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Specific Inactivation of Herpes Simplex Virus by Silver Nitrate at Low Concentrations and Biological Activities of the Inactivated Virus

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The infectivities of herpes simplex virus types 1 and 2 were inactivated by silver nitrate at concentrations of 30 μM or less, which did not affect at all the infectivities of hemagglutinating virus of Japan, vesicular stomatitis virus, poliovirus, vaccinia virus, and adenovirus. The inactivated virus retained the capability of adsorbing to the cell, with an adsorption kinetics quite similar to that of intact virus, and of inducing the concanavalin A agglutinability in the infected cells, whereas it lost completely the capability of producing viral antigens and other cytopathic changes.

Salts of heavy metal ions such as silver, mercury, or copper are bactericidal at very low concentrations, under which conditions they act by combining with sulfhydryl groups. Such sulfhydryl reagents have also been shown to inactivate the infectivity of many viruses. This reaction has been used by many previous investigators to obtain information on the superficial structure of viruses and the early stages of virus multiplication (1, 2, 4, 7).

It has been reported that the infectivity of herpes simplex virus (HSV) is almost completely diminished when the virus is treated with the compounds at concentrations as low as 10^{-4} to 10^{-5} M (7, 10). Thus, in vitro and in vivo effects of compounds such as silver nitrate (5) or silver sulfadiazine (3) on HSV infection have recently been investigated to evaluate them as local chemotherapeutic agents.

The present communication is concerned with further studies on the in vitro effect of silver nitrate on the infectivity of HSV. The inactivation kinetics of HSV by the compound and the specific inactivation of HSV at low concentrations of the compound, which did not affect the infectivity of other viruses, are described. It is also shown that the inactivated virus retained the capability to interact with the cellular membranes, although they did not undergo a growth cycle.

MATERIALS AND METHODS

Viruses. Two types of HSV (type 1, Miyama strain; type 2, UW 268 strain), vaccinia virus (IHD strain), adenovirus (type 5), hemagglutinating virus of Japan (HVJ; Fushimi strain), vesicular stomatitis virus (Indiana strain), and poliovirus (type 1, Mahoney strain) were used. Both types of HSV were propagated in African green monkey kidney cells and centrifuged at 1,400 × *g* for 15 min. Titers of the stock HSV-1 and -2 were 2 × 10^7 and 10^7 plaque-forming units/ml, respectively. Partially purified HSV-1 was prepared by centrifugation of the crude virus onto a cushion of 60% (wt/wt) sucrose in tris(hydroxymethyl)aminomethane(Tris) - hydrochloride buffer through a layer of 5% sucrose in Tris-hydrochloride at 64,000 × *g* for 90 min at 4 C. The viral preparation at the interface between 5 and 60% sucrose layers was rinsed at once and resuspended in the buffer. Adenovirus was propagated in HEp-2 cells, and the titer was $10^{6.8}$ 50% tissue culture infectious doses ($TCID_{50}$)/ml. Vesicular stomatitis virus and vaccinia virus were propagated in rabbit kidney cells (RK13), and their titers were 10^7 and $10^{7.5}$ $TCID_{50}$/ml, respectively. Poliovirus was prepared in human embryonic lung cells, and the titer was $10^{7.2}$ $TCID_{50}$/ml. HVJ was prepared in the chorioallantoic cavity of 11-day-old embryonated chicken eggs, and the titer was $10^{9.6}$ 50% egg infectious doses/ml. All the viruses were stored at −80 C until used.

Tissue culture cells. Monolayer cultures of chicken embryo, RK13, African green monkey kidney, HEp-2, and human embryonic lung cells were grown in Eagle minimum essential medium (MEM) supplemented with 10% heat-inactivated bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The growth medium for monolayer cultures of 3T3 mouse cells was MEM containing 10% calf serum, penicillin, and streptomycin.

Infectivity assay. All the viruses other than HVJ were assayed by counting plaque-forming units or $TCID_{50}$ per milliliter, using African green monkey kidney or human embryonic lung cells. HVJ was assayed by hemagglutinin titration.

Reagents and radioisotopes. The reagents used were obtained from the following sources. Silver nitrate ($AgNO_3$), silver iodide (AgI), and reduced glutathione (R-glutathione) were from Wako Chemical Industries, Tokyo. Potassium iodide (KI) was

from Junsei Chemicals, Tokyo. $AgNO_3$ was dissolved in distilled water at 100 μg/ml, and lower concentrations were prepared by diluting the solution with MEM. KI and AgI were diluted with MEM to the desired concentrations. R-glutathione was dissolved in Tris buffer, and the pH was adjusted to 7.4. [^{3}H]thymidine (specific activity, 10 Ci/mmol) was obtained from New England Nuclear Corp.

Inactivation of viruses by silver nitrate. In most experiments, inactivation of viruses with $AgNO_3$ was performed by adding 1.0 ml of 2×10^5 plaque-forming units or 2×10^5 50% egg infectious doses (HVJ) of viruses per ml to 1.0 ml of a 60 μM $AgNO_3$ solution and agitating the mixture at 37 C for varying times. For controls, 1.0 ml of phosphate-buffered saline (PBS), pH 7.2, was added to the virus suspension before agitation at 37 C. At intervals, serial dilutions into MEM were made, and their virus titers were assayed by counting plaque-forming units or $TCID_{50}$.

Preparation of labeled HSV. Monolayer cultures of 2×10^7 African green monkey kidney cells were infected with HSV-1 at an input multiplicity of 6. After a 2.5-h adsorption period at 37 C, cell sheets were washed three times with PBS to remove the unadsorbed virus and flooded with 30 ml of fresh medium. After 4 h of incubation, [^{3}H]thymidine in MEM was then added to monolayer cultures to a concentration of 4 μCi/ml. The cultures were incubated at 37 C until the cytopathic effect became evident (about 24 h). After centrifugation of the culture medium at 4,000 $\times$ g at 4 C for 15 min to remove cell debris, the supernatant fluid was harvested. The virus was purified by the cushion ultracentrifugation method in a sucrose gradient (20 to 70%), and 1.0-ml fractions of virus were collected from the top to the bottom. The fractions were assayed for infectivity and radioactivity.

ConA agglutination tests. The methods used were essentially those of Inbar and Sacks (6). For agglutination assays, chicken embryo cells were infected with HSV inactivated by 30 μM $AgNO_3$ or with noninactivated HSV. After an adsorption period of 90 min at 37 C, the cell sheets were washed three times with culture medium to remove the unadsorbed virus and flooded with 6 ml of fresh medium. Six hours after infection, the cell sheets were washed with Ca- and Mg-free PBS, prewarmed to 37 C, and then brought into single cell suspensions with 0.02% disodium versenate solution (ethylenediaminetetraacetate). The cells were then washed with saline and suspended with PBS at a concentration of 10^6 cells/ml, and 0.3-ml samples of cell suspension were transferred to 15-mm plastic trays to which was added either 0.3 ml of concanavalin A (ConA) or 0.3 ml of saline. The mixture was rocked gently several times by hand at room temperature and then examined for cell agglutination under an inverted microscope after 45 min.

Tests for hemadsorption of sensitized erythrocytes. Sensitization of sheep erythrocytes with rabbit anti-sheep erythrocyte serum and hemadsorption on cover slip cultures were carried out as described by Watkins (12). Briefly, a 1% suspension of erythrocytes was mixed with an equal volume of anti-erythrocyte serum (1 hemagglutination unit), and the mixture was incubated at 37 C for 30 min. The erythrocytes were washed three times with glucose veronal buffer and used as a 0.5% suspension for hemadsorption tests. After removing the culture medium, the cover slip cultures, in petri dishes, were gently rinsed three times with glucose veronal buffer. Two milliliters of a suspension of sensitized erythrocytes was then added. After incubation for 2 h at room temperature, the cover slip cultures were gently washed two times with glucose veronal buffer to remove nonadsorbed erythrocytes, and hemadsorption was then examined microscopically.

Fluorescent staining. The antibodies to HSV used were rabbit antisera from rabbits immunized with partially purified virus preparations of HSV-1, Miyama strain. The fluorescein isothiocyanate-conjugated sera used were sheep anti-rabbit gamma globulin (Behringwerke, Uppsala, Sweden).

Production of HSV antigens in the cells was detected by the indirect method. Smear preparations of infected cells fixed with acetone were first covered with an appropriate dilution of antiserum and incubated for 60 min in a moist chamber at 37 C. At this time the preparations were removed, washed with PBS, covered with fluorescein isothiocyanate-conjugated serum, and then reincubated at 37 C for 60 min.

To detect HSV-induced surface antigens, infected monolayer cultures were washed three times with glucose veronal buffer and incubated with the antiserum for 30 min at 4 C. The antiserum was removed by washing, and the cells were stained with a 1:8 dilution of fluorescein isothiocyanate-conjugated serum.

RESULTS

Kinetics of inactivation of HSV by $AgNO_3$. The first experiment was performed to investigate the rate of inactivation of HSV-1 during exposure to $AgNO_3$. A total of 10^5 $TCID_{50}$ of HSV per ml was treated with 30 μM $AgNO_3$ for up to 120 min at 37 C in a water bath and tested for the titers of residual virus. HSV was rapidly inactivated with time (Fig. 1). After 60 min, the virus was almost completely inactivated.

To investigate whether the inactivation of HSV by $AgNO_3$ was dependent upon the amounts of test virus, we exposed different infectivities of HSV to 30 μM $AgNO_3$ for 60 min at 37 C and tested for the titers of residual virus (Fig. 2). When the unpurified virus preparation was used as a test virus, less than 10^6 $TCID_{50}$ of HSV per ml was completely inactivated upon exposure to the compound, whereas 10^7 $TCID_{50}$ of the virus per ml lost no virus titers. Although the inactivation by $AgNO_3$ of unpurified HSV thus appeared to be dependent on the virus titers, partially purified virus at 10^7 $TCID_{50}$/ml was completely inactivated by the same concentration of the compound (Fig. 2). The protein concentrations of unpurified and purified prep-

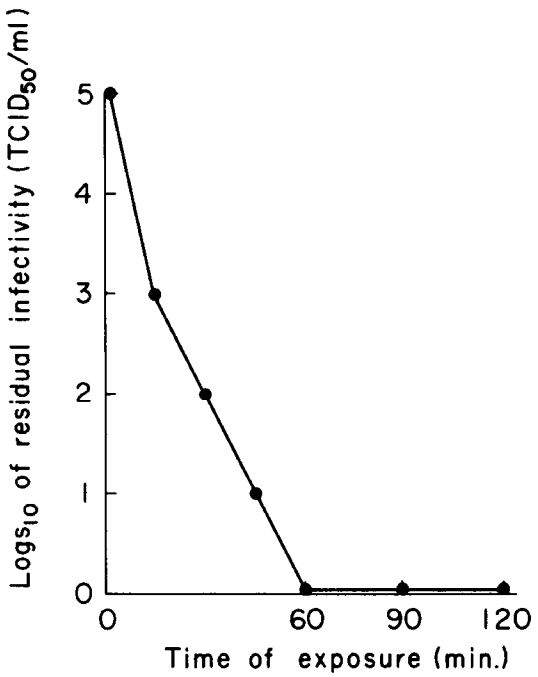

FIG. 1. *Effect of exposure time on inactivation of HSV-1 by silver nitrate.*

arations, both of which contained 10^7 $TCID_{50}$/ml, were 2.7 and 0.2 mg/ml, respectively, as determined by the method of Lowry et al. (8). These results, therefore, suggest that lack of inactivation by $AgNO_3$ of the unpurified virus at 10^7 $TCID_{50}$/ml may be due to the presence of the proteins at concentrations that would inhibit the effect of $AgNO_3$ on the virus. When the purified virus at 10^7 $TCID_{50}$/ml, after addition of bovine serum albumin at a final concentration of 0.8%, was tested for inactivation by $AgNO_3$, no inactivation was observed (Fig. 2). Thus, it was concluded that $AgNO_3$ at 30 μM can inactivate HSV independently of the virus titers, at least up to 10^7 $TCID_{50}$/ml.

Effect of $AgNO_3$ on different viruses. After studying the inactivation kinetics of HSV-1 by $AgNO_3$, the compound was tested for its effect on different viruses, including HSV-2. A total of 10^5 $TCID_{50}$ or 10^5 50% egg infectious doses (HVJ) of viruses per ml were treated with $AgNO_3$ at different concentrations (less than 30 μM) for 60 min at 37 C and then tested for the titers of residual virus. There was a marked contrast between the results for HSV (types 1 and 2) and those for the other viruses (Fig. 3). Both HSV-1 and -2 underwent at least a 10^4-fold loss of titer when treated with 15 or 30 μM $AgNO_3$. By contrast, the other viruses, irrespective of whether they were enveloped or non-enveloped and deoxyribonucleic acid or ribonucleic acid viruses, were not reduced in titer by treatment with the same concentrations of this reagent.

The protein concentrations contained in the test preparations were calculated for each virus by the estimation of the concentrations of undiluted samples and were found to be as low as 27, 28, 0.2, 70, 97, 83, and 140 μg/ml for HSV-1 and -2, HVJ, vesicular stomatitis virus, vaccinia virus, and polio- and adenoviruses, respectively. These results indicate that non-inactivation by $AgNO_3$ of the viruses other than HSV may be due to the insensitivity to the compound of the virus itself, in contrast to the case of an undiluted preparation of HSV-1 at 10^7 $TCID_{50}$/ml, in which non-inactivation of HSV is possibly due to the diminution of the $AgNO_3$ effect by the nonviral proteins. Thus, it is concluded that $AgNO_3$ at a concentration of 30 μM was specific to herpesviruses and could not inactivate other test viruses.

Inhibition of $AgNO_3$ effect by different reagents. An experiment was performed to test whether the inactivation effect of $AgNO_3$ is due to the binding of ionized Ag to the sulfhydryl groups in the virus (Table 1). The results clearly show that no inactivation of HSV was obtained with $AgNO_3$ after it was reacted with a sulfhydryl compound, R-glutathione, or with KI, the reaction of which with $AgNO_3$ results in formation of AgI. In addition, AgI per se, which is an extremely low-ionizing compound, could not inactivate HSV at all. These results indicate that this specific inactivation of HSV by

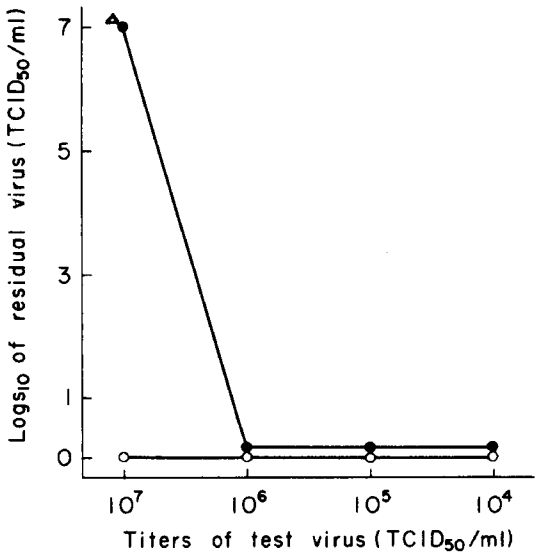

FIG. 2. *Loss of infectivity of HSV of various infectivities by silver nitrate. Symbols: ●, crude virus; ○, purified virus; △, purified virus after addition of bovine serum albumin.*

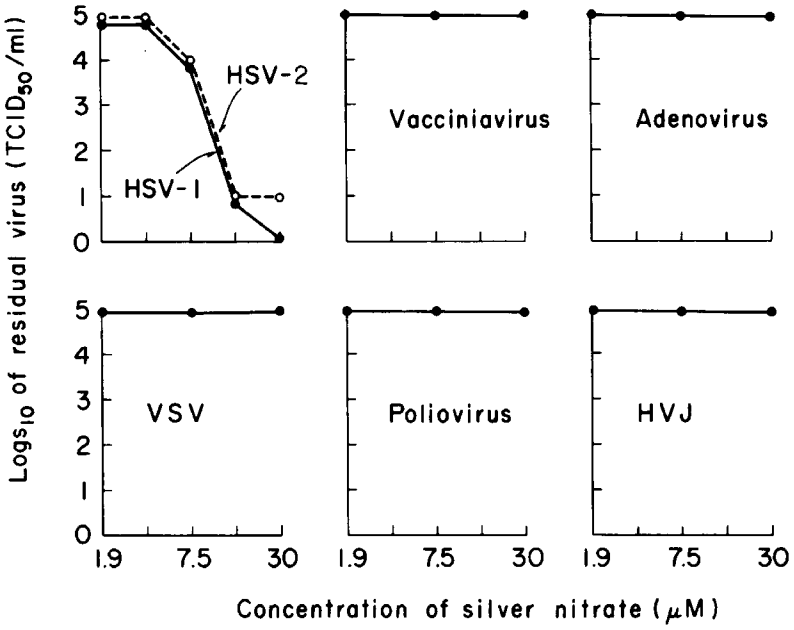

FIG. 3. *Effect of silver nitrate on various viruses.*

TABLE 1. *Inhibition by different reagents of the $AgNO_3$ effect on HSV-1*

Compounds added to HSV[a]	Residual virus[b]	
	PFU/ml	% of control
$AgNO_3$	<10	<0.02
$AgNO_3$ + R-glutathione[c]	4.8×10^4	88.9
$AgNO_3$ + KI[c]	4.3×10^4	79.5
AgI	3.9×10^4	72.2
MEM	5.4×10^4	

[a] Final concentrations of $AgNO_3$, R-glutathione, KI, and AgI in the virus preparations were 30, 10^3, 300, and 0.006 μM, respectively.

[b] Residual virus was assayed after exposure of the virus to the compound at 37 C for 60 min. PFU, Plaque-forming units.

[c] $AgNO_3$ and R-glutathione or $AgNO_3$ and KI were incubated at room temperature for 5 min after mixing and then were added to the virus preparation.

$AgNO_3$ may be due to the binding of ionizing Ag to the sulfhydryl groups in the virus.

Biological activities retained in $AgNO_3$-inactivated HSV. To investigate the steps by which replication of the $AgNO_3$-treated virus was prevented, we tested whether the inactivated virus retained some biological activities to the cells.

First, we tested the ability of the virus to adsorb to the cell surface. [^{3}H]thymidine-labeled HSV (12,000 counts/min per ml), which possessed an infectivity of 7×10^5, was divided into two equal portions. Infectivity of one portion of the virus was completely inactivated by exposure to 30 μM $AgNO_3$ at 37 C for 60 min. The other portion was incubated in PBS at 37 C for 60 min and served as an intact virus. Both virus preparations were diluted fourfold in MEM, and 1 ml of each was then inoculated into approximately 4×10^5 3T3 cells and incubated at 37 C. At intervals, radioactivities in the supernatant fluids and the cells were counted in a liquid scintillation spectrometer (Fig. 4). The results clearly show that the radioactivity of inactivated virus decreased in the supernatant fluids and increased in the cells, with time, at rates almost identical to those of intact virus. Simultaneously, the infectivity residues in the supernatant fluids were assayed in the cultures infected with intact virus (Fig. 4). The results show that the rates of decrease in radioactivities of both the intact and inactivated viruses were similar to that of the decrease in infectivity. This indicates that the HSV inactivated with $AgNO_3$ retained almost fully the activity of adsorbing to the cells.

A further attempt was made to test whether infection with the inactivated virus can induce the early surface changes and synthesis of viral antigens observed in the cells infected with intact HSV. The results clearly show that neither a hemadsorption phenomenon nor cell fusion and polykaryocytes occurred in the cells infected with the inactivated virus (Table 2). Neither surface nor intracellular viral antigens, as detected by immunofluorescence tests, were observed (Table 2).

Tevethia et al. have reported that, soon after

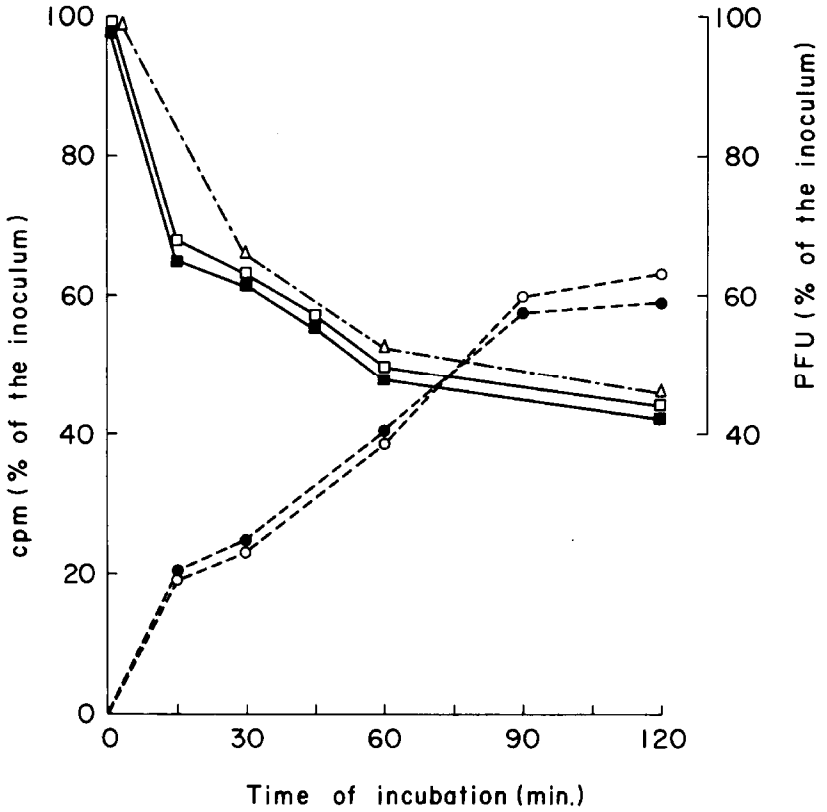

FIG. 4. *Adsorption of $AgNO_3$-inactivated HSV to the cells. Symbols: Circles represent the radioactivity in the cells—●, cells infected with $AgNO_3$-inactivated HSV; ○, cells infected with intact virus. Squares represent the radioactivity in the culture fluids—■, $AgNO_3$-inactivated HSV; □, intact virus. Triangles represent the infectivity in the supernatant fluids of the cultures infected with intact virus.*

infection, the cells infected with HSV showed agglutinability by ConA before production of infectious virus (11). We thus examined the agglutinability by ConA of cells infected with the $AgNO_3$-inactivated virus. The results clearly show that the chicken embryo cells infected with inactivated virus, as well as the cells infected with intact virus, were agglutinated in the presence of 250 μg of ConA per ml, by which the noninfected cells tested as a control were not agglutinated at all (Table 3). Hence, it would seem that infection of the inactivated virus underwent the early steps that produce certain cell surface changes responsible for ConA agglutinability, but not other surface changes and viral antigens.

TABLE 2. *Surface changes and antigen production in cells infected with intact and $AgNO_3$-inactivated HSV*[a]

Surface changes and antigen production	HSV[b]			
	Intact		$AgNO_3$-inactivated	
	6 h	20 h	6 h	20 h
Hemadsorption	1+	3+	0	0
Surface antigens	1+	4+	0	0
Intracellular antigens	1+	4+	0	0
Cell fusion and polykaryocytes	0	3+	0	0

[a] HSV-1 containing 5×10^5 $TCID_{50}$/ml, and its preparation inactivated by 30 μM $AgNO_3$ were diluted to one-fifth in MEM and inoculated into 10^5 3T3 cells. The infected cells were cultivated at 37 C and tested for their surface changes and production of viral antigens 6 and 20 h after infection.

[b] Positive reactions were graded form 1+ to 4+ depending upon the degree of reaction: 4+, almost all the cells were positive; 1+, less than one-fourth of the total cells were positive.

DISCUSSION

It is well known that many agents exist that have potent antiviral properties against extracellular HSV (7) and that mercaptide-forming sulfhydryl reagents are a group of potent inactivators (9). The present report has shown that the inactivation of HSV by $AgNO_3$ is also probably attributable to the formation of mercaptides of Ag with the SH base of the virion components. Previous investigators have reported that the infectivities of many viruses other than HSV were also inactivated by 10^{-3} to 10^{-5} M mercuric compounds such as mercuric

TABLE 3. *Agglutination by ConA of chicken embryo cells infected with $AgNO_3$-inactivated HSV-1*

Cells infected with:	Agglutination by ConA (μg/ml)		
	500	250	0
Intact HSV[a]	+++	++	−
$AgNO_3$-inactivated HSV[a]	++	++	−
Not infected (7.5 μM $AgNO_3$)[b]	−	−	−
Not infected	−	−	−

[a] A 1-ml amount of HSV-1 containing 2×10^6 $TCID_{50}$/ml and its preparation inactivated by 30 μM $AgNO_3$ were diluted to one-fourth in MEM. Two milliliters of each was inoculated into the monolayers of 10^6 chicken embryo cells in plastic petri dishes. The infected cells were then tested for agglutination by ConA as described in the text.

[b] To test whether $AgNO_3$ per se, of which trace amounts probably are residue in the $AgNO_3$-treated virus preparation, could induce ConA agglutinability, the cells were incubated in the presence of 7.5 μM $AgNO_3$ for 6 h at 37 C and then tested for their ConA agglutinability.

chloride, *p*-chloromercuribenzoate, or merthiolate (9). We have, however, demonstrated in this paper that 10^{-5} M $AgNO_3$ could inactivate specifically the infectivity of HSV at exposure times that did not affect the infectivity of other deoxyribonucleic acid or enveloped viruses or of ribonucleic acid or non-enveloped viruses tested so far. The biochemical basis for the differential inactivation of herpesviruses and other viruses is unknown. However, it was concluded that the sulfhydryl groups of the HSV virion may play an important role, more so than those in other viruses, in keeping the superficial structure required for the infectivity of the virus.

The present experiments have also demonstrated that $AgNO_3$ did not affect the rate of attachment of HSV to host cells. Philipson and Choppin (4, 9) have demonstrated that treatment with mercuric chloride and *p*-chloromercuribenzoate prevented the hemagglutination by many Echo- and Coxsackie viruses and the attachment of Echo-7 virus to host cells. In contrast, treatment with such sulfhydryl reagents have been shown not to have any effect on hemagglutination by fowl plague virus and other myxoviruses (9) and on attachment of fowl plague and vaccinia viruses to host cells (1). These results and our findings thus strongly suggest the possibility that the sulfhydryl groups in the enveloped viruses, in contrast to those in the non-enveloped viruses, are not involved in the adsorption of the virus to host cells.

It appears that $AgNO_3$ may prevent the multiplication of HSV at a certain step(s) of infection soon after attachment. The present paper does not indicate a precise step(s) after attachment responsible for the abortion of infection. Allison has presented some evidence indicating that after treatment with mercurials the uncoating of the vaccinia virus may not occur, although the virus has been taken up by the cells (1). Whether a similar mechanism exists for the inactivation of HSV by $AgNO_3$ is under investigation.

It is interesting to note that infection of the cells with $AgNO_3$-inactivated virus could induce ConA agglutinability, whereas it produced no virus-determined surface changes. Tevethia et al. have reported that the surface change leading to the agglutination of HSV-infected cells is caused by a virus-determined function but not by a nonspecific change due to virus-cell interaction (11). This may not be the case here, since it seems unlikely that the inactivated virus functioned as a genome determined only for the induction of ConA agglutinability and not for the other virus-specific changes. It seems more likely that certain nonspecific changes occurred in the steps of virus adsorption or penetration that were responsible for the induction of the ConA agglutinability obtained in our study.

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