Enhanced Utilization of Citrulline in Rabbitpox Virus-infected Mouse Sarcoma 180 Cells

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SUMMARY

The replication of rabbitpox virus has been studied in mouse sarcoma 180 cells in media containing either arginine or citrulline. The yield of infective virus depended on the concentration of each amino acid, and maximum yield was obtained with 0.1 mM-arginine or 0.5 mM-citrulline. In the presence of this concentration of citrulline, cell multiplication and protein synthesis in uninfected cells was suppressed markedly compared with cell cultures in medium containing 0.5 mM-arginine. Growth of a human citrullinaemia cell line was inhibited also when citrulline substituted for arginine yet the replication of vaccinia virus continued.

The incorporation of radioactivity available as [14C]-carbamoyl-citrulline into mouse sarcoma 180 cells maintained in citrulline-containing medium increased significantly following infection by rabbitpox virus. A similar, increased incorporation was observed with vaccinia virus-infected HeLa cells. Increased incorporation into rabbitpox virus-infected mouse sarcoma 180 cells occurred at 3 and 5 h post-infection and coincided with increased incorporation of [14C]-guanido-arginine into cell cultures infected in the presence of arginine. This enhanced utilization of citrulline was inhibited by actinomycin D, and rabbitpox virus replication in the presence of citrulline was inhibited by canavanine. It is concluded that virus-directed mechanisms determine arginine biosynthesis in mouse sarcoma 180 cells infected with rabbitpox virus in the presence of citrulline.

INTRODUCTION

Arginine has been shown to be essential for the replication of vaccinia virus in continuous cell lines (Holtermann, 1969; Singer et al. 1970). Two arginine-dependent events have been described with vaccinia-infected HeLa cells, one occurring before virus DNA synthesis and a second associated with the formation of mature virus particles (Archard & Williamson, 1971). In vaccinia-infected KB cells, although limited virus DNA synthesis proceeds in the absence of arginine, the synthesis of virus-specific, late mRNA is inhibited (Obert, Tripier & Guir, 1971). The replication of herpes simplex virus in HeLa cells shows a similar dependence, but virus growth in human embryo fibroblasts in the absence of arginine is unimpaired (Jeney, Gönçzöl & Vaczi, 1967). These different arginine requirements for herpes simplex virus replication in continuous and primary cell cultures reflect differences in the metabolic capabilities of host cells (Gönçzöl, Jeney & Vaczi, 1967).

Although mammals have a capacity for arginine biosynthesis through the urea cycle,
Eagle (1959) has shown the essential nature of this amino acid for the growth in vitro of many continuous cell lines derived from mammalian tissues. Citrulline can replace arginine for the growth of certain cell lines but ornithine is ineffective. However, a limited number of mammalian cell lines have been described that do not express this biosynthetic capability and show consequently a specific requirement for arginine in cell growth (Tytell & Neuman, 1960).

The present study describes poxvirus replication in continuous cell lines exhibiting differences in their ability to utilize citrulline as a substitute for arginine in cell growth. Detailed investigations have been made using mouse sarcoma 180 cells (Foley et al. 1960) and limited investigations using human citrullinaemia cells (Tedesco & Mellman, 1967); both cell lines are distinguished by their inability to grow in the presence of citrulline.

**METHODS**

**Virus.** The Lister strain of vaccinia virus or Utrecht strain of rabbitpox virus were used throughout this work. Infectivity titrations were made by plaque assay in cultures of RK 13 cells.

**Cell culture.** HeLa and mouse sarcoma 180 cells were grown in Eagle’s minimum essential medium (Eagle, 1959) supplemented with 5% (v/v) calf serum. Human citrullinaemia cells were grown in a modified Eagle’s medium (Macpherson & Stoker, 1962) supplemented with 10% (v/v) foetal calf serum. In this laboratory, growth of this cell line was sufficient to permit only a limited number of experiments. Confluent monolayers of cells were maintained in serum-free medium (Birch & Pirt, 1969) containing 14 mM-HEPES (Williamson & Cox, 1968). Depletion of intracellular pools of arginine was effected by incubation of cell monolayers in arginine-free maintenance medium. HeLa and human citrullinaemia cells were maintained for 18 h in this medium: mouse sarcoma 180 cells were maintained for 6 h under similar conditions.

All cell cultures were tested for mycoplasm contamination by inoculation on to suitable media (Hayflick, 1965). It was not possible to isolate mycoplasm from the cell lines used but, as an additional precaution, kanamycin sulphate (200 μg/ml) was added to experimental media.

**Determination of cell growth.** HeLa or mouse sarcoma 180 cells were seeded at a concentration of 1 x 10^6 viable cells/ml into 4 oz bottles in 10 ml of culture medium (Birch & Pirt, 1969) containing either 0.5 mM-arginine or 0.5 mM-citrulline. Additional cultures were prepared similarly but in medium lacking both amino acids. All media were supplemented with 5% (v/v) calf serum. Samples from each series of cell cultures were taken at various time intervals, the cells resuspended with 0.02% (w/v) ethylenediaminetetra-acetic acid and viable cell counts were made using trypan blue.

**Determination of protein synthesis.** Protein synthesis in uninfected and infected cell cultures was determined by the incorporation of [^14C]-phenylalanine (sp. act. 475 mCi/m-mol) or [^14C]-guanido-arginine (sp. act. 312 mCi/m-mol). Further experiments were made using [^14C]-carbamoyl-citrulline (sp. act. 57 mCi/m-mol). All labelled compounds were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. The procedures used for the separation of macromolecules and measurement of radioactivity have been described (Archard & Williamson, 1971). Protein estimations were made by the colorimetric method of Sutherland et al. (1949).

**Metabolic inhibitors and chemicals.** Actinomycin D (Calbiochem Ltd., London, England) was used at a concentration of 0.5 μg/ml: this concentration completely inhibited the growth.
Citrulline metabolism in poxvirus replication

Fig. 1. Growth rate of mouse sarcoma 180 cells in media containing arginine or citrulline. ●—●, cultures maintained in the presence of 0.5 mM-arginine; □—□, cultures maintained in the presence of 0.5 mM-citrulline; ○—○, cultures maintained previously in the presence of 0.5 mM-citrulline transferred on day 3 to medium containing 0.5 mM-arginine; △—△, cultures maintained in the absence of both arginine and citrulline.

...continued to support cell growth, only limited growth occurred in the presence of 5.0 mM-citrulline. This higher concentration of citrulline permitted cell growth at a rate that was 43% lower than that for similar cultures in medium containing 0.5 mM-arginine. Experiments with HeLa cell cultures gave similar growth rates in the presence of equimolar concentrations of either arginine or citrulline. These results
Table 1. Protein synthesis in mouse sarcoma 180 and HeLa cells maintained in media containing arginine or citrulline

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Medium</th>
<th>[14C]-Phenylalanine incorporated (d/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse sarcoma 180</td>
<td>0.5 mM-arginine</td>
<td>23313</td>
</tr>
<tr>
<td>Mouse sarcoma 180</td>
<td>0.5 mM-citrulline</td>
<td>6664</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.5 mM-arginine</td>
<td>45508</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.5 mM-citrulline</td>
<td>43294</td>
</tr>
</tbody>
</table>

show that, unlike HeLa cells, mouse sarcoma 180 cells are deficient in their ability to utilize citrulline for cell growth.

Protein synthesis in mouse sarcoma 180 cells in media containing arginine or citrulline

Incorporation of [14C]-phenylalanine during a period of 18 h was determined in cell cultures maintained in media containing either 0.5 mM-arginine or 0.5 mM-citrulline. There was a marked reduction in the level of incorporation into cell cultures maintained in the presence of citrulline compared with that in similar cell cultures maintained with arginine (Table 1). Protein synthesis in mouse sarcoma 180 cells was reduced by 71% on substitution of citrulline for arginine. Thus the inhibition of cell multiplication in the presence of 0.5 mM-citrulline is accompanied by a markedly reduced capacity for protein synthesis.

Further experiments with HeLa cell cultures showed that protein synthesis was not affected by maintenance in the presence of 0.5 mM-citrulline. The incorporation of label was similar to that for cultures maintained in medium containing 0.5 mM-arginine (Table 1).

Growth of poxviruses in mouse sarcoma 180 cells and human citrullinaemia cells in media containing arginine or citrulline

Rabbitpox virus failed to grow in mouse sarcoma 180 cells maintained in the absence of arginine. This was not due to irreversible effects on host cell metabolism since subsequent addition of arginine to deprived cultures resulted in the restoration of virus growth. A quantitative relationship was observed between the concentration of arginine supplied in the medium and the yield of infective virus measured at 18 h after infection. Maximum virus yield was reached at a concentration of 0.1 mM-arginine. Similar results were obtained with infected cell cultures maintained in the presence of citrulline but maximum virus yield was obtained with 0.5 mM-citrulline (Table 2).

The growth of vaccinia virus in human citrullinaemia cells was dependent also upon the availability of arginine. Again, substitution of citrulline for arginine permitted virus growth, maximum yield being reached with 0.5 mM-citrulline. Thus, this concentration of amino acid allowed yields of progeny virus from rabbitpox virus-infected mouse sarcoma 180 cells or vaccinia virus-infected human citrullinaemia cells which were comparable with those from similarly infected cultures maintained in the presence of optimal concentrations of arginine.

Growth curves of rabbitpox virus in mouse sarcoma 180 cells in the presence of arginine or citrulline

The kinetics of rabbitpox virus replication were determined under one-step growth conditions in infected cells maintained in the presence of 1.0 mM-arginine or 1.0 mM-citrulline (Fig. 2). In the presence of either amino acid, infective progeny virus was first detected 6 h
Citrulline metabolism in poxvirus replication

Table 2. Effect of arginine and citrulline on the replication of rabbitpox virus in mouse sarcoma 180 cells

<table>
<thead>
<tr>
<th>Arginine concentration (mM)</th>
<th>Virus yield log (p.f.u./cell)</th>
<th>Citrulline concentration (mM)</th>
<th>Virus yield log (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.005</td>
<td>0</td>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.78</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>1.66</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>0.1</td>
<td>2.36</td>
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<td>0.5</td>
<td>2.43</td>
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<td>2.34</td>
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<tr>
<td>1.0</td>
<td>2.50</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>3.0</td>
<td>2.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>5.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>-0.2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Fig. 2. One-step growth curves of rabbitpox virus replication in mouse sarcoma 180 cells maintained in media containing arginine or citrulline. ●—●, cultures infected in the presence of 1.0 mM-arginine; ○—○, cultures infected in the presence of 1.0 mM-citrulline.

After infection. The rates of subsequent production of virus were indistinguishable from those for infected cell cultures maintained in media containing either arginine or citrulline.

Incorporation of radioactivity available as \([^{14}\text{C}]-\)carbamoyl-citrulline or \([^{14}\text{C}]-\)guanido-arginine into rabbitpox virus-infected mouse sarcoma 180 cells

The growth of rabbitpox virus in mouse sarcoma 180 cells in the presence of 0.5 mM-citrulline, conditions that do not permit growth of the host cells, suggested changes in citrulline utilization following virus infection. This hypothesis was investigated by determining the amounts of radioactivity incorporated into uninfected and rabbitpox virus-infected mouse sarcoma 180 cells previously equilibrated for 18 h in medium containing 0.5 mM-citrulline before exposure to \([^{14}\text{C}]-\)carbamoyl-citrulline supplied in similar medium. Results obtained following virus infection showed enhanced incorporation of radioactivity...
Table 3. Effect of virus infection on the incorporation of radioactivity available as \(^{14}\text{C}\)-carbamoyl-citrulline into mouse sarcoma 180 and HeLa cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Virus</th>
<th>Incorporated radioactivity (d/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse sarcoma 180</td>
<td>Uninfected</td>
<td>1665</td>
</tr>
<tr>
<td>Mouse sarcoma 180</td>
<td>Rabbitpox</td>
<td>5661</td>
</tr>
<tr>
<td>HeLa</td>
<td>Uninfected</td>
<td>13364</td>
</tr>
<tr>
<td>HeLa</td>
<td>Vaccinia</td>
<td>21911</td>
</tr>
</tbody>
</table>

Fig. 3. Rates of incorporation of arginine into rabbitpox virus-infected mouse sarcoma 180 cells. ▲—▲, uninfected cultures; ■——■, infected cultures.

to a level over threefold higher than that for uninfected, control cultures (Table 3). In similar experiments with HeLa cells the level of incorporation into uninfected cultures was significantly higher than that into either uninfected or rabbitpox virus-infected mouse sarcoma 180 cells. However, there was a further increase in the level of incorporation following infection of HeLa cells with vaccinia virus.

Archard & Williamson (1971) have described changes in the rate of incorporation of arginine into HeLa cells following infection with vaccinia virus. Similar experiments were made with rabbitpox virus-infected mouse sarcoma 180 cells equilibrated to medium containing 0.5 mM-arginine and then pulse-labelled at different times for 20 min with \(^{14}\text{C}\)-guanido-arginine. The virus inoculum was adsorbed for 30 min from medium containing 0.5 mM-arginine and the first pulse commenced at the end of the virus adsorption period which was designated time zero. There was a marked increase in the rate of incorporation of arginine into infected cells within 50 min of initial exposure to virus (Fig. 3). After this early stimulation, the rate of incorporation declined rapidly to levels lower than for uninfected cells which incorporated label at a relatively constant rate throughout. Further, reproducible increases in rates of incorporation into infected cells were observed at 3 h and 5 h after infection. This pattern of incorporation is identical with that described for vaccinia-infected HeLa cells (Archard & Williamson, 1971).
Citrulline metabolism in poxvirus replication

Fig. 4. Rates of incorporation of radioactivity available as [14C]-carbamoyl-citrulline into rabbitpox virus-infected mouse sarcoma 180 cells. △—△, uninfected cultures; ■—■, infected cultures.

Similar experiments with [14C]-carbamoyl-citrulline showed that the rate of incorporation into uninfected cultures was relatively constant throughout the experiment. However, there were marked increases to higher levels of incorporation into infected cultures at 3 h and 5 h after first exposure of cells to virus (Fig. 4).

Effect of actinomycin D on the incorporation of radioactivity available as [14C]-carbamoyl-citrulline into rabbitpox virus-infected mouse sarcoma 180 cells

Infected cell cultures were pulse-labelled with [14C]-carbamoyl-citrulline as described previously in the presence of media containing actinomycin D at 0.5 µg/ml. There was no stimulation in the rate of incorporation of [14C]-carbamoyl-citrulline at any time after infection. These results indicate that the changes observed in the rate of incorporation of radioactivity (available as [14C]-carbamoyl-citrulline) following virus infection are dependent on the synthesis of new RNA species.

Effect of canavanine on rabbitpox virus replication in mouse sarcoma 180 cells

Canavanine, a homologue of arginine, has been shown to inhibit competitively the biosynthesis of arginine from citrulline (Walker, 1953). The requirement for arginine biosynthesis in rabbitpox virus replication in mouse sarcoma 180 cells maintained in the presence of citrulline was determined by examining the effect of canavanine on virus growth. Infected cell cultures were maintained in medium containing 0.5 mM-citrulline and in similar medium containing various concentrations of canavanine. Yields of infective virus were determined 18 h after infection. Growth of rabbitpox virus in mouse sarcoma 180 cells in the presence of citrulline was inhibited completely by 0.1 mM-canavanine. At this concentration there was no microscopic evidence of cytotoxic effects in similarly maintained, uninfected cell cultures. A similar inhibitory effect was observed upon the growth of vaccinia virus in HeLa cell cultures maintained in the presence of citrulline.
DISCUSSION

The results presented confirm earlier observations (Holtermann, 1969; Singer et al. 1970; Obert et al. 1971; Archard & Williamson, 1971) on the requirement for arginine in poxvirus replication. Further studies have been made to investigate the capacity of citrulline, a potential biosynthetic precursor of arginine, to satisfy this essential requirement. In HeLa cells, both cell growth and vaccinia virus replication occurred when citrulline replaced arginine in the culture medium. However, in two other cell lines, poxvirus replication occurred under conditions that did not permit cell growth. Both mouse sarcoma 180 and human citrullinaemia cell lines did not grow in the presence of citrulline, although these cells supported the replication of rabbitpox and vaccinia viruses, respectively, under identical nutritional conditions. Arginine biosynthesis from citrulline requires the presence of argininosuccinate synthetase and argininosuccinate lyase: the activity of both enzymes has been demonstrated in HeLa cells (Schimke, 1964). Tedesco & Mellman (1967) showed a deficiency in argininosuccinate synthetase activity in human citrullinaemia cells. The site of the metabolic lesion in mouse sarcoma 180 cells is not known but there is at least a deficiency in argininosuccinate lyase activity, since these cells are unable to grow in the presence of argininosuccinic acid (unpublished results).

Rates of protein synthesis in HeLa cells were essentially similar in the presence of either arginine or citrulline. However, in mouse sarcoma 180 cells, protein synthesis was reduced by 71% in the presence of 0.5 mM-citrulline compared with that of an equimolar concentration of arginine. Since the metabolic lesion in human citrullinaemia cells is not absolute (Tedesco & Mellman, 1967) these cells may have also a limited capacity for arginine biosynthesis. Although insufficient for cell multiplication, this may be sufficient to permit virus replication. The replication of rabbitpox virus in mouse sarcoma 180 cells was optimal in the presence of 0.5 mM-citrulline and the kinetics of virus growth were similar in the presence of arginine. Although protein synthesis in uninfected cells was suppressed significantly in medium containing 0.5 mM-citrulline, incorporation of label available as [14C]-carbamoyl-citrulline increased markedly following virus infection. These results indicate changes in the utilization of citrulline by mouse sarcoma 180 cells following rabbitpox virus infection. Similar changes were observed also in HeLa cells infected with vaccinia virus even though these cells are fully competent in their ability to utilize citrulline.

It has been shown previously that vaccinia virus infection results in changes in the rates of incorporation of arginine into HeLa cells (Archard & Williamson, 1971). Similar patterns were obtained with rabbitpox virus-infected mouse sarcoma 180 cells which showed an increased rate of arginine incorporation immediately after virus infection, followed by further increases at both 3 and 5 h post-infection. Apart from the absence of stimulated incorporation immediately following infection, the same incorporation pattern was obtained with infected cell cultures maintained in the presence of citrulline when pulse-labelled with [14C]-carbamoyl-citrulline. Unlike the incorporation of arginine, the levels of incorporation of radioactivity were higher than those of control, uninfected cultures maintained in the presence of citrulline. Thus, the enhanced utilization of citrulline by infected cultures coincides with events in the rabbitpox virus replication cycle exhibiting a requirement for arginine. However, both peaks of incorporation of radioactivity available as [14C]-carbamoyl-citrulline, which are characteristic of infected cultures, were suppressed by actinomycin D. These results indicate virus-determined events in rabbitpox virus-infected mouse sarcoma 180 cells resulting in enhanced citrulline utilization that is dependent upon the synthesis of new RNA species.
Citrulline metabolism in poxvirus replication

Archard & Williamson (1971) showed that radioactivity was associated with mature virus particles prepared from vaccinia virus-infected HeLa cell cultures labelled with [14C]-guanido-arginine. The presence of arginine in vaccinia virus particles has been demonstrated directly (Turner & Kaplan, 1968). Replication of polyoma virus is dependent similarly on the availability of arginine (Winters & Consigli, 1969). It has been shown that purified polyoma virus particles prepared from mouse embryo cell cultures infected in the presence of radioactively labelled citrulline have radioactivity associated exclusively with arginine (Winters, Consigli & Rogers, 1971). Although the prime function of citrulline is for intermediary metabolism, the amino acid has been detected in protein from the quill of the African porcupine and from guinea pig hair follicles (Steinart, Harding & Rogers, 1969). The direct utilization of citrulline for rabbitpox virus and vaccinia virus replication is unlikely in view of the inhibitory effect of canavanine. Inhibition of argininosuccinate lyase by this homologue prevents the anabolism of arginine from citrulline (Walker, 1953). It is concluded that arginine biosynthesis is essential for poxvirus replication in cell cultures maintained in the presence of citrulline. Further, it is concluded that in both rabbitpox virus-infected mouse sarcoma 180 cells and vaccinia virus-infected human citrullinaemia cells the utilization of citrulline for arginine biosynthesis is determined by virus-directed mechanisms.

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REFERENCES


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