AMINO ACID REQUIREMENTS OF HERPES SIMPLEX VIRUS IN HUMAN CELLS

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ABSTRACT

Tankersley, Robert W., Jr. (Medical College of Virginia, Richmond). Amino acid requirements of herpes simplex virus in human cells. J. Bacteriol. 87:609-613. 1964.—Progressive infection of human cells minimally infected with herpes simplex virus requires 11 of the amino acids of Eagle's medium, and glutamine. Lysine is not required, but rather exerts a partially inhibitory effect upon virus multiplication. Infected cells deprived of arginine support neither cytopathogenic effects nor virus replication; when arginine is replaced, a prompt and extensive infection follows. The effect of nutritional deficiencies on virus infection is discussed.

Medium constituents required for support of virus infection in cell culture vary markedly, depending upon the virus studied and the cell system employed. For example, foot-and-mouth disease virus and poliovirus have quite simple requirements; the former multiplies in calf kidney cells in the presence of inorganic salts and glucose (Pledger and Polatnick, 1962), and the latter proliferates readily in HeLa cells nourished by a medium consisting of balanced salts, glucose, and glutamine (Eagle and Habel, 1956). In contrast, psittacosis virus is much more demanding. Bader and Morgan (1958, 1960) showed that nine amino acids and five vitamins, in addition to balanced salts and glucose, are essential to active infection in Earle's L strain mouse fibroblasts by psittacosis. In the absence of these ingredients, a latent infection occurs (Morgan and Bader, 1957).

Cultures near the end point in herpes simplex tube titrations occasionally showed definite cytopathic effects (CPE) at one reading, and only normal, essentially intact cell sheets 24 to 48 hr later. Medium replacement in these tubes was followed by rapid and extensive infection. The similarity of these observations to some of the results with psittacosis virus indicated that a nutritional deficiency developing during infection might be responsible for the disappearance of virus effects from the system. Certain of the nutritional requirements of a herpes simplex-human cell system were examined, therefore, to determine their effects upon continuing infection.

MATERIALS AND METHODS

Virus. Herpes simplex virus (HSV) isolated from a generalized infection was propagated in Minn EE cells for over 4 years. Pools of virus for the present experiments were obtained as supernatant fluids from distilled water lysates of infected cells. The average pool titer was about 50 × 10⁴ plaque-forming units (PFU) per ml.

Cell culture. The Minn EE cell (Syverton and McLaren, 1957), a human esophageal epithelium derivative, was grown in Eagle's medium (Eagle, 1955) with 10% calf serum. Tubes for experimental work were inoculated with 150,000 cells each; plaque bottles for virus assay received 1.3 × 10⁴ cells. These cells were periodically treated with 100 μg/ml of kanamycin (Kantrex; Bristol Laboratories, Inc., Syracuse, N.Y.) for 1-week periods. Pleuropneumonia-like organisms (PPLO) were not present in this cell line, since cells carried on antibiotic-free medium for 3 months failed to show typical PPLO colonies on a yeast extract-Noble agar medium with 15% human serum, or on a commercial PPLO medium (PPLO-Eaton Agent Medium; Robbins Laboratories, Chapel Hill, N.C.).

Experimental media. Eagle's medium deficient in individual amino acids was made up from L-amino acids and vitamins (Calbiochem) and used without serum. To protect cells from effects produced by absence of serum, 0.3% Methocel (15 centipoise) was added as suggested by Merchant et al. (1962).

Experimental procedure. The basic procedure was designed to reproduce the conditions thought to prevail in the titration tubes described above. Established cell cultures were rinsed three times.
with Hanks' balanced salt solution and inoculated with 2 to 5 PFU of HSV in 0.1 ml of Eagle's medium. Adsorption of virus proceeded for 1 hr, and Eagle's medium with 3% horse serum was added. Tubes were incubated to allow infection to develop. At 48 to 72 hr, the cultures were screened, those showing definite infection were rinsed 2 to 3 times with Hanks' solution, and experimental media were added to groups of two or three tubes each. After 72 hr of further incubation to allow infection to progress, medium was discarded and tubes were rinsed with distilled water and frozen with 1 ml of distilled water until assayed. The assay procedure was a standard plaque method, with a fluid overlay of Eagle's medium plus 10% calf serum. As reported by Farnham (1958), results were linear and reproducible with this type of overlay.

Results

Amino acid requirements. By use of the basic procedure described, the effect of single amino acid deficiencies upon virus yield from infected Minn EE cells was examined. The results of four such experiments are shown in Fig. 1. Decreased virus yield is interpreted as reflecting increased requirement for a given amino acid; on this basis, histidine and arginine were the most important members of this group of compounds. The third basic amino acid, lysine, was not required; in fact, lysine-free medium supported greater virus production than did complete Eagle's medium. The remainder of the amino acids formed a spectrum of decreasing requirement between these extremes.

Effect of the basic amino acids upon infection. The results indicate that the lack of arginine or histidine, and possibly the presence of lysine, would interfere markedly with virus synthesis. To determine whether or not one or a combination of these factors was responsible for the disappearance of minimal virus infection, the original experiment was repeated. Only arginine and histidine deficiencies were examined, 3% dialyzed horse serum replaced Methocel in part of each series, and graded amounts of excess lysine were added. The results of one such experiment with arginine-deficient medium are recorded in Table 1.

Essentially no virus was recovered from any group of tubes containing arginine-deficient medium, whether Methocel or dialyzed horse serum was included. Cell sheets were excellent in both media, and no evidence of CPE was seen. As the results in the control tubes in complete medium indicate, a partial inhibition of virus yield occurred with increased lysine concentration. Similar results were obtained with histidine-deficient medium; in this instance, however, CPE were marked, and this deficiency was not studied further.

Sensitivity of cells and recovery of infection in an arginine-deficient medium. An arginine-deficient medium makes EE cells incapable of demonstrating CPE, or of replicating virus. The degree of cell insensitivity to infection, as well as the pos-

![Graph](http://jb.asm.org/)

**FIG. 1.** *Effect of single amino acid deficiencies on virus yield from infected cells in serum-free medium. Geometric means of four experiments.*
sible reactivation of the CPE, was examined with an experimental medium composed of arginine-deficient Eagle's medium with 0.18 mg/ml (2.5 times the usual concentration) of lysine and 3% dialyzed horse serum.

Groups of EE tubes were inoculated and adsorbed with tenfold dilutions of herpes virus in 0.1-ml volumes of Eagle's medium. After adsorption, part of each dilution group received 0.9 ml of Eagle's medium with 5% horse serum, and the remainder received 0.9 ml of the experimental medium containing 3% dialyzed horse serum. Tubes were examined for CPE at 24-hr intervals. Cells in the experimental medium were at least three logs less sensitive to viral effects than were control cells (Table 2).

To be certain that virus had infected cells in deficient medium, and also to determine whether virus was present in a recoverable form, medium in half of the experimental tubes above was replaced with complete medium 24 hr after infection. The third part of Table 2 indicates that CPE were detected in these tubes, although such effects appeared somewhat more slowly and to a lesser extent than did effects in control tubes.

**Role of arginine in recovery of infection.** In the previous section, the recovery of CPE was brought about by replacing arginine-deficient medium with complete medium. A final series of experiments was undertaken to confirm the importance of arginine to this system, and to rule out any effect that might result from changing

### Table 1. Effect of arginine deficiency and excess lysine on HSV multiplication in Minn EE cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Lysine conc (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>Arginine-free Eagle’s medium, 0.3% Methocel</td>
<td>&lt;10*</td>
</tr>
<tr>
<td>Arginine-free Eagle’s medium, 3% dialyzed HoS†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Eagle’s medium, 0.3% Methocel</td>
<td>1,080,000</td>
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</table>

* Virus yield in plaque-forming units.
† Horse serum.

### Table 2. Comparative sensitivity of Minn EE cells in complete and experimental media to herpes simplex virus infection and the recoverability of virus by replacing experimental medium with complete medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Log virus dilution</th>
<th>Time after infection (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse serum (5%) in Eagle’s medium (complete medium)</td>
<td>3</td>
<td>+++*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>Dialyzed horse serum (3%) in arginine-free Eagle’s medium with added lysine</td>
<td>3</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>Arginine-free medium replaced by complete medium at 24 hr</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
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<td>6</td>
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</table>

* Degree of CPE ranging from ++++, complete cell destruction, to —, no effect.
the medium (removal of inhibitor, for example). EE tubes were inoculated and adsorbed with about 0.5 PFU per cell of herpes virus. After 24 hr of incubation in the arginine-free medium described above, no CPE were seen. Pairs of tubes then received either a complete medium change with 5% horse serum in Eagle’s medium, or arginine or other compounds were added in amounts used in complete medium to the 24-hr-old experimental medium in the tubes. The cells were observed for CPE and were frozen 72 hr later for assay. A typical experiment is summarized in Table 3. Addition of arginine alone in a concentration equivalent to that in Eagle’s medium was sufficient to evoke viral CPE, and supported viral replication to a degree surpassing that in complete medium.

**Discussion**

These experiments were initiated to determine whether or not nutritional deficiencies developing in a minimally infected HSV-human cell system were responsible for spontaneous disappearance of viral CPE. Toward this end, 11 of the 12 amino acids studied, as well as glutamine, were shown to be required for continuing viral replication (Fig. 1). One of these required amino acids, arginine, was further found to affect appearance of viral CPE (Table 1). Lysine, the sole amino acid not required, actually inhibited viral proliferation to some degree.

Men EE cells deprived of arginine will not support proliferation of HSV, nor will CPE appear. Within the limits of the survival time of cells in arginine-free medium, a relationship resembling latent infection is observed. However, the finding that virus is increasingly difficult to recover with increasing incubation time (Table 2) makes it questionable whether a true latent state exists or not. Possibly the difficulty lies in creating the proper conditions for evoking active infection; this problem, and the long-term cell-virus relationship, are being studied.

There have been a number of reports of the importance of arginine to normal or virus-infected cells in culture. For example, Thomas et al. (1958) demonstrated that cell growth could be maintained at an optimal level for up to 10 days simply by adding fresh arginine periodically, indicating that arginine was a limiting factor. The enhancement of adenovirus plaque formation by adding arginine was reported by Bonifas and Schlesinger (1959), who more recently (Rouse, Bonifas, and Schlesinger, 1963) showed that not only CPE but also adenovirus multiplication is dependent upon this amino acid. In their studies, the arginine requirement was particularly marked in PPLO-contaminated cells. As was indicated by Kenny and Pollock (1963), PPLO specifically deplete arginine stores from the medium to the point where cell activity becomes limited by arginine content. The effect of PPLO contamination is to lend emphasis to the importance of arginine in these systems.

Lysine has a partially inhibitory effect upon replication of HSV in the system described. This effect by an amino acid shown to be essential to normal cells in culture (Eagle, 1955) is not unique. A similar reduction in virus yield by lysine was observed by Pearson, Lagerborg, and Winzler (1952), with GD VII mouse encephalomyelitis virus. Glycine, demonstrated to be necessary to monkey kidney cells in a synthetic medium, was found to inhibit poliovirus replication in these cells (Melnick et al., 1957). More striking was the effect of glutamine on HSV in HeLa cells as reported by Lewis and Scott (1962), who found that, while this compound was required for virus multiplication, under certain conditions glutamine actually effected a reduction in virus yield. No ready explanation is available for any of these observations. Certainly virus synthetic mechanisms are different from cell mechanisms, and would be expected to have somewhat different requirements. Identification of these mechanisms may be aided by the recognition of different metabolic requirements for the normal and the infected systems.
ACKNOWLEDGEMENTS

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LITERATURE CITED


