Inhibition of Vaccinia Virus Replication by Canavanine and Canaline

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(Accepted 3 May 1974)

SUMMARY

Both L-canavanine, the oxyguanidinium analogue of arginine, and L-canaline, the equivalent oxyamine analogue of ornithine, inhibit the replication of vaccinia virus in HeLa cells. Inhibition by canavanine is reversed progressively by the simultaneous addition of increasing amounts of arginine but inhibition by canaline is not reversed by addition of ornithine. However, the inhibitory effect of canaline is reversed progressively by the simultaneous addition of increasing amounts of pyridoxal phosphate. The effect of canavanine on virus production is suppressed completely by the presence of a tenfold greater concentration of arginine: the effect of canaline is suppressed completely by the presence of an equimolar concentration of pyridoxal phosphate. Additionally, the inhibition of virus growth in canaline-treated cells is reversed partially by the subsequent addition of pyridoxal phosphate. These observations suggest that canavanine inhibits competitively as a result of its structural analogy to arginine but that canaline does not act as a structural analogue of ornithine. It is proposed that canaline inhibits by its interaction with pyridoxal phosphate which results in a deficiency of this cofactor. The effect of each inhibitor on the incorporation of precursors of DNA, RNA and protein into both infected and control cells is reported. These results are compatible with the mode of action proposed for each inhibitor.

INTRODUCTION

Previous work from this laboratory shows that arginine is essential for the replication of vaccinia virus in HeLa cells. This amino acid is required for virus specific events involved in both DNA replication and the synthesis of structural proteins (Archard & Williamson, 1971). Additionally, it was shown that products of arginine metabolism are associated with mature virus particles. More recent work has shown that the biosynthesis of arginine from citrulline in vaccinia infected cells is effected by virus-specific enzymes (Cooke & Williamson, 1973; Williamson & Cooke, 1973).

In addition to functioning as a constituent of proteins, arginine, together with products of its metabolism, plays an important role in other biological processes. Ornithine is a direct product of arginine metabolism produced on hydrolysis by arginase. It functions predominantly in intermediary metabolism and is found rarely in proteins. Ornithine, derived directly from hydrolysis of arginine in the Krebs–Henseleit urea cycle, can be utilized in proline biosynthesis (Kruse, 1961) and in the synthesis of polyamines (Tabor, Rosenthal & Tabor, 1958). Canavanine is the oxyguanidinium analogue of arginine and is susceptible also to hydrolysis by arginase. The product, canaline, is the oxyamino analogue of ornithine. The structural relationships of canavanine and canaline suggest that these analogues may be
competitive inhibitors of biological processes involving arginine and ornithine. Canavanine has been shown to inhibit the replication of several viruses including adenovirus type 7 (Neurath et al. 1970), Semliki Forest virus (Ranki & Kaariainen, 1969) and herpes simplex virus (Bell, 1974). However, the effect of canaline on virus replication has not been investigated previously. The present report describes inhibitory effects of both canavanine and canaline on vaccinia virus replication in HeLa cells.

**METHODS**

**Virus.** The Lister strain of vaccinia virus was used throughout this work. Infectivity titres were determined by plaque formation in cultures of Vero cells.

**Cell culture.** The laboratory line of HeLa cells used was grown and maintained in a modified Eagle's medium containing various concentrations of arginine as described previously (Archard & Williamson, 1971).

**Inhibition of virus growth.** HeLa cell monolayers in test tubes (1 to 2 × 10^6 cells/tube) were equilibrated in the presence of 0.1 mM-arginine before infection with vaccinia virus suspended in similar medium at an input multiplicity of 5 p.f.u./cell. After adsorption for 1 h, the inocula were removed, the monolayers washed with Hanks's balanced salt solution and 1 ml samples of maintenance medium containing various concentrations of the inhibitor were added. All procedures were carried out at 37 °C. After incubation for 18 h, cells were disrupted by two cycles of freezing and thawing followed by ultrasonic treatment and infectivity titres were determined as described above.

**Measurement of macromolecular synthesis.** The synthesis of DNA in infected and control cultures was examined by incorporation of [³H]-thymidine (sp. act. 50 Ci/mmol), synthesis of RNA by incorporation of [¹⁴C]-uridine (sp. act. 492 mCi/mmol) and protein synthesis by incorporation of either [¹⁴C]-arginine (sp. act. 20 to 40 mCi/mmol) or [¹⁴C]-amino acid mixture (sp. act. 10 to 30 mCi/mmol) was measured also. All labelled compounds were obtained from The Radiochemical Centre, Amersham, Buckinghamshire. Infected and sham-infected HeLa cell monolayers were maintained in medium containing the appropriate labelled precursor together with a suitable concentration of the inhibitor. After 4 h, the amount of incorporated radioactivity was determined as described previously (Archard & Williamson, 1971). The incorporation of [³H]-thymidine into the cytoplasmic fraction only of infected cells was measured. Cells were resuspended using 0.02 % (w/v) EDTA in phosphate-buffered saline (PBS) and disrupted with the non-ionic detergent Nonidet P40 (Shell; 0.25 % (w/v) Nonidet P40; 0.14 M-KCl; 0.01 M-tris-HCl buffer, pH 7.4). After exposure to the detergent at room temperature for 5 min, nuclei were sedimented at 500 g for 3 min and the supernatant, cytoplasmic fraction recovered.

**Chemicals.** L-canavanine (chromatographically homogenous) and pyridoxal phosphate were obtained from Sigma London Chemical Co. Ltd, Norbiton, Surrey. L-canaline was prepared enzymically (Williamson & Archard, 1974) and DL-canaline was synthesized by the method of Karpeiskii, Khomutov & Severin (1962).

**RESULTS**

**Inhibition of virus growth**

The results show that both L-canavanine and L-canaline are potent inhibitors of vaccinia virus growth. In the presence of 0.1 mM-arginine, the addition of 1.0 mM-L-canavanine
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resulted in complete inhibition of production of infectious progeny virus (Fig. 1). A similar effect was observed following the addition of either 2 mM-L-canaline prepared enzymically or 2 mM-DL-canaline prepared by organic synthesis (Fig. 2). However, a 50% reduction of virus yield was observed in the presence of about 0.1 mM concentrations of either canavanine or canaline. Microscopic examination of uninfected, control cultures maintained in medium containing up to either 2 mM-L-canavanine or 5 mM-L-canaline did not reveal any c.p.e.

Further experiments were made to determine the ability of L-arginine to reverse the inhibitory effect of canavanine and of L-ornithine to reverse the effect of canaline. In the presence of 1.0 mM-canavanine, the inhibition of virus growth was reversed progressively

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Fig. 1. The effect of L-canavanine on the production of infectious vaccinia virus in HeLa cell monolayers infected under one-step growth conditions. Arginine concentration = 100 μM.

Fig. 2. The effect of either L-canaline or DL-canaline on the production of infectious vaccinia virus in HeLa cell monolayers infected under one-step growth conditions. Slope = -45.

Fig. 3. The reversal of canavanine inhibition of vaccinia virus growth by addition of arginine. Canavanine concentration = 1000 μM.
Table 1. The effect of pyridoxal phosphate on the production of infectious vaccinia virus in HeLa cell monolayers maintained in the presence of 2 mM-L-canaline

<table>
<thead>
<tr>
<th>Pyridoxal phosphate (mM)</th>
<th>Virus yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.33</td>
<td>3.5</td>
</tr>
<tr>
<td>0.67</td>
<td>5.5</td>
</tr>
<tr>
<td>1.00</td>
<td>15.0</td>
</tr>
<tr>
<td>1.33</td>
<td>22.5</td>
</tr>
<tr>
<td>1.67</td>
<td>39.5</td>
</tr>
<tr>
<td>2.00</td>
<td>100</td>
</tr>
</tbody>
</table>

by the simultaneous addition of increasing concentrations of arginine. Complete reversal was obtained with 10 mM-arginine (Fig. 3). However, the inhibitory effect of 2 mM-canaline was not reversed in the presence of either 10 mM-ornithine or 10 mM-arginine. These results indicate that canavanine inhibits vaccinia virus growth by competition with arginine whereas canaline does not appear to act as a structural analogue of ornithine.

Reversal of canaline inhibition of virus growth by pyridoxal phosphate

Previous work has demonstrated that canaline interacts with pyridoxal phosphate (Kekomaki et al. 1969a) to produce an addition compound (Williamson & Archard, 1974). Further experiments were made to determine the effect of pyridoxal phosphate on the inhibition by canaline of virus growth. The simultaneous addition of increasing concentrations of pyridoxal phosphate to medium containing either 2 mM-L-canaline or 2 mM-DL-canaline resulted in increasing virus yields. At equimolar concentrations, the virus yield was comparable with that obtained in control cultures containing 2 mM-pyridoxal phosphate alone (Table 1). This suggests an equimolar interaction between canaline and pyridoxal phosphate. On this basis, residual canaline concentrations were computed by subtraction of the pyridoxal phosphate concentrations from the 2 mM-canaline present originally. A plot of virus yield versus residual canaline concentration shows that the kinetics of the reversal by pyridoxal phosphate are similar to those of the original inhibition observed in the presence of canaline alone (Fig. 4).
Table 2. Effect of L-canavanine on macromolecular synthesis in uninfected and vaccinia virus-infected HeLa cells

<table>
<thead>
<tr>
<th>Labelled precursor</th>
<th>Control</th>
<th>Inhibited</th>
<th>% control</th>
<th>Control</th>
<th>Inhibited</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]-thymidine</td>
<td>475</td>
<td>391</td>
<td>83</td>
<td>202</td>
<td>151</td>
<td>75</td>
</tr>
<tr>
<td>[14C]-uridine</td>
<td>386</td>
<td>278</td>
<td>72</td>
<td>364</td>
<td>258</td>
<td>71</td>
</tr>
<tr>
<td>[14C]-amino acids</td>
<td>158</td>
<td>91</td>
<td>58</td>
<td>129</td>
<td>92</td>
<td>71</td>
</tr>
<tr>
<td>[14C]-arginine</td>
<td>1274</td>
<td>603</td>
<td>47</td>
<td>891</td>
<td>500</td>
<td>56</td>
</tr>
</tbody>
</table>

* Radioactivity incorporated into cytoplasmic fractions only.

In additional experiments, infected cultures were maintained in the presence of 2 mM-L-canavanine for 5 h after infection. After this time, the medium was replaced by maintenance medium lacking the inhibitor but containing 0.004 mM-pyridoxal phosphate. This change resulted in a partial reversal of canavanine inhibition, the final yield of infectious progeny virus being 23 % of that from control cultures. However, supplementation of the maintenance medium with 0.25 mM-pyridoxal phosphate increased the virus yield to 43 % of that from appropriate controls. Higher concentrations of pyridoxal phosphate are cytotoxic.

Effects of canavanine and canaline on macromolecular synthesis

At a concentration of canavanine which inhibits virus growth completely, the incorporation of thymidine or uridine into whole, uninfected cells was reduced by 17 % and 28 %, respectively, compared with non-inhibited controls. Under similar conditions, thymidine incorporation into the cytoplasmic fraction of infected cells was reduced by 25 % and uridine incorporation into whole, infected cells by 29 %, compared with appropriate controls. These results indicate that canavanine does not have a marked inhibitory effect on nucleic acid synthesis in either uninfected or infected cells. Protein synthesis in uninfected cells, examined by the incorporation of either [14C]-arginine or a [14C]-amino acid mixture, was reduced more markedly to about 50 % of the values in non-inhibited cultures. In infected cells, however, a similar reduction was observed using [14C]-arginine but a reduction of only 29 % using the [14C]-amino acid mixture (Table 2).

Similar experiments with canaline showed that the incorporation of thymidine or uridine into whole, uninfected cells was reduced by 31 % and 29 %, respectively. Thymidine incorporation into the cytoplasmic fraction of infected cells was reduced by 41 % and uridine incorporation into whole, infected cells showed a 22 % reduction. These results indicate that nucleic acid synthesis is relatively unaffected in the presence of this inhibitor also. Protein synthesis examined by the incorporation of the amino acid mixture into either uninfected or infected cells showed a reduction of about 50 % compared with non-inhibited controls. The incorporation of radioactivity supplied as [14C]-ornithine was inhibited markedly, showing about an 80 % reduction. The incorporation of radioactivity supplied as [14C]-arginine was reduced similarly (Table 3).

In further experiments, the effect of canaline on the uptake of the labelled amino acid mixture, arginine or ornithine into the acid-soluble, intracellular pool of uninfected cells was determined. Cultures were exposed to labelled amino acids in the presence or absence of 2 mM-L-canaline. After 4 h, the media were removed, the cultures washed three times with ice-cold PBS and the recovered cells precipitated with trichloroacetic acid. The incorporation
Table 3. Effect of L-canaline on macromolecular synthesis in uninfected and vaccinia virus-infected HeLa cells

<table>
<thead>
<tr>
<th>Labelled precursor</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Inhibited</td>
</tr>
<tr>
<td>$[^3]$H-thymidine</td>
<td>81</td>
<td>56</td>
</tr>
<tr>
<td>$[^14]$C-uridine</td>
<td>47</td>
<td>34</td>
</tr>
<tr>
<td>$[^14]$C-amino acids</td>
<td>182</td>
<td>85</td>
</tr>
<tr>
<td>$[^14]$C-ornithine</td>
<td>107</td>
<td>18</td>
</tr>
<tr>
<td>$[^14]$C-arginine</td>
<td>96</td>
<td>17</td>
</tr>
</tbody>
</table>

* Radioactivity incorporated into cytoplasmic fractions only.

Table 4. Effect of L-canaline on the uptake of amino acids into the acid-soluble, intracellular pool of uninfected HeLa cells

<table>
<thead>
<tr>
<th>Labelled precursor</th>
<th>Radioactivity (d/min x 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>$[^14]$C-amino acids</td>
<td>14</td>
</tr>
<tr>
<td>$[^14]$C-arginine</td>
<td>67</td>
</tr>
<tr>
<td>$[^14]$C-ornithine</td>
<td>871</td>
</tr>
</tbody>
</table>

of amino acids into the acid-precipitable material showed reductions similar to those described above. The uptake of the amino acid mixture into the acid-soluble, intracellular pool was reduced by 34% in the presence of the inhibitor. Under similar conditions, the uptake of arginine was reduced by 14% and that of ornithine by 65% (Table 4).

DISCUSSION

The present study shows that canavanine inhibits the formation of infectious progeny virus in vaccinia-infected HeLa cells. This inhibition is reversed progressively by the addition of increasing amounts of arginine indicating that canavanine inhibits vaccinia virus replication by direct competition with arginine. In the presence of a concentration of canavanine which inhibits completely the formation of infectious progeny virus, the incorporation of arginine into infected and control cultures is reduced by 44% and 53%, respectively. As arginine is essential, the fact that virus growth is inhibited completely under conditions effecting a partial reduction only of arginine incorporation indicates that there are particular arginine requiring events in the virus growth cycle upon which subsequent production of infectious progeny depends. Previous studies have shown that the rate of arginine incorporation into infected cells is stimulated at two points during the first 4 h (Archard & Williamson, 1971; Cooke & Williamson, 1973). However, the total amount of arginine incorporated into infected cells during this period is less than that into control cells. Since the rate of protein synthesis examined using labelled leucine or labelled phenylalanine is similar in infected and control cells (Archard & Williamson, 1971), the overall frequency of arginyl residues in virus-induced proteins must be less than that in host cell proteins. This is supported by the observation that the extent of inhibition by canavanine of arginine incorporation into infected cells is less than that into control cells. The fact that supplying canavanine at a molar concentration tenfold greater than that of arginine results in but a
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partial reduction of arginine incorporation suggests either that canavanine competes inefficiently with arginine for incorporation into nascent polypeptides or that its transport into the intracellular pool is restricted.

It has been demonstrated previously that canavanine competes with arginine for association with the arginyl-tRNA (Allende & Allende, 1964; Schachtele & Rogers, 1965) leading to the formation of canavanyl proteins, as demonstrated directly by incorporation of [14C]-canavanine (Neurath et al. 1970). The studies of Neurath et al. (1970) have shown also that proteins in which canavanine substitutes for arginine can be completed and can function in the assembly of adenovirus type 7 particles. In the present study, the incorporation of the amino acid mixture into either infected or control cultures is reduced compared to similar cultures maintained in the absence of canavanine but to a lesser extent than the reduction of arginine incorporation. These results show that overall protein synthesis is not reduced greatly in the presence of the inhibitor. If canavanine could substitute efficiently for arginine, inhibition of incorporation of the labelled amino acid mixture commensurate with exclusion of labelled arginine only should be observed. The degree of inhibition measured indicates that canavanine is not incorporated efficiently in lieu of arginine but reduces the rate of synthesis of such proteins. This also is supported by the fact that the inhibitory effect of canavanine is greater in uninfected than in infected cells. Additionally, inhibition of the synthesis of arginine-containing proteins may account for the small inhibition of virus-specific DNA synthesis observed. It has been demonstrated previously that small amounts of arginine, not involved directly in nucleic acid synthesis, are required for the production of vaccinia virus DNA (Archard & Williamson, 1971).

Unlike canavanine, canaline does not appear to act as a structural analogue since inhibition by canaline of vaccinia virus growth is not reversed by the addition of ornithine. The fact that canavanine inhibition is reversed completely by the addition of arginine indicates that canavanine is not metabolized to canaline in vivo to any great extent. Nucleic acid synthesis in either infected or uninfected cells is not reduced greatly in the presence of a concentration of canaline which inhibits completely the formation of progeny virus. Similarly, protein synthesis, examined by the incorporation of the [14C]-amino acid mixture showed a reduction of about 50% only. However, the incorporation of radioactivity supplied as [14C]-ornithine showed an 80% reduction in both infected and uninfected cells compared with non-inhibited controls. The incorporation of radioactivity supplied as [14C]-arginine was reduced similarly. These results are remarkable in that neither ornithine nor arginine reverses the canaline inhibition of virus growth. This suggests that either the ability to utilize these amino acids or their availability within the cells is reduced. Whilst the uptake of the amino acid mixture into the intracellular pool of uninfected cells was reduced by 34%, the uptake of ornithine was reduced by 65%. However, the uptake of arginine was relatively unaffected. Thus, in the presence of canaline, both uptake and utilization of ornithine are inhibited but utilization only of arginine is reduced.

It has been shown previously that canaline reacts with the cofactor, pyridoxal phosphate (Kekomaki et al. 1969a; Rahiala et al. 1971). This mechanism appears to be an equimolar reaction resulting in the formation of a Schiff base (Williamson & Archard, 1974). The addition of increasing amounts of pyridoxal phosphate to medium containing the inhibitor reverses progressively the subsequent canaline inhibition of vaccinia virus replication. Complete reversal is achieved by the addition of an equimolar amount of pyridoxal phosphate, the kinetics of this reversal being similar to those of the original inhibition. The tissue culture medium employed in the present study contains only 0.004 mM-pyridoxal phosphate, whereas 2 mM-canaline is required to inhibit virus replication completely. This
indicates that reversal does not result exclusively from an extracellular interaction but either that pyridoxal phosphate is accumulated within the cells or that the uptake of canaline is restricted. Further, the enhanced reversal of inhibition of virus growth following the transfer of infected cultures pretreated with canaline to a medium supplemented with pyridoxal phosphate indicates that canaline inhibition results from an \textit{in vivo} interaction with pyridoxal phosphate.

The effects of canaline reported here can be correlated with the known physiological roles of pyridoxal phosphate. This cofactor may function in active transport across mammalian cell membranes (Christensen, 1955) accounting for the selective inhibition of amino acid uptake. However, Kekomaki, Rahiala & Raiha (1969b) demonstrated that canaline inhibits the activity of certain pyridoxal phosphate-dependent enzymes within intact liver cells indicating that, in spite of the inhibition of transport mechanisms, some canaline must be available within the cells. Pyridoxal phosphate is required also in decarboxylation and transamination reactions of amino acids (Wagner & Folkers, 1964). Therefore, removal of pyridoxal phosphate by intracellular reaction with canaline may result in inhibition of the metabolism of ornithine and arginine. Decarboxylation of ornithine is the initial step in the synthesis of polyamines which are required for the regulation of macromolecular synthesis (Stevens, 1970). As ornithine can be derived by hydrolysis of arginine, inhibition of ornithine metabolism by canaline may lead to feed-back inhibition of arginine metabolism. An important transamination reaction which may be inhibited by pyridoxal phosphate deficiency is the biosynthesis of proline, an amino acid not usually supplied in the tissue culture medium employed here. Attempts to reverse canaline inhibition by supplementing the medium with proline and various polyamines were unsuccessful (our unpublished results). This does not result solely from the inability of canaline-inhibited cells to accumulate these supplements as equilibration prior to the addition of canaline did not reverse the inhibition. It is concluded that there must be many pyridoxal-phosphate dependent events which are required for the production of progeny virus.

We are grateful to Dr D. J. Bauer and Dr S. Wilkinson for providing DL-canaline and to Professor K. R. Dumbell for many helpful discussions.

\textbf{REFERENCES}


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(Received 4 March 1974)