Argininosuccinate Synthetase-lyase Activity in Vaccinia
Virus-infected HeLa and Mouse L Cells

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SUMMARY

Argininosuccinate synthetase-lyase activity in HeLa or mouse L cells increased markedly following infection by vaccinia virus. A repression mechanism determines specific activities in uninfected cells and greater activity was obtained in the presence of 0.2 mm-citrulline than of 0.6 mm-arginine. However, maximum activity in infected cells was obtained with 0.6 mm-arginine. The development of increased enzyme activity in virus-infected cells was inhibited by puromycin or actinomycin D. Treatment of infected cells with FUdR resulted in higher levels of activity than in similar cells in the absence of the inhibitor. The Michaelis constants of enzymes in HeLa and mouse L cells were $0.11 \times 10^{-3}$ M and $0.07 \times 10^{-3}$ M, respectively; the same value of $1.0 \times 10^{-3}$ M was determined for both species of infected cells. Amino acid analysis of purified vaccinia virus prepared from HeLa cell cultures infected in the presence of $[^{14}C]$-carbamoyl-citrulline showed that radioactivity was associated exclusively with arginine. It is concluded that the anabolism of arginine from citrulline in vaccinia virus-infected cells is determined by virus-coded enzymes.

INTRODUCTION

The accompanying paper (Cooke & Williamson, 1973) has described enhanced utilization of citrulline in certain continuous cell lines following poxvirus infection. In particular, the replication of rabbitpox virus in mouse sarcoma 180 cells and of vaccinia virus in human citrullinaemia cells in the presence of citrulline has been shown to occur under conditions not permissive for cell growth. Inhibition by canavanine of rabbitpox virus replication under such conditions indicated the necessity for arginine biosynthesis. Anabolism of arginine from citrulline is determined by the activity of two enzymes, argininosuccinate synthetase (E.C. 6.3.4.5.) and argininosuccinate lyase (E.C. 4.3.2.1.). This paper describes changes in the activity of these enzymes during vaccinia virus replication and presents evidence showing that these changes depend specifically upon virus infection.

METHODS

Virus. The Lister strain of vaccinia virus was used throughout this work. Infectivity titrations were made by plaque formation in cultures of RK19 cells.

Cell cultures. Methods used for the growth and maintenance of HeLa cells and their equilibration to medium containing either arginine or citrulline have been described (Cooke & Williamson, 1973). The same methods were used with mouse L cells.
Enzyme assay. The procedures used were modified from those described previously for the separate determination of argininosuccinate synthetase and argininosuccinate lyase (Schimke, 1964). Due to the unavailability of a preparation of suitably labelled argininosuccinic acid, no attempt has been made in the present study to make separate assays. All data presented in this paper, therefore, refer to the determination of the combined enzyme activities. The assay depends upon the conversion of arginine, the product of the action of the enzymes in the cell extract, to urea in the presence of excess arginase. Using $[^{14}C]$-carbamoyl-citrulline as the substrate, this results in the liberation of $^{14}$CO$_2$ when the urea is hydrolysed by the subsequent addition of urease. Determination of the amount of gaseous radioactivity produced, therefore, enables the amount of arginine synthesized enzymatically to be determined.

Confluent monolayers of either HeLa or mouse L cells in 40 oz roller bottles ($1 \times 10^8$ cells/bottle) were maintained for 18 h in media containing appropriate concentrations of either arginine or citrulline. After this time, the cell cultures were either sham-infected or infected with vaccinia virus using 5 p.f.u./cell and maintained for a further 18 h in similar media. Uninfected and infected cells were then detached from the glass using 0.02 % (w/v) ethylenediaminetetra-acetic acid (EDTA), resuspended in 4.0 ml 0.05 M-tris-HCl buffer, pH 8.0, and subjected to two cycles of freezing and thawing. This cell extract was used routinely for enzyme assays and the protein content of such extracts was determined by the method of Sutherland et al. (1949).

The assay medium comprised 2.5 $\mu$-mol aspartic acid, 1.25 $\mu$-mol ATP, 2.5 $\mu$-mol phosphoenolpyruvate, 20 $\mu$g pyruvate kinase, 0.6 $\mu$-mol MgCl$_2$, 5 $\mu$-mol KCl, 250 units purified arginase, 25 $\mu$-mol citrulline, and 1 $\mu$-mol $[^{14}C]$-carbamoyl-citrulline. This mixture was added in a vol. of 1.0 ml to a glass scintillation vial together with 1.0 ml cell extract. A second vial was connected immediately to the vial containing the enzyme assay mixture by means of a short rubber collar joining the necks of each vial. The upper vial was lined with a cylinder 4 x 8 cm of Whatman No. 1 filter paper soaked with 0.4 ml 0.1 N-KOH. The assembled apparatus was placed in a shaking water-bath (Grant Cambridge Instruments, Cambridge, England) and incubated at 37 °C with constant agitation for 1 h. After this time, the reaction was stopped by placing the apparatus in a boiling water-bath for 3 min and the assay mixture adjusted to pH 7.0 by the addition of 0.5 ml 1 N-HCl and 1.0 ml 1.6 N-disodium hydrogen phosphate. The addition of reagents was made by means of a canula inserted through the rubber collar. The canula was connected externally to plastic tubing that was sealed at all other times with a roller clamp. After the adjustment of pH, 0.5 ml urease solution at 10 units/ml in 0.01 M-phosphate buffer, pH 7.0, was added. Incubation at 37 °C was continued for a further 20 min and the release of $^{14}$CO$_2$ was completed by the addition of 1.0 ml 1 N-HCl followed by incubation for 1 h under similar conditions.

The vials containing filter paper were then dried in vacuo over phosphorus pentoxide before the addition of 20 ml scintillation liquid (4 g 2,5-diphenyl oxazole; 50 mg 1,4-di(2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l of toluene). Radioactivity was measured in a Packard liquid scintillation spectrometer. The efficiency of counting was 60 % and was determined by adding known amounts of $[^{14}C]$-NaHCO$_3$ directly to the KOH-soaked filter paper. Further control experiments showed that the recovery of $^{14}$CO$_2$ was greater than 95 % and that there was a 95 % correlation between replicate samples. Enzyme activity was calculated after correction for activity obtained with boiled cell extracts and is expressed as $\mu$-mol arginine synthesized/mg cell protein/h. The detection of this activity depended upon the addition of both arginase and urease to the assay mixture.

Arginase purification. Commercial arginase preparations were contaminated with both
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argininosuccinate synthetase and argininosuccinate lyase. A substantial reduction in this contaminating activity was achieved by the purification of arginase essentially by the method of Bach, Hawkins & Swaine (1963).

Preparation of radioactively-labelled vaccinia virus. Purified vaccinia virus particles were prepared by methods described previously (Archard & Williamson, 1971) from HeLa cells infected in the presence of [14C]-carbamoyl-citrulline.

Amino acid analysis. Purified, labelled vaccinia virus preparations were dialysed against distilled water before hydrolysis with 6 N-HCl for 48 h in sealed glass ampoules in a boiling water-bath. After hydrolysis the acid was removed over KOH in vacuo and the samples dissolved in a mixture of unlabelled amino acids. The dissolved samples were applied in 0.03 ml amounts to 45 × 45 cm Whatman No. 1 chromatography paper and developed by a two-dimensional procedure. The first solvent system was butanol:acetic acid:water (4:1:5) and the second phenol-ammonia. After development, the chromatogram was cut into 4 cm wide strips and examined for radioactivity using a Packard chromatogram strip scanner. Further chromatograms developed simultaneously were used to detect ninhydrin-positive areas.

Radiochemicals. The enzyme assay substrate [14C]-carbamoyl-citrulline (sp. act. 0.1 mCi/m-mol) and the similarly labelled preparation (sp. act. 57 mCi/m-mol) used for preparation of radioactively-labelled vaccinia virus were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Metabolic inhibitors, enzymes and chemicals. 5-fluorodeoxyuridine (FUdR, a gift from Roche Products Ltd., Welwyn Garden City, Hertfordshire) was used at a concentration of 0.01 mM, actinomycin D (Calbiochem Ltd, London, England) at 0.5 µg/ml and puromycin (Sigma London Chemical Company Ltd, Norbiton, Surrey) at 1.5 µg/ml. These concentrations of inhibitors prevented the replication of vaccinia virus in HeLa cells without overt cytotoxic effects on uninfected cell cultures. All other enzymes and chemicals were obtained from Sigma London Chemical Company Ltd.

RESULTS

Enzyme activity in HeLa and mouse L cells

Schimke (1964) has described an arginine repression mechanism in continuous cell lines affecting the levels of activity of both argininosuccinate synthetase and argininosuccinate lyase. The highest levels of enzyme activity were obtained in the presence of growth-limiting concentrations of either arginine or citrulline. Preliminary experiments were made, therefore, to determine if a similar mechanism controlled the enzyme activities of the two cell lines used in the present study. The concentrations of amino acids in the maintenance medium allowed maximum virus yields to be obtained following infection. These yields were obtained with both cell lines in the presence of 0.1 mM concentrations of either arginine or citrulline.

Maximum activity was obtained with both HeLa and mouse L cells in the presence of 0.6 mM-arginine or 0.2 mM-citrulline; the levels of enzyme determined are shown in Table 1. As observed by Schimke (1964), enzyme activity was higher in the presence of citrulline at the concentrations of amino acids used. Maintenance of HeLa cells in the presence of 0.2 mM-citrulline resulted in a sevenfold increase in activity compared with that for cultures in the presence of 0.6 mM-arginine. Similarly, mouse L cells showed a twofold increase in activity when maintained under the same conditions.
Table 1. Argininosuccinate synthetase-lyase activity in uninfected and infected HeLa and mouse L cells maintained in medium containing arginine or citrulline

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Medium</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.6 mM-arginine</td>
<td>0.011</td>
<td>0.115</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.2 mM-citrulline</td>
<td>0.079</td>
<td>0.262</td>
</tr>
<tr>
<td>Mouse L</td>
<td>0.6 mM-arginine</td>
<td>0.032</td>
<td>0.090</td>
</tr>
<tr>
<td>Mouse L</td>
<td>0.2 mM-citrulline</td>
<td>0.070</td>
<td>0.179</td>
</tr>
</tbody>
</table>

*μ-mol arginine synthesized/mg protein/h.

Table 2. Effect of metabolic inhibitors on argininosuccinate synthetase-lyase activity in uninfected and infected HeLa cells

<table>
<thead>
<tr>
<th>Inhibitor concentration</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>1.5 μg/ml puromycin</td>
<td>0.016</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0.5 μg/ml actinomycin D</td>
<td>0.011</td>
<td>0.012</td>
</tr>
<tr>
<td>0.01 mM-FUdR</td>
<td>0.021</td>
<td>0.024</td>
</tr>
</tbody>
</table>

*μ-mol arginine synthesized/mg protein/h.

Enzyme activity in vaccinia virus-infected HeLa and mouse L cells

Enzyme activity was determined in vaccinia virus-infected HeLa and mouse L cells maintained in media containing either 0.6 mM-arginine or 0.2 mM-citrulline. Marked increases in the levels of enzyme activity were observed with all cell cultures following virus infection (Table 1). Vaccinia virus-infected HeLa cells showed a tenfold increase in the presence of arginine and a 3.3-fold increase in the presence of citrulline compared with similarly maintained, uninfected cultures. Infected mouse L cells showed 2.8-fold and 2.5-fold increases, respectively. Since the degree of stimulation of enzyme activity following virus infection was maximal in HeLa cells maintained in the presence of 0.6 mM-arginine, further studies were carried out using this system.

Effect of metabolic inhibitors on enzyme activity in virus-infected cells

Experiments were made to determine the roles of translation, transcription and DNA synthesis in the increase in enzyme activity following virus infection. Cultures of uninfected and vaccinia virus-infected HeLa cells were maintained for 18 h in media containing 0.6 mM-arginine supplemented with 1.5 μg/ml puromycin, 0.5 μg/ml actinomycin D or 0.01 mM-FUdR. Additional uninfected or infected cultures were maintained in similar medium without inhibitors before all cultures were processed for the enzyme assay.

Treatment with puromycin resulted in reduced enzyme activity in both uninfected and virus-infected cells compared with controls (Table 2). There was an 87% reduction in the enzyme activity of uninfected cells but residual activity in infected cells was barely detectable. Thus, protein synthesis is essential for increased enzyme activity following virus infection. The almost complete inhibition of activity in puromycin-treated, infected cells indicates also an increased turn-over rate of host-specified enzymes in virus-infected cells.
Enzyme activity in infected cells did not increase in the presence of actinomycin D (Table 2). The level of enzyme activity was similar to that found in uninfected cells both in the presence and absence of the inhibitor. These results indicate that the increased activity in infected cells requires RNA synthesis. Further experiments were made to determine the stability of mRNA for argininosuccinate synthetase and argininosuccinate lyase in uninfected HeLa cells. Confluent cultures were maintained for 18 h in arginine-deficient medium. The withdrawal of arginine has been shown to result in the inhibition of protein synthesis (Archard & Williamson, 1971). After starvation, the cultures were transferred either to complete medium containing 0.6 mM-arginine or to similar medium containing 0.5 μg/ml actinomycin D. Enzyme activities in cells maintained in each medium were determined after further incubation for 18 h. The level of activity in cells maintained in the presence of actinomycin D was only 20% lower than that for cells maintained in the absence of the inhibitor. These results show that the mRNA’s for argininosuccinate synthetase and argininosuccinate lyase in uninfected cells have half-lives in excess of 18 h. Increased enzyme activity following virus infection, therefore, requires the synthesis of new mRNA species.

The addition of FUdR to the maintenance medium did not inhibit the increase in enzyme activity in infected cells (Table 2). However, the level of activity in such cells was twofold higher than that in cells infected in the absence of the inhibitor. Enzyme activity in uninfected cell cultures was relatively unaffected. These results show that although the increase in enzyme activity following virus infection was independent of DNA synthesis, such synthesis determined the level of activity attained.

**Michaelis constant (K<sub>m</sub>) values for enzymes in uninfected and vaccinia virus-infected HeLa and mouse L cells**

Cell extracts were prepared from uninfected and virus-infected cultures of HeLa or mouse L cells equilibrated to medium containing 0.6 mM-arginine. The reaction velocities of enzyme activity in such extracts were determined in the presence of increasing citrulline concentrations and K<sub>m</sub> values were calculated by the method of Lineweaver & Burk (1934). In each case replicate determinations were made using separately prepared extracts of either uninfected or virus-infected cells; typical results are shown in Fig. 1.

Comparison of K<sub>m</sub> values determined with HeLa and mouse L cells shows significant differences between enzyme activities in uninfected and vaccinia virus-infected cells (Table 3). The K<sub>m</sub> value for enzymes in both species of infected cells was 1.0 × 10<sup>-3</sup> M but different values of 0.11 × 10<sup>-3</sup> M and 0.07 × 10<sup>-3</sup> M were obtained with control HeLa and mouse L cells, respectively. Thus, although enzymes in uninfected cells were distinguishable, enzymes of identical characteristics were produced following virus infection.

**Utilization of citrulline for the synthesis of arginine-containing, virus structural protein**

Further experiments were made to establish that arginine, the product of argininosuccinate synthetase and argininosuccinate activity, was incorporated into virus-specified protein. Purified virus particles were prepared from vaccinia virus-infected HeLa cells maintained in the presence of [14C]-carbamoyl-citrulline. The radioactively-labelled virus obtained was hydrolysed in 6 N-HCl and the hydrolysate dissolved in an unlabelled amino acid mixture containing both citrulline and arginine before examination for the presence of labelled amino acids by the methods described. A single peak of radioactivity was detected with an R<sub>f</sub> value corresponding to arginine on control chromatograms. No radioactivity above background levels was detected in the area of the chromatogram containing citrulline.
Fig. 1. Reaction rates of argininosuccinate synthetase-lyase activity from uninfected and infected HeLa and mouse L cells determined at different citrulline concentrations. The data are plotted according to the method of Lineweaver & Burk (1934) where \( v \) is m-mol of arginine synthesized/h and \( S \) is the concentration of citrulline (mM). (a) •, enzyme activity in uninfected HeLa cells; ■, enzyme activity in uninfected mouse L cells. (b) O, enzyme activity in infected HeLa cells; □, enzyme activity in infected mouse L cells.

Table 3. Michaelis constants \( (K_m) \) of argininosuccinate synthetase-lyase activity in uninfected and infected HeLa and mouse L cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.11 ± 0.002</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>Mouse L</td>
<td>0.07 ± 0.005</td>
<td>1.0 ± 0.06</td>
</tr>
</tbody>
</table>

* Means of six determinations on three different preparations.

These results show that in cell cultures infected in the presence of citrulline, this amino acid is metabolized to arginine for the synthesis of virus-specific proteins.

**DISCUSSION**

Arginine is an essential requirement for the replication of vaccinia virus in continuous cell lines (Holtermann, 1969; Singer et al. 1970; Archard & Williamson, 1971; Obert, Tripier & Guir, 1971). The present study has shown that infection of such cells is accompanied by...
marked increases in the activity of two enzymes involved in arginine biosynthesis. It is important, therefore, to consider the possible mechanisms determining this increased activity.

A repression phenomenon has been described in continuous cell lines affecting the specific activities of both argininosuccinate synthetase and argininosuccinate lyase (Schimke, 1964). The levels of enzyme activity are determined by the concentration of arginine available either directly or derived from the metabolism of citrulline. A similar effect was observed with the HeLa and mouse L cells used in the work described. In view of this, concentrations of each amino acid were used that gave maximal enzyme activity in uninfected cells together with the capacity to support optimal virus growth. In uninfected cells maintained under these conditions the level of enzyme activity in the presence of 0.2 mM-citrulline was higher than that in 0.6 mM-arginine. Argininosuccinate synthetase-lyase activity in both HeLa and mouse L cells in similar media increased markedly following virus infection. However, the level of activity in virus-infected cells maintained with 0.6 mM-arginine was greater than that for uninfected cells maintained with 0.2 mM-citrulline. These results indicate that the enhanced enzyme activity in infected cells is determined by a different mechanism to the repression operative in uninfected cells.

Enhanced expression of host-specific argininosuccinate synthetase and argininosuccinate lyase activity by virus-determined mechanisms would require either the presence of an activator of enzyme activity or increased de novo synthesis. No evidence has been found for the presence of an activator in uninfected cells (Schimke, 1964) and infection in the presence of puromycin resulted in an almost complete suppression of enzyme activity. Increased activity depends, therefore, upon protein synthesis, but an additional requirement for RNA synthesis is shown by the inhibition of increased enzyme activity by actinomycin D. Since the mRNA for argininosuccinate synthetase and argininosuccinate lyase in uninfected cells is stable, the dependence of increased enzyme activity following virus infection upon RNA synthesis indicates either quantitative or qualitative changes in transcription. Evidence for a qualitative change is provided by the effect of FUdR on enzyme activity in uninfected and virus-infected cells. Exposure to FUdR had little effect on levels of activity in uninfected cells. However, the increased activity following infection was twofold higher in the presence of FUdR than in the absence of the inhibitor. These results show that argininosuccinate synthetase-lyase activity in infected cells depends upon both protein and RNA synthesis but is controlled to some extent by DNA synthesis.

These different effects of metabolic inhibitors resemble the similar effects upon other enzyme activities that are induced by vaccinia virus infection. The enzymes, all associated with nucleic acid metabolism, include thymidine kinase, DNA polymerase and three deoxyribonucleases (McAuslan & Joklik, 1962; Magee, 1962; McAuslan, 1965). Treatment of infected cells with actinomycin D or puromycin prevented increased activity (Kit, Dubbs & Piekarski, 1962; McAuslan, 1963a; Jungwirth & Joklik, 1965). Enzyme activity in infected cells was higher in the presence of FUdR than in the absence of the inhibitor (Jungwirth & Joklik, 1965). These results show that expression of these enzymes requires both transcription and translation and is regulated by virus-specific DNA synthesis. Identical criteria determine increased argininosuccinate synthetase-lyase activity in vaccinia virus-infected cells. A similar conclusion may be drawn that this enhanced activity arises from the expression of virus-induced enzymes.

The virus-induced enzymes associated with nucleic acid metabolism have properties differing from similar activities in the host cell (McAuslan, 1963b; Kit & Dubbs, 1965; McAuslan & Kates, 1967). This suggests that the induced enzymes are coded by the virus...
genome or are derepressed, host-coded isoenzymes. Comparison of the $K_m$ values for argininosuccinate synthetase-lyase activity shows differences between the enzymes in uninfected and vaccinia virus-infected cells. The $K_m$ values for enzymes in HeLa and mouse L cells were $0.11 \times 10^{-3}$ M and $0.07 \times 10^{-3}$ M, respectively reflecting their derivation from different host species. However, the same $K_m$ value of $1.0 \times 10^{-3}$ M was obtained with enzymes from infected cells of both species suggesting common genetic expression arising from the virus genome. Further, it has been shown that two cell lines deficient in argininosuccinate synthetase or argininosuccinate lyase activity support poxvirus replication (Cooke & Williamson, 1973). The properties of argininosuccinate synthetase in human citrullinaemia cells have been determined and a $K_m$ value of between $10$ and $100 \times 10^{-3}$ obtained compared with $0.4 \times 10^{-3}$ M for normal human cells (Tedesco & Mellman, 1967). The repression of isoenzymes with higher substrate affinity in the citrullinaemic cells cannot afford any selective advantage. Although virus-determined modifications of host-coded functions cannot be excluded, these results suggest strongly that increased argininosuccinate synthetase-lyase activity in vaccinia virus-infected cells depends upon the synthesis of virus-coded enzymes.

The survival of particular virus-coded functions indicates advantages attendant upon possession of appropriate genes. Although one cannot extrapolate between different virus-host systems, such an advantage is suggested by certain features in the pathogenesis of poxvirus infections. In the natural transmission of mousepox virus, the portal of entry is the skin (Fenner, 1947). Smallpox infection is characterized by growth of the virus in the skin, particularly the sebaceous glands (Dixon, 1962), leading to contamination of the immediate environment (Downie et al., 1965). Analysis of the amino acid composition of human sweat has shown that citrulline is present at the highest concentration, sufficient to support virus replication, whereas arginine is at a lower concentration (Coltman, Rowe & Atwell, 1966). Citrulline was detected consistently but arginine was present in only 37% of the 155 samples tested. The authors drew attention to the virtual absence of citrulline from other body fluids indicating metabolism peculiar to the skin. Therefore, to take advantage of the ready dissemination of virus from the skin, the milieu may demand virus-coded enzymes to synthesize arginine necessary for poxvirus replication.

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