The Effect of Canaline on Some Events in Vaccinia Virus Replication

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SUMMARY

Canaline, a pyridoxal phosphate antagonist, is shown to inhibit two distinct events in the replication of vaccinia virus in HeLa cells. Initial events proceed in the presence of the inhibitor leading to the formation of DNA-containing, cytoplasmic inclusions. However, further DNA synthesis is required for the subsequent production of infectious progeny following the reversal of canaline inhibition by pyridoxal phosphate. Inhibition of a separate, maturation event is shown by the delayed addition of canaline resulting in the failure to coat virus-specific DNA synthesized previously in the absence of the inhibitor. Thus, the replication of vaccinia virus is sensitive to inhibition by canaline at an early and a late stage in the replication cycle. Reversal is accomplished alternatively by the addition of ‘non-essential’ amino acids suggesting that the effects of canaline result from inhibition of specific protein functions.

INTRODUCTION

A previous communication from this laboratory shows that L-canaline (2-amino-4-aminoxybutyric acid), an oxyamino analogue of ornithine, is a potent inhibitor of vaccinia virus replication in HeLa cells (Archard & Williamson, 1974). This inhibition is not reversed by ornithine indicating that the effect does not result from structural analogy with ornithine. However, the addition of pyridoxal phosphate to canaline-treated, infected cultures results in a partial reversal of inhibition of virus growth. An in vitro reaction between canaline and pyridoxal phosphate resulting in the formation of a single addition compound has been demonstrated by both physical and chemical techniques (Rahiala, 1973; Williamson & Archard, 1974). These results indicate that canaline inhibits by its interaction with pyridoxal phosphate which results in a deficiency of this cofactor.

This action of canaline does not result in a drastic reduction of overall macromolecular synthesis. It has been shown previously that a concentration of canaline which inhibits completely the production of infectious, progeny virus results in but a partial inhibition of virus-specific DNA synthesis and of the uptake and incorporation of amino acids into infected cultures. Under these conditions, the synthesis of RNA is relatively unaffected (Archard & Williamson, 1974). The present paper investigates the effect of canaline on certain stages in the replication of vaccinia virus and describes the inhibition of two separate events. One of these is related to the inability of virus-specific DNA synthesized in the presence of the inhibitor to function in infectious progeny following reversal of inhibition. In contrast, a second event is demonstrated by the late addition of canaline resulting in a failure to coat virus-specific DNA synthesized previously in the absence of the inhibitor.
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 in Eagle’s minimum essential medium (Eagle, 1959) containing 5% calf serum. Confluent monolayers were maintained in similar medium containing 2% calf serum.

Infection of cultures. HeLa cell monolayers in either test-tubes (1×10⁶ cells/tube) or 5 cm plastic Petri dishes (5×10⁶ cells/dish) were infected with purified vaccinia virus suspended in Eagle’s medium at a concentration of 5×10⁷ p.f.u./ml to give an input multiplicity of 5 p.f.u./cell. After adsorption for one hour the inocula were removed, the monolayers washed with Hanks’ balanced salt solution and suitable volumes of appropriate maintenance media were added. All procedures were carried out at 37 °C.

In certain experiments, HeLa cells grown as monolayer cultures were resuspended at a concentration of 5×10⁶ cells/ml in an Eagle’s medium modified for suspension cultures (Flow Laboratories, Irvine, Scotland) and infected with [¹H]-thymidine-labelled vaccinia virus at a multiplicity of 10 p.f.u./cell. After adsorption for 30 min at 37 °C, the infected cells were washed and recovered by centrifuging and resuspended at a concentration of 1×10⁶ cells/ml in the suspension medium containing also 2% calf serum, 14 mM-N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and 0.1% (w/v) sodium carboxymethylcellulose. Infected cells were maintained in suspension at 37 °C in a shaking water-bath.

The yield of infectious, progeny virus was determined, as appropriate, at various times after infection. Infected cells were disrupted with two cycles of freezing and thawing followed by ultrasonic treatment and infectivity titres were determined as described above.

Preparation and purification of virus. Confluent monolayers of HeLa cells in 40 oz bottles (1×10⁶ cells/bottle) were infected with vaccinia virus at a multiplicity of 0.1 to 1.0 p.f.u./cell. After adsorption for 2 h, the inocula were removed and maintenance medium containing 2% calf serum was added. When confluent c.p.e. was established, the medium was removed and the infected cells resuspended in Hanks balanced salt solution. The virus was extracted from the cells and purified essentially as described by Joklik (1962).

Virus radioactively labelled in the DNA was prepared by addition of 1.0 μCi/ml [³H]-thymidine (5.0 Ci/mmol; Radiochemical Centre, Amersham, Buckinghamshire) to the maintenance medium following infection. After purification as described, radioactively-labelled virus preparations were treated with 150 μg/ml DNase-I (DN-CL; Sigma London Chemical Company Ltd, Norbiton, Surrey) for 1 h at 37 °C, then washed and recovered by centrifuging. Radioactivity was measured by methods described previously (Archard & Williamson, 1971).

Acridine orange staining of infected cultures. HeLa cell monolayers were grown on 1 cm glass coverslips and infected at a multiplicity of 5 p.f.u./cell as described above. At appropriate times after infection the maintenance media were removed, the monolayers fixed with absolute alcohol for 15 min and stained with 0.5% (w/v) acridine orange in 4 mM-McIlvaine’s buffer, pH 3.0 for 20 min. After washing in the same buffer the stained preparations were differentiated by rinsing in phosphate-buffered saline and examined by ultraviolet microscopy.

Chemicals. L-canaline was prepared enzymically by methods described previously (Williamson & Archard, 1974). 5-Fluorodeoxyuridine (FUDR) was a gift from Roche Products Ltd, Welwyn Garden City, Hertfordshire. All other chemicals were obtained from Sigma London Chemical Company Ltd, Norbiton, Surrey.
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RESULTS

Effect of canaline on the uncoating of vaccinia virus

HeLa cell cultures were infected with [3H]-thymidine-labelled virus and maintained in suspension in the presence or absence of 1 mM-canaline. Samples of suspensions were taken at various times after infection and the cells disrupted with freezing and thawing followed by ultrasonic treatment. Samples of disrupted cells were treated with 150 μg/ml DNase-I at 37 °C for 1 h before the addition of 10% (w/v) trichloroacetic acid final concentration to both enzyme-treated and untreated samples. The resulting precipitates were removed by centrifuging and the supernatant fractions analysed for the presence of acid-soluble radioactivity.

There was little acid-soluble radioactivity associated with DNase-digested samples until 1 h post-infection (Fig. 1). After this time the amount of acid-soluble radioactivity increased progressively and was about 20% of the total activity supplied by 4 h post-infection (p.i.). Acid-soluble radioactivity before DNase digestion remained at a low level throughout the experiment. These results demonstrate an increasing susceptibility of input virus DNA to degradation by DNase which has been taken as a measure of the degree of uncoating of the virus genome (Joklik, 1964). This process is unaffected by the presence of canaline.

Effect of delayed reversal by pyridoxal phosphate of canaline inhibition of virus growth

Previous studies have shown that the inhibition of virus growth in canaline-treated cells is reversed partially by the subsequent addition of pyridoxal phosphate at 5 h p.i. (Archard & Williamson, 1974). In the present study, infected cultures were maintained in the presence of 1 mM-canaline for various times after infection before transfer to maintenance medium supplemented with 0.25 mM-pyridoxal phosphate. Yields of infectious virus were determined at 18 h p.i. (Table 1). These results show that canaline inhibition may be reversed by the addition of pyridoxal phosphate at any time up to 10 h p.i. Thus, prolonged exposure to the
Table 1. The effect of the delayed reversal of canaline inhibition of virus growth on the subsequent yield of infectious virus

<table>
<thead>
<tr>
<th>Time of reversal* (h p.i.)</th>
<th>Infectivity titre at 18 h p.i. (log₁₀ p.f.u./ml)</th>
<th>Virus yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.11</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>6.71</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>6.83</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>6.79</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>6.49</td>
<td>24</td>
</tr>
<tr>
<td>18</td>
<td>5.09</td>
<td>0</td>
</tr>
</tbody>
</table>

* Infected cultures were maintained in the presence of 1 mM-canaline before transfer at the times indicated to maintenance medium supplemented with 0.25 mM-pyridoxal phosphate.

inhibitor does not affect irreversibly any canaline-sensitive event either early or late in the virus replication cycle.

In further experiments the kinetics of the production of progeny virus after reversal by pyridoxal phosphate were studied. The titre of infectious virus at various times after reversal was determined in infected cultures maintained previously in the presence of canaline for 12 h p.i. Infectious progeny virus was detected initially at 2 h after reversal and increasing virus yields were measured until 12 h after reversal. Thus, the lag phase preceding the formation of infectious progeny virus is reduced, compared with the normal growth curve, on reversal of canaline inhibition (Fig. 2). This indicates that certain events after uncoating of the infective virus which are related to the subsequent production of progeny virus can proceed in the presence of the inhibitor.
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Table 2. The effect of FUdR on the reversal of canaline inhibition of virus growth

| Medium supplied until 12 h p.i. | Medium supplied from 12 to 24 h p.i. | Infectivity titre at 24 h p.i. | Virus yield (%)
<table>
<thead>
<tr>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MM*</td>
<td>RM†</td>
<td>7.48</td>
<td>100</td>
</tr>
<tr>
<td>MM + 5 × 10^-5 M-FUdR</td>
<td>RM + 5 × 10^-5 M-FUdR</td>
<td>5.13</td>
<td>0</td>
</tr>
<tr>
<td>MM</td>
<td>RM + 5 × 10^-5 M-FUdR</td>
<td>7.42</td>
<td>86</td>
</tr>
<tr>
<td>MM + 1 mM-canaline</td>
<td>RM</td>
<td>6.98</td>
<td>32</td>
</tr>
<tr>
<td>MM + 1 mM-canaline</td>
<td>RM + 5 × 10^-5 M-FUdR</td>
<td>5.08</td>
<td>0</td>
</tr>
</tbody>
</table>

* MM = minimum essential medium (Eagle, 1959) + 2 % calf serum.
† RM = MM supplemented with 0.25 mM-pyridoxal phosphate.

Table 3. The effect of canaline on the frequency and morphology of cytoplasmic, DNA-containing inclusions in vaccinia virus-infected HeLa cells at 12 h post-infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells showing inclusions (%)*</th>
<th>Morphology of inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Discrete (%)</td>
<td>Diffuse (%)</td>
</tr>
<tr>
<td>Without canaline</td>
<td>30.1</td>
<td>11.6</td>
</tr>
<tr>
<td>With 1 mM-canaline</td>
<td>11.4</td>
<td>87.5</td>
</tr>
</tbody>
</table>

* A total of 500 cells was examined in each series.

Reversal in the presence of FUdR of canaline inhibition

Similar reversal experiments were made at 12 h p.i. except that 5 × 10^-5 M-FUdR was added to some cultures. Infectivity titres were determined at 24 h p.i. (Table 2). These results show that the addition of FUdR at the time of reversal inhibits completely the subsequent formation of infectious, progeny virus. The addition of FUdR at 12 h p.i. to cultures infected in the absence of the inhibitor does not affect virus growth significantly indicating that virus DNA synthesis is normally completed at this time. Thus, some further DNA synthesis is required for the production of progeny virus after reversal of canaline inhibition.

Acridine orange staining of canaline-treated, infected cultures

In a previous study the effect of canaline on the synthesis of virus DNA was measured by the incorporation of thymidine into the cytoplasmic fraction of vaccinia virus-infected HeLa cells maintained in the presence of the inhibitor. The amount of thymidine incorporated during the period of maximum virus DNA synthesis was reduced to about 60 % of that found in non-inhibited, infected controls (Archard & Williamson, 1974). The synthesis of vaccinia virus DNA is accompanied normally by the formation of cytoplasmic, DNA-containing inclusions. In the present study, the effect of canaline on the association of virus DNA with such inclusions was visualized by acridine orange staining. In the presence of canaline, cytoplasmic inclusions were observed by 4 h p.i., confirming that some virus DNA is synthesized under these conditions. Their frequency was about 40 % of that in non-inhibited, infected cultures. Cytoplasmic inclusions were not distinguishable in every control, infected cell because the HeLa cell nucleus occupies a large proportion of the total cell volume. The inclusions in inhibited cells remained small and discrete in appearance even at 12 h p.i. whereas inclusions in control cells were large and diffuse by this time (Table 3). These results demonstrate that virus DNA synthesized in the presence of canaline becomes associated with cytoplasmic inclusions but their further development is arrested.

Reversal of inhibition by the addition of pyridoxal phosphate at 12 h p.i. resulted in a marked change in the frequency and morphology of the inclusions. By 4 h after reversal the
number of inclusions had increased to a value similar to the maximum observed in non-
inhibited cultures and by 8 h after reversal their appearance was predominantly diffuse. This
stage of development, associated with the maturation of virus particles in unstained cultures
(Loh & Riggs, 1961), was not observed in control cultures until 12 h p.i. The addition
of $5 \times 10^{-5}$ M-FUdR at the time of reversal prevented this change in the frequency and
morphology of inclusions. These results confirm that some virus-specific events take place
in the presence of canaline and that further synthesis of virus DNA occurs after reversal of
inhibition.

Reversal of canaline inhibition by 'non-essential' amino acids

The growth medium employed in the experiments described above contains only those
amino acids which cannot be synthesized by HeLa cells (Eagle, 1959). The synthesis of other,
'non-essential' amino acids is dependent on transamination reactions which require
pyridoxal phosphate as a cofactor. This suggests that, in the presence of canaline, the syn-
thesis of such amino acids may be inhibited as a result of pyridoxal phosphate deficiency.

In further experiments, infected cultures were maintained in the presence of 1 mM-
canaline for 5 h p.i. before transfer to medium lacking the inhibitor. Some cultures were then
transferred to medium supplemented with 1.0 mM-alanine, 0.2 mM-glycine and 0.1 mM-
serine and the yields of infectious, progeny virus compared at 18 h p.i. Transfer of cultures
to unsupplemented medium resulted in a $25\%$ yield of virus compared with non-inhibited
controls. Transfer of cultures to medium supplemented with amino acids as described
resulted in a complete reversal of canaline inhibition of virus growth. These results confirm
that pyridoxal phosphate deficiency resulting from interaction with canaline inhibits the
synthesis of 'non-essential' amino acids within the cells.

Effect of delayed addition of canaline on production of infectious virus

Infected cultures were transferred to medium containing 1 mM-canaline at various times
after infection. Yields of infectious virus were determined at 18 h p.i. (Table 4). Exposure of
infected cultures to medium containing the inhibitor at any time before 6 h p.i. resulted in
complete inhibition of virus yield. Addition of the inhibitor at progressively later times
resulted in a concomitant increase of virus yield. In non-inhibited, infected cultures in-
fected progeny virus was detectable from 6 h p.i. At this time, virus-specific DNA
synthesis is complete (Archard & Williamson, 1971). These results demonstrate a further
inhibitory effect of canaline upon a late event in the replication cycle.

Table 4. The effect of the delayed addition of 1 mM-canaline on virus growth

<table>
<thead>
<tr>
<th>Time of addition (h p.i.)</th>
<th>Infecitvity titre (log_{10} p.f.u./ml)</th>
<th>Virus yield (%)</th>
<th>Infecitvity titre (log_{10} p.f.u./ml)</th>
<th>Virus yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.40</td>
<td>0</td>
<td>5.07</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5.10</td>
<td>0</td>
<td>5.01</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5.38</td>
<td>0</td>
<td>4.96</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>5.94</td>
<td>3.0</td>
<td>5.48</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>6.71</td>
<td>17.8</td>
<td>6.40</td>
<td>8.7</td>
</tr>
<tr>
<td>12</td>
<td>7.23</td>
<td>59.1</td>
<td>6.78</td>
<td>20.8</td>
</tr>
<tr>
<td>18</td>
<td>7.46</td>
<td>100</td>
<td>7.46</td>
<td>100</td>
</tr>
</tbody>
</table>
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Fig. 3. The effect of canaline on the coating of progeny virus DNA. Acid-precipitable radioactivity recovered after DNase-I digestion of disrupted, infected cells, labelled with $[^{3}H]$-thymidine from 2 to 5 h p.i. and maintained subsequently in the absence (●——●) or in the presence (○——○) of 1 mM-canaline.

### Effect of canaline on the coating of progeny virus DNA

Infected HeLa cultures were maintained as described until 2 h p.i. when 1 μCi/ml $[^{3}H]$-thymidine was added to the medium. At 5 h p.i. the medium was removed, the cultures washed with unlabelled medium and maintained further in similar medium alone or containing 1 mM-canaline. Subsequently, cultures from each series were taken at various times, the disrupted cells treated with DNase-I and the radioactivity associated with acid-precipitable material measured as described above.

In non-inhibited, infected cultures the amount of acid-precipitable radioactivity resistant to enzyme degradation increased from 6 to 13 h p.i. (Fig. 3). These kinetics are similar to the kinetics of production of infectious progeny virus. In uninfected, control cultures labelled similarly with $[^{3}H]$-thymidine, the amount of acid-precipitable radioactivity after DNase digestion was not significant. This indicates that the DNA synthesized between 2 and 5 h p.i. and becoming resistant subsequently to enzyme degradation is virus-induced. The progressive production of DNase-resistant, vaccinia virus DNA has been shown previously to represent the coating of progeny virus genomes by structural proteins (Joklik & Becker, 1964). In the present study, acid-precipitable DNA synthesized in infected cultures in the presence of canaline remained susceptible to DNase digestion. These results indicate that coating of the virus genome is prevented in the presence of the inhibitor.

### DISCUSSION

The present study identifies two events in the replication of vaccinia virus that are inhibited by canaline. Initial events leading to the uncoating of infective virus are unaffected by the presence of the inhibitor. This is demonstrated by the appearance of DNase-sensitive input virus DNA shortly after infection of canaline-treated cells. Additionally, a previous study shows that, in the presence of a concentration of the inhibitor which prevents completely the formation of infectious progeny virus, virus-specific DNA synthesis was reduced
by 40% only (Archard & Williamson, 1974). Under similar conditions, the present investigation describes the formation of DNA-containing, cytoplasmic inclusions in infected cells although the frequency is reduced compared with that in untreated controls. These observations indicate also that the characteristic association of virus DNA with cytoplasmic inclusions is relatively unaffected by canaline. Thus, events in the virus replication cycle leading to DNA synthesis proceed in the presence of the inhibitor.

This hypothesis is supported by the ability of pyridoxal phosphate to reverse canaline inhibition of virus growth at any time up to 12 h p.i.; reversal at this time results in a reduction of the lag phase preceding the first appearance of infectious, progeny virus compared with the normal growth curve. Under these conditions, cytochemical studies demonstrate a rapid increase in the frequency of cytoplasmic, DNA-containing inclusions followed by the morphological development of such inclusions which is associated with the formation of mature virus particles. If, however, FUDR is added to such cultures at the time of reversal, these changes are not observed and no infectious progeny virus is detected subsequently.

This effect indicates that some further DNA synthesis is required after reversal and that virus DNA synthesized in the presence of canaline does not function normally in the production of infectious progeny. It is suggested that inhibition of this virus DNA function does not result from an intrinsic defect in the nature of the DNA molecules synthesized but from an effect on other concurrent events upon which subsequent DNA function depends. This phenomenon resembles the effect of p-fluorophenylalanine on the replication of variola virus in HeLa cells in which case the synthesis of virus DNA was not accompanied by a parallel accumulation of ‘functional’ DNA (Bedson & Cruikshank, 1968). In the present system, complete reversal of canaline inhibition is effected by supplementation with ‘non-essential’ amino acids. This suggests that the pyridoxal phosphate-dependent, transamination reactions leading to the synthesis of these amino acids are inhibited in the presence of canaline. It is proposed that this compound has an effect similar to that of p-fluorophenylalanine, arising from the inhibition of either the synthesis or function of specific protein(s).

It has been postulated previously that, in addition to the virus-specified enzymes involved in DNA synthesis, there is a requirement for the synthesis of protein related stoichiometrically rather than catalytically with virus DNA (Kates & McAuslan, 1967). The present study suggests that a further protein function is required because some virus DNA is synthesized in canaline-treated cells.

The inhibition by canaline of a further event later in the virus replication cycle is indicated by the effect of the delayed addition of the inhibitor on the subsequent production of infectious virus. Under conditions where virus-specific DNA synthesis has preceded the addition of the inhibitor, the eventual yield of virus is increased as the addition is delayed further. This indicates that a later maturation event, presumably related to protein synthesis, is affected by the inhibitor. This is confirmed by the failure to coat progeny virus DNA molecules synthesized previously in the absence of the inhibitor. This effect is distinct from the failure of virus DNA synthesized in the presence of the inhibitor to function subsequently in formation of infectious, progeny virions after reversal of inhibition. It is concluded that the replication of vaccinia virus in HeLa cells is sensitive to inhibition by canaline at both an early and a late stage in the replication cycle.
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REFERENCES


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