

## NOTES

### Irreversible Inhibition of Herpes Simplex Virus Replication in BSC-1 Cells by Zinc Ions

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Zinc sulfate added to the medium of herpes simplex virus-infected BSC-1 cells, at a concentration of 0.1 mM, inhibited the synthesis of infectious virus progeny by 95 to 96%. A concentration of 0.2 mM zinc sulfate inhibited herpes simplex virus synthesis by 99.8% as determined by centrifugation in sucrose gradients and by plaque assay on BSC-1 monolayers. The inhibition of herpes simplex virus replication in BSC-1 cells was found to be irreversible.

Zinc chloride has been shown to inhibit the growth of rhinoviruses (5) and to inhibit the post-translational cleavage of rhinovirus and picornavirus polypeptides (1). It has also been reported (3) that zinc ions, added at 3.5 h postinfection (p.i.) at concentrations of  $10^{-4}$  to  $2 \times 10^{-4}$  M, inhibit giant cell formation due to herpes simplex virus type 1 (HSV-1) infection of rabbit kidney cells. The present study deals with the effect of zinc ions on the replication of HSV in BSC-1 cells and demonstrates that zinc ions irreversibly inhibit virus replication.

Monolayers of BSC-1 cells were infected with 1.1 ml of the HF strain of HSV-1 ( $10^7$  plaque-forming units [PFU]/ml) (6). After adsorption, at 3 h p.i. (4), or at different time intervals thereafter, excess virus and media were removed, and 0.5 ml of  $ZnSO_4$  in varying concentrations (0.1, 0.2, or 0.3 mM) was added along with 4.5 ml of Dulbecco modified Eagle medium containing 0.5  $\mu$ Ci of tritiated thymidine/ml (specific activity 12.3 Ci/mM; Nuclear Research Center, Negev) and incubated at 37 C. At the end of a one-step growth cycle (18 or 21 h after infection), the cells were harvested into TBS buffer [0.2 M tris(hydroxymethyl)amino-methane-hydrochloride, pH 8.3, 0.85% (wt/vol) NaCl], counted, and sonicated, and a portion of infectious virus was removed for plaque assay on BSC-1 monolayers (6). The virus preparations were also centrifuged on sucrose gradients (12 to 52% [wt/wt] prepared in TBS buffer) at 45,000 rpm in the Beckman SW50.1 rotor for 15 min at 4 C. Fractions were collected and precipitated with 10% trichloroacetic acid and counted in a Packard Tri-Carb scintillation counter.

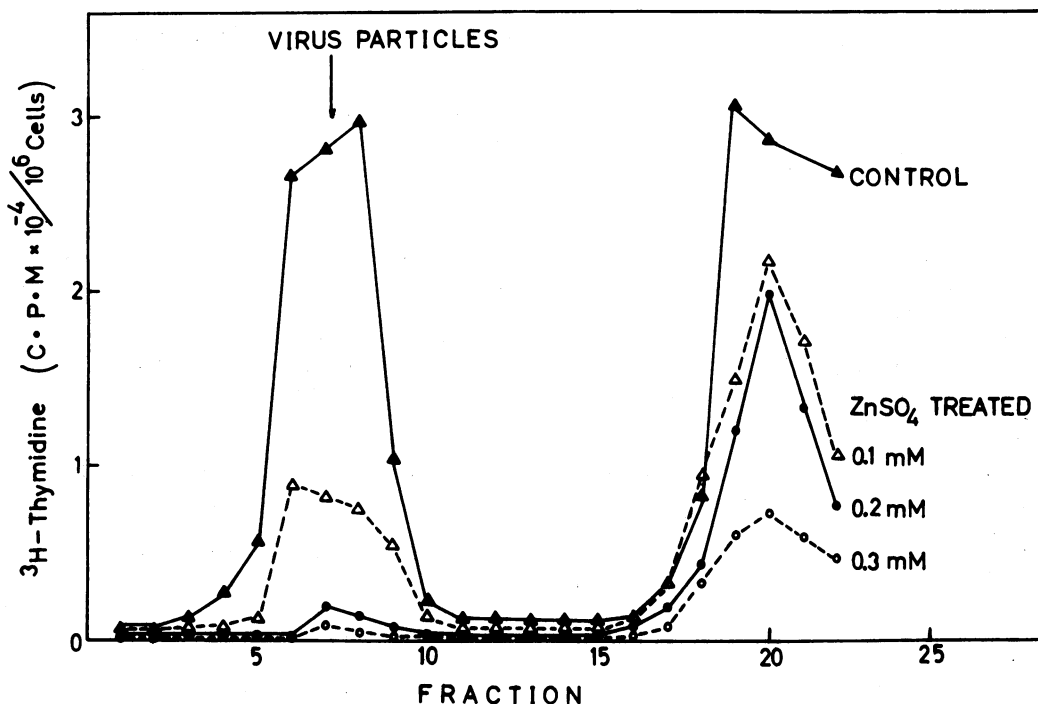
#### Effect of zinc sulfate on the formation of

**HSV-1 particles.** HSV-1-infected BSC-1 cells, without or with 0.1, 0.2, or 0.3 mM zinc sulfate as well as [ $^3H$ ]thymidine added at 3 h p.i., were harvested at 21 h after infection. The cells were counted, sonicated, and titrated for virus infectivity. Table 1 shows that zinc sulfate at a concentration of 0.1 mM inhibited the synthesis of infectious virus progeny in HSV-infected BSC-1 cells by 95 to 96%. At a concentration of 0.2 mM, the synthesis of infectious HSV progeny was inhibited by 99.8%. The infectious virus ( $5.92 \times 10^5$  PFU/ml = 0.22 PFU/cell) obtained after treatment with 0.2 mM  $ZnSO_4$  was the original virus inoculum that had not undergone replication. Thus, 0.2 mM  $ZnSO_4$  completely inhibited the production of new virus progeny.

These results were confirmed by sucrose gradient analyses of the radioactive virus particles in the zinc-treated and untreated HSV-1-infected cells (Fig. 1). There was a marked reduction in the amount of labeled virus particles isolated from HSV-infected BSC-1 cells treated with 0.1 mM zinc sulfate. However, at this concentration, there was only a partial reduction in the amount of radioactive deoxyribonucleic acid (DNA), mostly uncoated viral DNA, which appeared at the top of the sucrose gradient. At a concentration of 0.2 mM zinc sulfate, no labeled virus particles were isolated from infected BSC-1 cells, whereas the amount of radioactive DNA at the top of the gradient resembled that obtained from infected BSC-1 cells treated with 0.1 mM zinc. Treatment of HSV-infected BSC-1 cells with 0.3 mM zinc sulfate also resulted in complete inhibition of virus particle formation along with a drastic

TABLE 1. Effect of  $Zn^{2+}$  on yield of infectious virus progeny

Expt. no.	0 <sup>a</sup>		0.1 mM <sup>a</sup>		0.2 mM <sup>a</sup>	
	PFU/ml	PFU/cell	PFU/ml	PFU/cell	PFU/ml	PFU/cell
1	$4.25 \times 10^8$	155	$0.835 \times 10^7$	2.8	$7 \times 10^6$	0.15
2	$10^8$	51	$2.50 \times 10^7$	13.0	$4.67 \times 10^6$	0.23
3	$5.18 \times 10^8$	215	$1.86 \times 10^7$	7.6	$3.67 \times 10^6$	0.24
4	$8.65 \times 10^8$	240			$8.35 \times 10^6$	0.24
Avg	$4.77 \times 10^8$	165	$1.73 \times 10^7$	7.8	$5.92 \times 10^6$	0.22
% of control	100	100	3.7	4.8	0.2	0.2
% Inhibition	0	0	96.3	95.2	99.8	99.8

<sup>a</sup> Zinc concentration.FIG. 1. Sucrose gradient analysis of HSV-1 labeled with [<sup>3</sup>H]thymidine in BSC-1 cells, in the absence and presence of 0.1 to 0.3 mM zinc sulfate.

reduction in the amount of radioactive DNA which banded at the top of the sucrose gradient. This was due to cell death caused by 0.3 mM zinc sulfate, in contrast to 0.2 mM zinc sulfate which produced no decrease in the uptake of tritiated thymidine into cellular DNA or morphological changes in uninfected BSC-1 cells.

**Effect of ZnSO<sub>4</sub> concentration on the uptake of [<sup>3</sup>H]thymidine into HSV-1 particles.** Different concentrations of ZnSO<sub>4</sub> (0.1 to 0.5 mM) were added at 3 h p.i. to HSV-1-infected BSC-1 cells. The cells were incubated for 18 h at 37 C in the presence of [<sup>3</sup>H]thymidine, harvested, sonicated, and centrifuged in 12 to 52% (wt/wt) sucrose gradients. The total radioactive

counts appearing in the virus particles (see Fig. 1) were calculated for each concentration of zinc sulfate and plotted as percentage of the untreated control. The results of five experiments, summarized in Fig. 2, show that 0.2 mM zinc sulfate completely prevents the replication of HSV-1. Subsequent experiments were done using 0.2 mM ZnSO<sub>4</sub>.

**Effect of ZnSO<sub>4</sub> added at different stages of the virus growth cycle.** HSV-1-infected cells labeled with [<sup>3</sup>H]thymidine at 3 h p.i. received 0.2 mM zinc sulfate at 3, 7, and 11 h after infection. The infected zinc-treated cells were harvested at 18 h p.i. and sonicated, and then the virus particles were isolated by centrifuga-

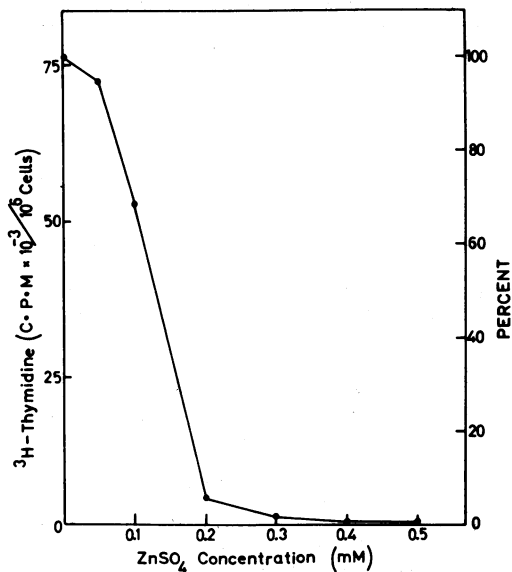


FIG. 2. Effect of zinc sulfate concentration on the uptake of [<sup>3</sup>H]thymidine into HSV-1, calculated from the total radioactive counts appearing in the virus particles after centrifugation in sucrose gradients.

tion in sucrose gradients (Fig. 3). Addition of zinc sulfate at 3 h p.i. completely inhibited the formation of herpesvirus particles, whereas addition at 7 h p.i. only had a partial inhibitory effect. Addition of zinc sulfate at later time periods had no effect on the formation of virus particles. These results suggest that zinc ions interfere with a process which occurs early in the virus replication cycle.

**Irreversible inhibition with zinc sulfate.** To determine whether the inhibitory effect of zinc sulfate is reversible, HSV-infected cultures, treated with 0.2 mM zinc sulfate for 18 h, were washed to remove the zinc ions and subsequently reincubated in the absence of the inhibitor. Virus infectivity titers were determined after 3, 6, and 24 h. Removal of zinc sulfate did not result in renewed virus replication (Table 2). This finding suggests that zinc ions irreversibly inhibit the replication of HSV in BSC-1 cells.

The results of the present study reveal that 0.2 mM zinc sulfate, added to HSV-infected cells at the end of the virus adsorption period, effectively inhibits virus replication. This result is in agreement with Falke and Kahl (3) who

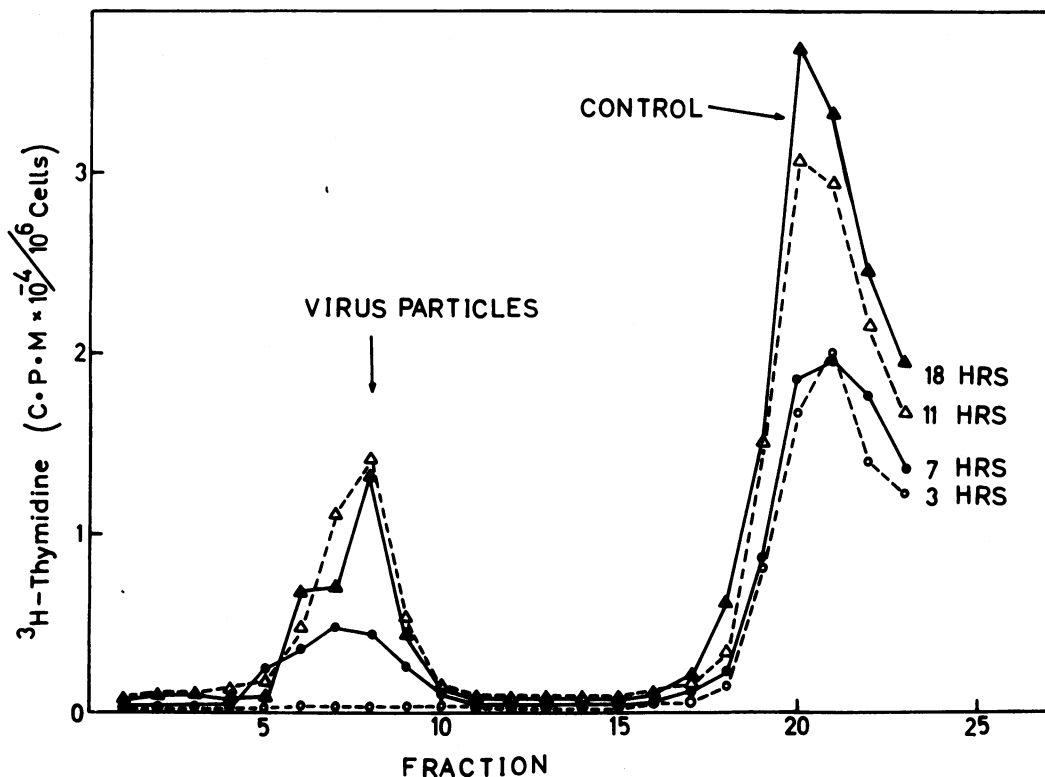


FIG. 3. Sucrose gradient analysis of [<sup>3</sup>H]thymidine-labeled HSV-1 grown in the absence (18 h) or the presence of 0.2 mM zinc sulfate added at 3, 7, and 11 h p.i.

TABLE 2. Irreversible inhibition of herpesvirus synthesis due to treatment with 0.2 mM ZnSO<sub>4</sub>

Expt.	HSV titer at 18 h p.i. (PFU/ml)		HSV titer after removal of 0.2 mM ZnSO <sub>4</sub> (PFU/ml)		
	Untreated control	Treated with 0.2 mM ZnSO <sub>4</sub>	+ 3 h	+ 6 h	+ 24 h
Expt. I	20 × 10 <sup>7</sup>	3.0 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>	2.0 × 10 <sup>6</sup>	4.0 × 10 <sup>5</sup>
Expt. II	3.0 × 10 <sup>7</sup>	2.0 × 10 <sup>6</sup>	7.0 × 10 <sup>5</sup>	9.0 × 10 <sup>4</sup>	1.5 × 10 <sup>5</sup>

also found that zinc ions inhibited giant cell formation by herpesvirus when added at an early stage after infection. The mechanism by which zinc ions inhibit herpesvirus replication is unknown, but is currently under investigation. The therapeutic use of zinc sulfate as an antiviral agent should also be considered. It is of interest that zinc sulfate (0.5% wt/vol) has already been reported to be an effective drug in the treatment of herpetic keratitis in man (2).

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#### ADDENDUM IN PROOF

It was found that zinc ions selectively inhibit the activity of herpes simplex virus-specified DNA poly-

merase (J. Shloma, Y. Asher, Y. J. Gordan, U. Olshevsky, and Y. Becker. *Virology* **66**: 330, 1975).

#### LITERATURE CITED

1. Butterworth, B. E., and B. D. Korant. 1974. Characterization of the large picornaviral polypeptides produced in the presence of zinc ions. *J. Virol.* **14**:282-291.
2. de Roeth, A. 1963. Treatment of herpetic keratitis. *Am. J. Ophthalmol.* **56**:729-731.
3. Falke, B., and G. F. Kahl. 1967. Ca<sup>++</sup>, Histidine und Zn als Faktoren bei der Riesenzellbildung durch das herpesvirus hominus. *Z. Med. Mikrobiol. Immunol.* **153**:175-189.
4. Hochberg, E., and Y. Becker. 1968. Adsorption, penetration and uncoating herpes simplex virus. *J. Gen. Virol.* **2**:231-241.
5. Korant, B. D., J. C. Kaner, and B. E. Butterworth. 1974. Zinc ions inhibit replication of rhinoviruses. *Nature (London)* **248**:588-590.
6. Rosenkranz, H. S., and Y. Becker. 1973. Reversible inhibition of herpes simplex virus replication by hydroxyurea. *Antimicrob. Agents Chemother.* **3**:325-331.