The Effect of Canavanine on Herpes Simplex Virus Replication

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SUMMARY

The arginine analogue canavanine strongly inhibited the replication of herpes simplex virus in BSC-1 cells. Virus absorption and entry of the virus DNA into the cell nucleus were unaffected. However, changes qualitatively similar to those produced by arginine deprivation did occur. There was little inhibition of protein synthesis but the transport of proteins from cytoplasm to nucleus was substantially reduced. Virus DNA synthesis which was extremely sensitive early in the growth cycle was little affected at late times although virus maturation was still prevented. Possible modes of action of the drug are discussed.

INTRODUCTION

It has been known for some time (Tankersley, 1964; Sharon, 1966) that the replication of herpes viruses in tissue culture is particularly dependent on the presence of arginine in the culture medium. However, the arginine-requiring function has not yet been positively identified. (Becker, Olshevsky & Levitt, 1967; Roizman, Spring & Roane, 1967; Inglis, 1968; Courtney, McCombs & Benyesh-Melnick, 1970; Courtney, McCombs & Benyesh-Melnick (1971) using herpes simplex virus and Mark & Kaplan (1971) with pseudorabies virus, obtained evidence which suggested that the reduction in virus yield was primarily due to the failure to transport virus structural proteins from the cytoplasm to their site of assembly in the nucleus. This, however, still leaves open the question as to why the migration of virus proteins fails to occur. It has been suggested (Mark & Kaplan, 1971) that migration might be dependent on the synthesis, in the nucleus, of a condensing protein which is especially sensitive to arginine deprivation.

The reduction in DNA synthesis resulting from arginine deprivation, which has been variously reported as 0% (Becker et al. 1967) to 65% (Mark & Kaplan, 1971), has generally been thought to be of secondary importance.

Also of interest in this regard is a recent paper (Raška, Prage & Schlesinger, 1972), which demonstrated that arginine deprivation of adenovirus type 2 infected cells affects not only the rate of virus (and cellular) DNA replication but also the ability of the DNA which has been synthesized to function in the virus assembly process.

The present study was undertaken to ascertain the effect of the arginine analogue canavanine (H₂NC(NH)NHOCH₂CH₂CH(NH₂)COOH) on herpes-virus replication in the hope that it might be inhibitory and help to shed light on the problems outlined above.

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METHODS

Cells. BSC-1 cells, a line of African green monkey kidney cells (originally obtained from Flow Laboratories, Irvine, Scotland) were used throughout. The cells were grown in Eagle's medium (Glasgow modification) supplemented with 10% bovine foetal serum (EF) and gave monolayers of large epithelioid cells at a density of approx. 10^6 cells/cm^2.

Virus. Virus stocks were grown by infecting rotating 80 oz bottles of confluent BSC-1 cells with herpes simplex virus (HSV) strain x at a multiplicity of 1 p.f.u./200 cells. After 3 days at 37 °C the cells were shaken off and collected by centrifuging. The cell released virus was recovered from the supernatant fluid by centrifuging at 15,000 g for 2 h. The cell pellet was disrupted by ultrasonic vibration to release cell associated virus and the cell debris removed by low-speed centrifuging. [3H] labelled virus was grown as above in medium containing 5 μCi/ml [3H]-thymidine.

Virus assay. Infectivity was determined by plating 0.2 ml of appropriate dilutions on to confluent cell monolayers in 5 cm Petri dishes absorbing for 1 h at 37 °C and then overlaying with 5 ml of medium containing 10% pooled human sera (EH). Plaques were counted after 2 days at 37 °C.

Eclipse of virus infectivity. Cells were infected, at an input multiplicity of 10 p.f.u./cell, in normal medium or medium containing canavanine. After 1 h at 37 °C the cells were washed twice with 3 ml and overlayed with 5 ml EH. At 1, 2 and 3 h.p.i. cells were washed twice with 3 ml EF, scraped off into a further 2 ml, treated in a sonifier and assayed for infectious virus.

Entry of virus into the cell nucleus. Cells were infected with [3H] labelled virus (2 x 10^7 p.f.u. = 40000 ct/min/culture) in the usual way. At 30, 60 and 90 min p.i. duplicate cultures were washed twice with 2 ml cold PBS, and the cytoplasm separated from the nuclei by two treatments with 1 ml ice-cold 0.5% Nonidet NP-40/0.01 M-KCl/0.0015 M-MgCl_2/0.01 M-tris-HCl, pH 7.5. Nuclear DNA was extracted as described below and the radioactivity of the DNA and the cytoplasmic extract determined.

Single cycle growth curves and yield experiments were performed in 5 cm Petri dishes at an input multiplicity of 10 p.f.u./cell (inoculum 0.2 ml). After absorption for 1 h at 37 °C the cells were washed twice with 3 ml of the appropriate medium and then overlayed with a further 2 ml. At appropriate times the cells were scraped into the medium, treated in a sonifier and the virus assayed as above.

In all experiments involving the removal of amino acids or the addition of canavanine, media were supplemented with sera which had been extensively dialysed.

Protein synthesis and transport. Confluent cells were infected at an input multiplicity of 10 p.f.u./cell in medium containing no amino acids. After absorption for 1 h at 37 °C cells were washed with one of the following media: EF with (a) 1/5 normal concentration of amino acids; (b) (a) minus lysine; (c) (a) minus arginine; (d) (a) plus canavanine; (e) (a) minus arginine, plus canavanine.

At 6½ h.p.i. the cells were pulsed for 15 min with [3H]-leucine (10 μCi/ml) after which they were washed twice and overlaid with media corresponding to (a) to (e) but containing the full concentration of amino acids, plus 5 μg/ml emetine (Sigma Chemical Co., London) to prevent further protein synthesis.

After a further 1½ h incubation the cells were scraped off and spun down in the cold.

Nuclei and cytoplasm were separated by gentle mixing in ice-cold 0.5% Nonidet NP-40/0.01 M-KCl/0.0015 M-MgCl_2/0.01 M-tris-HCl, pH 7.5, followed by low-speed centrifuging. The nuclear pellet was washed with 0.5% NP-40 and the wash and cytoplasm
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The rate of entry of \([\text{H}]\)-virus DNA into the cell nucleus in the presence (\(\times-\times\)) and absence (\(\bullet-\bullet\)) of canavanine.

Precipitated with 5% trichloroacetic acid. This precipitate and the nuclear pellet, which appeared to be free of cytoplasm, were dissolved in 0.5 M-NaOH. Acid-insoluble radioactivity was determined by scintillation counting utilizing the filter paper disc method of Bollum (1959).

**DNA synthesis.** Confluent cells were infected at an input multiplicity of 10 p.f.u./cell, or mock-infected, in (a) normal medium; (b) normal minus lysine; (c) normal minus arginine; (d) normal plus canavanine; and (e) normal minus arginine, plus canavanine. After 1 h the cells were washed and overlaid with the appropriate medium and at 3 h p.i. \([\text{H}]\)-thymidine (10 \(\mu\)Ci/ml) was added. At 7 h p.i. total DNA was extracted by treatment with 0.5% sodium lauryl sulphate/standard saline citrate/0.005 M-EDTA/0.1 M-tris-HCl, pH 8.0 for 10 min at 65°C, followed by digestion with pronase (500 \(\mu\)g/ml) for 18 h at 37°C.

The DNA was analysed by isopycnic sedimentation in CsCl together with \([^{32}\text{P}]\)-labelled bacteriophage T4 DNA as marker.

For comparison, the DNA of growing uninfected cells was also labelled over a 4 h period. In order to establish whether the canavanine inhibition of virus DNA synthesis could be prevented by the simultaneous addition of an equal quantity (500 \(\mu\)g/ml) of arginine, cells were infected as above in (a) normal medium; (b) normal medium plus arginine; (c) normal medium plus canavanine; and (d) normal medium plus arginine and canavanine. The DNA was labelled, extracted and analysed as before.

**RESULTS**

The effect of canavanine on the growth of herpes virus

Plaque formation in normal medium (containing 50 \(\mu\)g/ml arginine) is completely inhibited by the addition of 500 \(\mu\)g/ml canavanine. At 250 \(\mu\)g/ml extremely small plaques can just be seen under the assay conditions, while 50 \(\mu\)g/ml causes only a slight reduction in plaque size. Higher arginine concentrations moderate the effect.

After several days in the presence of 500 \(\mu\)g/ml canavanine some thinning of the cell sheet occurs.

In order to establish whether canavanine interfered with virus uptake, the number of plaques formed, when canavanine was present only during the absorption period, was...
Fig. 2. Single cycle growth curve for herpes simplex virus in BSC-1 cells and the effect on the 24 h yield, of adding canavanine at the times indicated. □, plus canavanine; ○—○, controls.

compared with the numbers produced when absorption took place in normal medium, or when virus penetration was prevented by keeping the cells at 0 °C during the absorption period. The number of plaques obtained after canavanine treatment was the same as in control cultures.

Canavanine was also shown to have no effect on the rate of ‘eclipsing’ of virus infectivity after absorption, nor does it prevent entry of the virus DNA into the cell nucleus (Fig. 1).

A virus growth curve under single cycle conditions is shown in Fig. 2 together with the effect of adding canavanine at 3, 5, 7 and 9 h p.i. Very little infectious virus seems to be produced after canavanine addition. However, the 24 h values, for canavanine added at 7 and 9 h p.i. may be falsely low because of inactivation of preformed virus during the subsequent incubation period (15 to 17 h) at 37 °C. The values obtained for canavanine addition at 3 and 5 h p.i. probably indicate that some virus growth has occurred, although these values are subject to considerable error due to variation in the efficiency with which input virus is removed.

The 24 h yield of virus in the presence of canavanine, or in the absence of various amino acids (the test medium being added at the end of the absorption period), was compared to that obtained in normal medium and the results are listed in Table 1. The removal of arginine or the addition of canavanine reduces the yield to around 0.5%, the background under these conditions. The removal of histidine or methionine has a lesser effect whereas the removal of lysine consistently increased the virus yield by 40 to 90%. Canavanine (at 500 μg/ml) also reduces the yield of adenovirus type 5 by a factor of about 10⁶ (J. F. Williams, personal communication). This would be expected for a virus which has long been known to be very sensitive to arginine deprivation (Bonifas & Schlesinger, 1959 and others).
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Table 1. The effect of amino acid deprivation, or canavanine addition, on virus yield under conditions of single cycle infection

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>24 h yield (p.f.u./culture)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$3.0 \times 10^7$</td>
<td>100</td>
</tr>
<tr>
<td>− Lysine</td>
<td>$4.2 \times 10^7$</td>
<td>140</td>
</tr>
<tr>
<td>− Methionine</td>
<td>$7.2 \times 10^6$</td>
<td>24</td>
</tr>
<tr>
<td>− Histidine</td>
<td>$2.1 \times 10^6$</td>
<td>7</td>
</tr>
<tr>
<td>− Arginine</td>
<td>$2.4 \times 10^5$</td>
<td>0.8</td>
</tr>
<tr>
<td>+ Canavanine*</td>
<td>$1.2 \times 10^5$</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Canavanine at 500 μg/ml.

Fig. 3. The effect of amino acid deprivation, or canavanine addition, on the synthesis and transport of proteins in infected cells. Cells were infected in various media and labelled with [3H]-leucine, for 15 min at 6½ h p.i. Half the cells were harvested immediately and the distribution of acid-precipitable radioactivity between cytoplasm and nucleus was determined (p). The other half were washed and returned to non-radioactive medium. After incubation for a further 105 min the distribution of radioactivity was measured again (p/c).

The effect of canavanine on protein synthesis and transport

As can be seen from Fig. 3 under the experimental conditions canavanine caused only a slight reduction in protein synthesis in infected cells, in contrast to the 50% reduction caused by arginine deprivation. However, in both these situations transport of protein from the cytoplasm to the nucleus is severely inhibited. Removal of lysine from the growth medium has no effect on protein synthesis or transport. It should be noted, however, that even when the movement of protein from cytoplasm to nucleus is most restricted, over 30% of the radioactivity incorporated ends up in the nucleus. It seems unlikely that this is due to simple contamination of the nuclei with cytoplasm, but how much of this is due to virus proteins is not known.

The effect of canavanine on DNA synthesis

Canavanine affects DNA synthesis more than does the deprivation of arginine (Fig. 4; Table 2). The removal of arginine combined with canavanine addition eliminates even the small
Table 2. The effect of amino acid deprivation or canavanine addition, on DNA synthesis in confluent herpes virus infected, or mock-infected cells, and in growing cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Infected cells [3H]-thymidine incorporated into virus DNA 3 to 7 h p.i. (%)</th>
<th>Mock-infected cells [3H]-thymidine incorporated into cell DNA 3 to 7 h p.m.i. (%)</th>
<th>Growing cells [3H]-thymidine incorporated into cell DNA over a 4 h period (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>- Lysine</td>
<td>42</td>
<td>73</td>
<td>-</td>
</tr>
<tr>
<td>- Arginine</td>
<td>6</td>
<td>73</td>
<td>-</td>
</tr>
<tr>
<td>+ Canavanine*</td>
<td>0</td>
<td>48</td>
<td>98</td>
</tr>
<tr>
<td>- Arginine + canavanine*</td>
<td>70</td>
<td>73</td>
<td>-</td>
</tr>
</tbody>
</table>

* Canavanine at 500 μg/ml.

amount of virus DNA synthesized when only canavanine is added. This can also be achieved without arginine starvation by having canavanine present for 1 h before, as well as during, virus infection.

In mock-infected stationary cultures (Table 2) arginine deprivation has much the same
**Effect of canavanine on herpes virus**

Fig. 5. The effect of exposing HSV-infected cells to canavanine 7 to 9 h p.i. (a) The production of infectious virus. ×—×, canavanine treated; ○—○, untreated. (b) The rate of DNA synthesis as measured by 2 h pulses of [\textsuperscript{3}H]-thymidine. ——, canavanine treated; ----, untreated.

...effect on cellular DNA synthesis as it has on virus DNA in infected cells, whereas canavanine is much less inhibitory. In growing cultures (i.e. which have not reached stationary phase) canavanine has virtually no effect on [\textsuperscript{3}H]-thymidine incorporation over a 4 h period (Table 2).

If cells are infected and maintained in medium containing 500 μg/ml arginine as well as 500 μg/ml canavanine, the canavanine inhibition of virus DNA synthesis is greatly reduced. However, the level of synthesis is still only about 70% of control cultures.

**Exposure to canavanine for short periods at various times during the virus growth cycle**

*Canavanine present from 7 to 9 h p.i.*

Cells, infected under single-cycle growth conditions, were exposed to canavanine (500 μg/ml) from 7 to 9 h p.i. washed carefully with normal medium and the rate of virus production compared with that of control cultures (Fig. 5). In the same experiment replicate cultures were subjected to 2 h pulses of [\textsuperscript{3}H]-thymidine throughout the growth cycle to determine the effect of exposure to canavanine on subsequent DNA synthesis (Fig. 5b).

The rate of production of active virus was reduced while canavanine was present but quickly recovered after its removal to give virus yields which were equal to, or in excess of, the control values.

The rate of DNA synthesis (which at this time is more than 95% virus DNA synthesis) was relatively unaffected by canavanine at this time. It was, if anything, slightly accelerated in the presence of canavanine although it subsequently fell below the control levels, especially in the 2 h period immediately after canavanine has been removed.
Canavanine present from 3 to 5 h p.i.

When infected cells were exposed to canavanine 3 to 5 h p.i. the rate of virus production never again reached that of untreated cultures and the final virus yield was about 70% of the control value (Fig. 6a).

The effect of this treatment on virus and cellular DNA synthesis was also studied. Treated and untreated cultures were labelled with $^3$H-thymidine and the synthesis of virus and cellular DNA followed by fractionating the DNA on CsCl gradients and measuring the radioactivity accumulated by each species (Fig. 6b).

Cellular DNA synthesis appears to be unaffected by canavanine treatment but subsequent virus DNA synthesis only reached about 70% of that in control cultures.

Canavanine present from 1 to 2 h p.i.

Infected cultures were exposed to canavanine 1 to 2 h p.i., washed thoroughly and labelled with $^3$H-thymidine in normal medium 2 to 3 h and 3 to 4 h p.i. The effect of adding canavanine along with $^3$H-thymidine at 2 to 3 h p.i. was also checked. The labelled DNA was analysed on CsCl gradients and compared with that obtained from control cultures labelled at the same times (Fig. 7). Addition of canavanine at the time of labelling (i.e. 2 to 3 h p.i.) causes some reduction in DNA synthesis (about 25%). However, in the cultures which were exposed to canavanine 1 to 2 h p.i. very little virus DNA is made 2 to 3 h p.i. and even at 3 to 4 h p.i. virus DNA synthesis is only about 40% of the control value. Cellular DNA, on the other hand, is the same as in the controls.
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Fig. 7. The effect of canavanine 1 to 2 h p.i. on the incorporation of $[^3H]$-thymidine into virus and cellular DNA at 2 to 3 h and 3 to 4 h p.i. CsCl gradient profiles. Density increases from right to left and virus DNA is the denser band. Gradients aligned by means of marker. (a), (b) DNA from cells infected and maintained in normal medium and labelled 2 to 3 h and 3 to 4 h p.i., respectively. (c), (d) DNA from cells exposed to canavanine 1 to 2 h p.i. and labelled 2 to 3 h and 3 to 4 h p.i., respectively. (e) DNA from cells to which both canavanine and $[^3H]$-thymidine were added 2 to 3 h p.i.

DISCUSSION

Canavanine has been shown to be a potent inhibitor of herpes-virus replication, capable, under the right circumstances, of reducing the yield of active virus to virtually zero. Absorption of the virus and penetration of the virus DNA into the cell nucleus appear to be unaltered but changes in macromolecular synthesis and transport, qualitatively similar to those known to occur during arginine deprivation, are produced. These changes differ, however, in the extent to which certain functions are affected. For example, although both treatments reduce the flow of newly synthesized proteins from the cytoplasm to the nucleus, the absence of exogenous arginine causes a substantial reduction in protein synthesis, whereas canavanine has relatively little effect (Fig. 3). This is compatible with the finding in Escherichia coli that canavanine can be readily incorporated into proteins in place of arginine (Schactele, Anderson & Rogers, 1968; Schactele & Rogers, 1968). In the case of
virus DNA synthesis the opposite is true. Arginine deprivation in this system reduces DNA synthesis by about 60% but canavanine almost eliminates it (Fig. 4; Table 2). In fact, if cells are incubated with canavanine for 1 h prior to, as well as during, infection no virus DNA synthesis can subsequently be detected. It is also of interest to note that arginine deprivation reduces DNA synthesis in mock-infected cells to almost the same extent as it does virus DNA synthesis in infected cells, while canavanine reduces cellular DNA synthesis by only 25 to 30% in stationary cultures and has virtually no effect on growing cultures over a comparable (4 h) period (Table 2).

The results of adding canavanine to infected cells for short periods at various times in the virus growth cycle highlight the complex nature of the canavanine effect. If it is added for 2 h late in infection (see Fig. 5a, b), when a great many progeny DNA molecules have already been produced and the rate of virus DNA synthesis is slowing down, canavanine has little effect on DNA labelling either at that time, or subsequently, although the slight reduction in the synthetic rate immediately after its removal may be significant. On the other hand, the production of active virus is inhibited by over 50% during the time that canavanine is present but recovers quickly, when the cells are returned to normal medium, giving titres initially above, but ultimately similar to control values. This suggests some synchronization of virus maturation, perhaps due to an accumulation of virus components during the canavanine block.

If, however, canavanine is added early in infection (3 to 5 h p.i., see Fig. 6a, b) both DNA synthesis and virus production are permanently reduced. Furthermore, if the DNA synthesized at 2 to 3 h and 3 to 4 h p.i. in cells which have been exposed to canavanine from 1 to 2 h p.i. is analysed on CsCl (Fig. 7), the profile obtained for 3 to 4 h p.i. resembles that normally found for 2 to 3 h p.i. in control cells. In addition, much less virus DNA is synthesized 2 to 3 h p.i. in cells which had previously been treated with canavanine (Fig. 7c) than in those to which canavanine was added at that time (Fig. 7e).

These results seem to indicate that canavanine can prevent virus replication in at least two ways. At early times DNA synthesis is inhibited in such a way as to suggest interference with a factor necessary for the continuation of synthesis (perhaps, initiation) rather than by direct inhibition of the synthetic process. At late times this effect is of little importance and the inhibitory effect of canavanine is presumably due to non-assembly of the virus, in which the reduced flow of virus structural proteins from the cytoplasm is probably an important factor.

Now, arginine is a constituent of virtually all proteins and participates in many metabolic pathways. It is therefore hardly surprising that in its absence several functions are affected, or that these functions should be affected to different degrees by the presence of an arginine analogue. Especially one which is almost certainly incorporated into proteins.

Thus the effects of arginine deprivation and canavanine inhibition may be due to the failure to synthesize, or the synthesis of defective proteins essential for, say, the initiation of DNA synthesis, or the transport of structural proteins from cytoplasm to nucleus. However, a possible alternative explanation for these effects could lie in the inability of the arginine-starved or canavanine treated, infected cells to maintain polyamine synthesis. The polyamines, spermine and spermidine, are known to interact with nucleic acids and to play an important role in the stabilization of biological membranes (Tabor & Tabor, 1964; Gfeller & Russell, 1970; Russell, 1970). Furthermore, Gibson & Roizman (1971) have demonstrated substantial quantities of both these polyamines in purified herpes virus–spermine within the nucleocapsid, probably in close association with the DNA, and spermidine in the virus envelope.
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In *Escherichia coli* two synthetic routes to putrescine (and hence the polyamines) have been demonstrated (Morris & Pardee, 1966), (a) from ornithine by simple decarboxylation:

\[
\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{(NH}_2\text{)} \cdot \text{COOH} \rightarrow \text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{NH}_2
\]

ornithine

putrescine

and (b) from arginine by decarboxylation to agmatine followed by the elimination of urea:

\[
\text{H}_2\text{N} \cdot \text{C(NH)} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{(NH}_2\text{)} \cdot \text{COOH} \rightarrow \text{H}_2\text{N} \cdot \text{C(NH)} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{NH}_2
\]

arginine

agmatine

\[
\text{H}_2\text{N} \cdot \text{CO} \cdot \text{NH}_2 + \text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{NH}_2
\]

The energetically less favourable route (b) assumes importance under circumstances where route (a) is blocked. Gibson & Roizman (1971) have shown that after herpes infection just such a situation does seem to occur. They found that in infected cells labelled ornithine could no longer be incorporated into polyamines. They further concluded that although pre-existing polyamines were used, polyamine synthesis continued after virus infection.

However, more recent data (B. Roizman, personal communication) show that almost all the polyamines in the herpes-virus particle are drawn from pre-existing cellular pools, making it seem unlikely that this is the source of the arginine requirement of herpes viruses. Whether this is also true for the effects of canavanine is less clear. There is no obvious reason why canavanine should not be converted at least to the agmatine analogue, and probably to the putrescine derivative also, i.e. \(\text{H}_2\text{N} \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}_2\). However, it is perhaps more plausible to suggest that this compound might not be converted to the spermine analogue, or alternatively that this analogue then could not function. This remains to be determined.

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REFERENCES


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