of Giemsa staining (Cruickshank, 1965). Nuclear changes were studied by acridine orange staining (Pollard & Starr, 1962). The fluorescent antibody staining technique was used as an indicator of viral protein synthesis (Nairn, 1964). The human antiherpes serum was previously absorbed with a powder of RK 13 cells in order to reduce non-specific fluorescence. The complement-fixation test also was used as an indicator of virus-induced protein synthesis (Cruickshank, 1965). The samples used were the water-lysed infected cells harvested for the assay of virus yield.

(e) Electron microscopy. Samples of infected cells were removed for examination by electron microscopy at 0, 1, 2, 3, 6, 9, 11 and 23 hr after inoculation. A double-fixation technique, using glutaraldehyde and 1% osmium tetroxide in veronal buffer at pH 7.4, was employed as this was known to give good preservation (Cruickshank, 1965). Sections were stained with lead citrate for 2 min. (Reynolds, 1963) and with a saturated solution of uranyl acetate in 70% aqueous ethanol for 10 min., and examined in an A.E.I. electron microscope type E.M. 6.
RESULTS

The effect of some amino acid omissions on the growth of herpes virus in RK 13 cells was examined. Monolayers were inoculated with virus and incubated in complete Eagle's medium for 24 hr at 37°. The cultures were then washed, test media added, and incubation continued for a further 48 hr. The relative virus yields are given as a percentage of the control.

Table 1. Percentage virus yields in cells growing in deficient media

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Lysine</td>
<td>75</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.1</td>
<td>0.3</td>
<td>5</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.8</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
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Table 2. Growth of RK 13 cells in media containing normal or restricted amounts of arginine

<table>
<thead>
<tr>
<th>Incubation period in days</th>
<th>Number of cells x 10^-4 per culture when grown in:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>'199'</td>
</tr>
<tr>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>1</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
</tr>
<tr>
<td>3</td>
<td>7.4</td>
</tr>
<tr>
<td>3rd subculture: inocula were</td>
<td>2.5*</td>
</tr>
<tr>
<td>9</td>
<td>3.3</td>
</tr>
<tr>
<td>2nd subculture: inocula were</td>
<td>0.7†</td>
</tr>
</tbody>
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All media were supplemented by 5% calf serum.
* Cells appeared healthy and gradually multiplied though much more slowly in the arginine deficient medium.
† Cells did not thrive; they became vacuolated and granular and were discarded after 8 days' incubation.

The omission of arginine or of histidine but not of lysine from Eagle's medium resulted in a marked reduction in virus yield (Table 1). Cytopathic change did not develop when the medium lacked arginine but did so when histidine was omitted. Eagle's medium lacking arginine will be referred to as Arg^- medium.

The effect of arginine deficiency on RK 13 cells was examined. Cell growth in Eagle's and Arg^- media was compared with growth in medium '199' (Table 2). In Arg^- medium without calf serum, cells remained morphologically normal for 3 days. Between 3 and 4 days they became thinner, more spindle-shaped and then died rapidly. When Arg^- medium was supplemented by 10% calf serum the cells remained morphologically normal for up to 16 days and multiplied to form sparse cell sheets which could be maintained with changes of medium. Cultures recovered when the Arg^- medium was replaced by optimal medium; the normal growth rate was resumed and the cells could then be subcultured. Cells growing in arginine deficient media were not easily
subcultured; the populations which resulted contained many swollen and abnormal cells. These cultures did not recover when optimal medium was replaced.

The effect of substituting calf serum for arginine in media on virus infected cells is shown in Table 3. Plaque size and virus yield in Eagle's medium was increased as the

<table>
<thead>
<tr>
<th>Concentration of calf serum (%)</th>
<th>Eagle's</th>
<th>Arg-</th>
<th>Eagle's</th>
<th>Arg-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large inoculum</td>
<td></td>
<td>Small inoculum</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>162,000</td>
<td>4,600</td>
<td>22,200</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>60,000</td>
<td>29</td>
<td>16,600</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>62,000</td>
<td>9</td>
<td>10,800</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>12,400</td>
<td>0</td>
<td>2,700</td>
<td>0</td>
</tr>
</tbody>
</table>

* 1,200 p.f.u./tube culture; total incubation period was 24 hr.
† 150 p.f.u./Petri dish culture; total incubation period was 60 hr.

Fig. 1. The yield of herpes virus per culture of RK 13 cells in media containing different concentrations of arginine. The concentration of arginine in routine Eagle's medium was R; five times this was 5R; one half was ½R, etc.

concentration of calf serum was increased. At low multiplicities of infection neither virus replication nor cytopathic effect was detected in cells in Arg- medium containing even 20% calf serum. With a large inoculum there was no virus proliferation in cells in Arg- medium supplemented by even 10% calf serum although some small syncytia were detected 24 hr after inoculation. With 20% calf serum there was slight reproduction of virus and small syncytia were observed at 12 hr. Analysis of undialysed calf serum by two-way paper chromatography indicated that a 1% solution in water
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contained approximately as much arginine or arginine-like material as complete Eagle's medium.

In modified media without calf serum but containing different concentrations of arginine, there was a threshold level of arginine, \( \frac{3}{4}R \), below which no virus was produced (Fig. 1). Virus yield was increased when the concentration of arginine was increased to \( 2R \). Greater excesses failed to increase the yield further.

Very low virus yields resulted (Table 1) when complete medium was replaced by arginine-free medium on established infections. Multiple and one-step growth experiments were made to determine if even one virus replication cycle could be completed in arginine-free medium. At both low (Fig. 2) and high (Fig. 3) multiplicities of infection in arginine-deficient cells no increase in infective virus was detected. Further, these results suggested that the virus adsorbed, penetrated and entered eclipse at the normal rate even in the absence of arginine. Similar results were obtained when the media contained 100 \( \mu \)g./ml. kanamycin, an antibiotic active against a wide range of pleuropneumonia-like organisms.

The rate of virus attachment was also investigated by comparing adsorption from '199' and from Arg- suspending fluids for periods of 1, 2 and 3 hr, and found to be independent of the presence or absence of arginine. The intracellular fate of the virus particle and the progress of viral biosynthesis in the arginine-deficient cell were investigated. Unless specifically stated the cells had no previous history of arginine starvation. Cultures were examined up to 24 hr after infection. No viral inclusions were revealed by Giemsa staining. No viral protein synthesis was detected by either the fluorescent antibody staining technique or the complement-fixation test. No virus-induced nuclear...
changes were observed after acridine orange staining. In all these tests positive results were obtained in control Eagle's medium cultures within 24 hr of infection.

In electron microscopic studies of infected cells in Arg− medium, virus adsorption and penetration were observed. However, no increase in the number of virus particles was observed; nor were any nuclear or cytoplasmic changes seen. The results of the control series of electron micrographs prepared from cultures in Eagle's medium were in agreement with those of Holmes & Watson (1963). Virus particles were observed inside the nuclei of infected cells by 9 hr after infection.

![Graph](image)

**Fig. 4**. Single-cycle growth curves of herpes virus in RK 13 cells maintained 24 hr before infection either in complete, ○, or in Arg−, O, medium. Adsorption was for 1 hr at 4°. After inoculation all cultures received complete medium.

**Fig. 5**. The recovery of viral activity in herpes virus infected cells in Arg− medium when the deficient medium was exchanged for complete medium. The number of foci of infection decreased as the period of arginine deprivation was extended. After 7 days it took progressively longer for viral activity to resume. At 21 days the system broke down with spontaneous viral outgrowth.

An experiment was designed to estimate the time in the virus growth cycle in cells in Arg− medium at which replication was arrested (Fig. 3). From the first Eagle's medium growth curve (Fig. 3A) new infective virus was seen to be produced between 6 and 12 hr after infection. In other experiments, e.g. Fig. 4, virus was first detected at 9 hr. After the addition of complete medium, Fig. 3C, a more synchronous culture resulted and new infective virus was produced between 9 and 11 hr. Experiments in which more frequent samples were taken showed resumption of virus growth after 9 hr. These results did not indicate any curtailment in the eclipse phase. There may have been a lag before resumption of metabolism due to the effect on the cells of previous arginine starvation. To examine this possibility parallel growth experiments were made with cultures that had been maintained in Arg− or Eagle's media for 24 hr at 37° before
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infection. Any differences found could be ascribed to the different pre-inoculation histories. The results obtained (Fig. 3) suggested that the eclipse phase in previously starved cells was about 2 hr longer. Therefore part of the lag shown in Fig. 3C can probably be ascribed to a cellular effect of arginine starvation.

Tests were made to determine the maximum interval between infection in Arg− medium and successful resumption of viral growth. Arg− medium containing 5% calf serum was used since this enhanced cell survival while remaining inadequate for virus multiplication and the inoculum was large to allow for a decrease in the number of infective foci surviving (see Fig. 5). For the first 7 days of suppression, virus could be recalled and stimulated to form standard-size plaques in 2 days. The time required to attain standard plaque size increased as the duration of suppression increased. After 16 days plaques took 6 days to develop. After 21 days incubation in Arg− medium widespread syncytia developed without the addition of complete medium. It is significant that areas of the cell sheet had completely disintegrated without any sign of viral cytopathic effect in a manner similar to that seen in uninfected cultures grown in deficient media for long periods. Throughout this experiment the medium was changed every 3 or 4 days. This was unlikely to cause any secondary foci of infection since supernatant fluids from suppressed cultures were found to contain no infective virus.

DISCUSSION

The presence of arginine and histidine in the medium was found to be essential for the replication of herpes virus in RK 13 cells whereas omission of lysine did not decrease the virus yield. Viral cytopathic changes continued in the absence of histidine but could not be observed when arginine was omitted. These results confirm those of Tankersley (1964) working with a different strain of herpes virus in human cells.

Herpes virus in cells in arginine-free medium was unable to complete a single replication cycle. Biological experiments and electron microscopy showed that successful adsorption, penetration and eclipse of the virus particle occurred, and at the normal rate, in the absence of arginine. In Arg− medium, synthesis of virus-induced protein was not observed and neither visible nuclear changes nor viral inclusions were detected. Roizman, Spring & Roane (1967) also have found that the intranuclear and cytoplasmic granules characteristic of herpes virus infected cells were not formed in Arg− medium. However, Becker, Olshevsky & Levitt (1967) have been able to show that the synthesis of viral DNA was not affected by the absence of arginine from the culture medium. The normal growth cycle was of 9 hr duration, but, owing to asynchrony of infection, the maximum virus yield was not attained until 18 to 24 hr after inoculation. When infected cells in Arg− medium were stimulated to resume viral reproduction, by exchanging the deficient for a complete medium, a near-synchronous virus growth resulted with progeny virus first appearing 9 hr later. Becker et al. (1967) found an immediate stimulation of protein synthesis followed by formation of virus on the addition of arginine. Cells which had been starved for 24 hr before inoculation produced new infective virus approximately 2 hr later than did normal cells (Fig. 3). This lag may have been due to the necessity of cells to replenish the stock of enzymes required in the initial stages of viral replication. From a comparison of these results it appeared that the time required between restimulation of infected cultures in Arg− medium and the completion of viral synthesis was not 9 but 7 hr. Therefore the viral
growth cycle appears to have continued for 2 hr only, before stopping due to shortage of arginine. Roizman et al. (1967) have suggested that arginine was not necessary in the first 4 hr but was required after 6 hr. However, Roizman, Borman & Rousta (1965) have found with this strain of herpes virus that most of the viral protein synthesis starts about 2 to 3 hr after inoculation. It may be that there is a high demand for arginine in one or some of the early virus-induced proteins which the intracellular amino acid pool is inadequate to supply. Small pools of arginine have been found, in fact, in many types of cells (Piez & Eagle, 1958).

The arginine requirement for normal cellular metabolism was considerably less than for viral synthesis. In Arg− medium RK 13 cells remained morphologically normal and even multiplied slowly for 3 to 4 days, whereas herpes virus replication was found to stop after about 2 hr. The addition of undialysed calf serum to Arg− medium greatly enhanced the survival of non-infected cells. However, deficient medium containing up to 20 % calf serum was inadequate for herpes virus replication when the cells were infected at low multiplicities. At high multiplicities of infection a small amount of virus was produced in deficient medium supplemented by 20 % but not 10 % calf serum. In experiments of long duration or with cells infected at high multiplicities many cells died. The concomitant liberation of arginine and other nutrients may greatly influence the surviving cells. Calf serum could be substituted for arginine more successfully in the replication of vaccinia virus and even more successfully in the replication of coxsackie B3 virus. Experiments (not reported) showed that both these viruses continued to multiply, but with diminished yields, in medium without arginine or without lysine.

The herpes virus genome survived in a recoverable form in infected cells kept in Arg− medium. Up to the seventh day after infection virus proliferation resumed without noticeable delay when Arg− medium was replaced by complete medium. After this the condition of the infected cultures deteriorated and there was an increasing delay before resumption of virus growth. Finally, after 21 days in deficient medium, a spontaneous outbreak of virus growth occurred. Probably the very large number of dead cells in the culture yielded enough arginine to activate and support virus growth. It is of interest that the virus had remained in a recoverable state as long as the cells survived. Thus the decrease in the number of surviving infective foci (Fig. 5) was due more likely to the death of infected cells, than to cell survival with differential loss of the virus genome. By removal and subsequent replacement of arginine from the medium of herpes virus-infected cells a condition is created somewhat resembling latency followed by an overt infection. Perhaps the fine balance of extracellular and intracellular metabolites is involved in the phenomenon of latent herpes virus infection.

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