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Inhibition of Herpesvirus Deoxyribonucleic Acid and Protein Synthesis by Tilorone Hydrochloride

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Tilorone hydrochloride at a concentration of 10 μg/ml very efficiently inhibited herpes simplex virus growth in BSC1 cells when the virus is infected at a low multiplicity of infection. The adsorption of the virus was not affected by the drug, and the penetration of the deoxyribonucleic acid of the input virus into the cytoplasm and nuclei proceeded normally when tilorone hydrochloride was present. However, newly synthesized viral deoxyribonucleic acid was not detectable under these conditions, there was a remarkable decrease in the rate of viral polypeptide synthesis, and virus particles were not formed. The inhibition of herpesvirus growth by tilorone hydrochloride was absolutely dependent on the presence of the drug in the cultures. Pretreatment of the cells with the drug did not result in resistance to herpesvirus infection after the removal of the drug.

Tilorone hydrochloride, a dihydrochloride salt of 2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one (2), reduces infection of mice with Semliki Forest, vesicular stomatitis, encephalomyocarditis, mengo, influenza A and B, and herpes simplex viruses. It also inhibits tail lesions by nonlethal doses of vaccinia virus (6, 14). It has been suggested that in vivo antiviral activity caused by tilorone results from induction of interferon (6, 14, 22). Tilorone also has antitumor activity (1, 3, 18, 19) and reduces the deoxyribonucleic acid (DNA) polymerase activity associated with ribonucleic acid tumor viruses (5).

Recently, we showed that tilorone hydrochloride (10 μg/ml) very efficiently inhibits the growth of herpes simplex virus type 1 in BSC1 cells (E. Katz, E. Margalith, and B. Winer, J. Gen. Virol., in press). Since no direct inactivation of virus infectivity was observed after incubation of virus suspension with the drug, an intracellular mechanism of antiviral action was suggested. The inhibition of herpesvirus growth was more effective when the multiplicity of infection was less than 1 and when the drug was added early in the course of infection.

The aim of the present study was to examine the intracellular antiviral action of tilorone hydrochloride and to observe the synthesis of macromolecules of herpes simplex virus in BSC1 cells in the presence of the drug.

MATERIALS AND METHODS

Virus. Herpes simplex virus type 1 HF was obtained from ATCC. Virus stock was kept at 4 C.

Cell cultures. BSC1 cells were grown in M199 supplemented with 10% inactivated calf serum in 60-mm plastic petri dishes (Nunc, Denmark). The dishes were incubated at 37 C in a humidified atmosphere supplied with 5% CO_2.

Infection procedure. Monolayers of BSC1 cells were infected with 0.3 ml of virus suspension at a multiplicity of infection of 0.5. After 1 h at 37 C the monolayers were washed with saline buffer, and 5 ml of M199 supplemented with 2% calf serum was added.

Plaque assay. Monolayers of BSC1 cells were infected with 0.2 or 0.3 ml of virus dilution. After 1 h at 37 C, the cells were overlaid with Eagle medium containing 1% special Agar Noble (Difco Laboratories, Detroit, Mich.) and 5% inactivated calf serum. Neutral red, 0.0025%, was added 3 days after infection, and plaques were counted on the following day.

CsCl gradient analysis of DNA. Infected cells were labeled with 2 μCi of [^{3}H]thymidine per ml starting at 3 h after infection. The cells were harvested 22 h postinfection, centrifuged at low speed (600 × *g*), washed, and suspended in SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.2). Sodium dodecyl sulphate (SDS) and Pronase solution (Calbiochem, B grade, preheated at 37 C for 1 h) were added at a final concentration of 1% and 0.3 mg/ml, respectively. After incubation for 5 h at 37 C, the DNA was centrifuged in CsCl density gradient in buffer containing 0.01 M tris(hydroxymethyl)aminomethane and 0.001 M ethylenediaminetetraacetic acid, pH 8.0) in a 50 Ti rotor at 35,000 rpm at 20 C for 48 h. Fractions (15 drops) were collected from the bottom of the tube. The density of the fractions and radioactivity after trichloroacetic acid precipitation were determined.

Virus particle isolation. To infected cultures, 2 μCi of [^{35}S]methionine per ml was added 3 h after infection. The cultures were harvested 22 h postinfection, the cells were suspended in 0.6 ml of retic-

ulocyte standard buffer (RSB) (0.01 M KCl, 0.0015 M $MgCl_2$, and 0.01 M Tris, pH 7.7) and sonicated for 60 s in a Bransonic model 12 ultrasonic cleaner. The sonicated cells were layered on a 12 to 52% (wt/wt) sucrose gradient in Tris-buffered saline (0.085% NaCl and 0.2 M Tris, pH 7.3) and centrifuged in an SW50.1 rotor at 26,000 rpm for 1 h at 4 C. Fractions (10 drops) were collected from the bottom of the tube. Radioactivity was measured in a Tri-Carb scintillation counter after trichloroacetic acid precipitation.

Polyacrylamide gel electrophoresis. The details of the method used for polyacrylamide gel electrophoresis have been previously described (23). Gels (10 by 0.6 cm) were prepared from 7.5% acrylamide, 0.2%, *N-N'*-methylene bisacrylamide in 0.1 M sodium phosphate (pH 7.1), and 0.1% SDS. Before use, excess catalyst was removed from the gels by electrophoresis at 5 mA/gel for 1 h. Purified virus or cell cytoplasm was solubilized by incubation in 2% SDS and 1% mercaptoethanol at 100 C for 1 min. Sucrose was then added to 10%, and 200 μliters were applied to each gel. Electrophoresis was carried out at 3 mA/gel for 20 h. Bromphenol blue served as a marker dye. After electrophoresis, the gels were placed in 10% trichloroacetic acid, stained with 0.1% Coomassie blue in trichloroacetic acid for 5 h, and washed in 7.5% acetic acid for 1 day. The gels were sliced longitudinally, dried, and placed in contact with X-ray film (7).

Chemicals and isotopes. Tilorone hydrochloride (batch 19538) was kindly donated by R. F. Krueger, Merrell National Laboratories, Cincinnati, Ohio. [^{3}H]thymidine (14.5 Ci/mmol) was obtained from Nuclear Research Center, Negev, Israel, and L-[^{35}S]methionine (>100 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Herpes simplex virus growth in the presence of tilorone hydrochloride. The kinetics of virus growth in the absence and presence of tilorone hydrochloride (10 μg/ml) were observed. Monolayers of BSC1 cells were infected with herpes simplex virus at a multiplicity of infection of 0.5. One set of cultures was treated with tilorone hydrochloride from the time of infection. The cultures were harvested at various time intervals, and virus infectivity was determined by plaque assay. The results presented in Fig. 1 demonstrate the significant inhibition by 10^6-fold of virus infectivity caused by tilorone hydrochloride at 24 h after infection.

Virus adsorption to BSC1 cells. To determine the step in herpesvirus growth that is inhibited by tilorone hydrochloride, we first examined the adsorption of the virus in the presence of the drug. The method was previously described by Hochberg and Becker (12). Adsorption was studied with a crude suspension of virus at a low multiplicity of infection

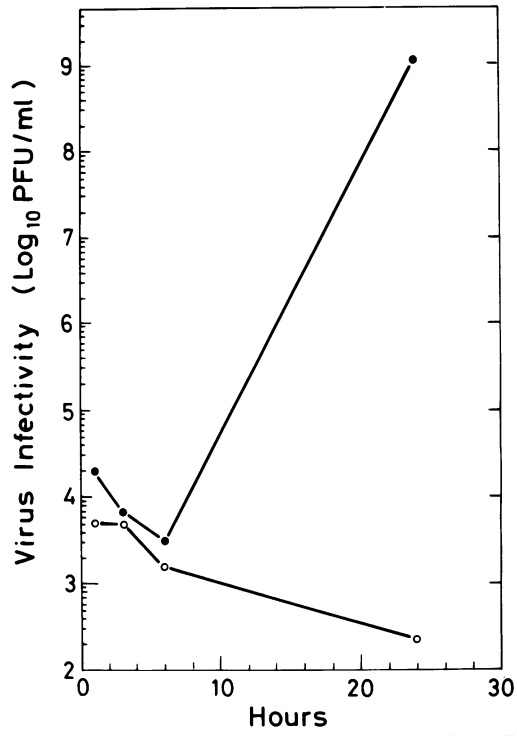

FIG. 1. *Growth curve of herpesvirus. BSC1 cell monolayers in 60-mm-diameter plastic petri dishes were infected with herpes simplex virus type 1 at a multiplicity of infection of 0.5. After 1 h at 37 C the cultures were washed with buffered saline, and M199 containing 2% calf serum was added. To one set of dishes tilorone hydrochloride (10 μg/ml) was added at the time of infection. At various time intervals, cultures were harvested and virus infectivity was determined by plaque assay on BSC1 cells. Symbols: ●, control cultures; ○, tilorone-treated cultures.*

(1 plaque-forming unit (PFU) per 3 × 10^4 cells) and a highly purified [^{3}H]thymidine-labeled virus at a higher multiplicity of infection (2 PFU/cell). The adsorption of the labeled virus reached its highest level within the first 15 min, whereas the crude virus preparation showed a slower rate of adsorption (Fig. 2). However, the presence of tilorone (10 μg/ml) did not affect significantly the rate of adsorption of the two virus preparations tested (Fig. 2).

Penetration of the infecting virus DNA into the cytoplasm and nucleus. The fate of the parental herpesvirus DNA in BSC1 cells was determined after infection with [^{3}H]thymidine-labeled virus in the presence of tilorone hydrochloride. BSC1 cells in monolayers were infected with herpesvirus at a multiplicity of in-

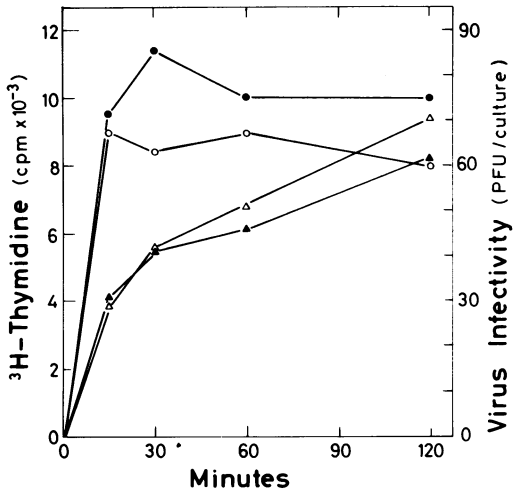

FIG. 2. *Herpesvirus adsorption to BSC1 cells. Infectivity test: Monolayers of BSC1 cells in petri dishes were infected with 100 PFU of crude virus stock in the absence or presence of 10 μg/ml of tilorone hydrochloride. At various time intervals the cultures were washed with buffered saline and agar overlay was added. Virus plaques were counted on day 4. Symbols: ▲, control; △, tilorone hydrochloride added. Radioactivity test: Monolayers of BSC1 cells were infected with [^{3}H]thymidine-labeled purified herpesvirus at a multiplicity of infection of 2. One set of cultures was treated with tilorone hydrochloride (10 μg/ml). At various time intervals the plates were washed with cold buffered saline and the cells were dissolved in SDS buffer (0.005 M Tris, 0.2 M NaCl, and 0.5% SDS, pH 7.3). The radioactivity was determined after precipitation with trichloroacetic acid. Symbols: ●, control; ○, tilorone hydrochloride added. (The values at zero time were deduced from the values obtained.)*

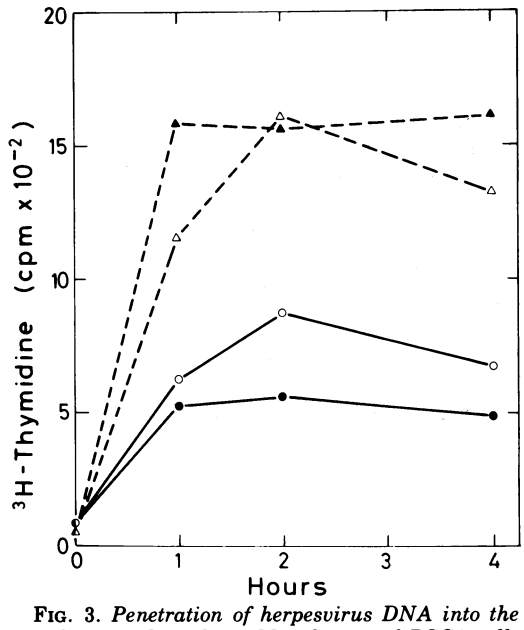

FIG. 3. *Penetration of herpesvirus DNA into the cytoplasm and nucleus. Monolayers of BSC1 cells were infected with [^{3}H]thymidine-labeled purified virus at a multiplicity of infection of 5 in the absence and presence of tilorone hydrochloride (10 μg/ml). After 1 h at 37 C, the cells were washed and fresh medium was added. At various time intervals the cells were scraped off the dishes, washed with cold buffered saline, and resuspended in RSB. NP-40 was added to a final concentration of 0.1%. The disrupted cells were centrifuged at 1,000 × g for 2 min. The radioactivity in the supernatant (cytoplasmic fraction) and in the pellet (nuclear fraction) was determined after trichloroacetic acid precipitation. Symbols: ●, cytoplasm, control; ○, cytoplasm, tilorone hydrochloride added; ▲, nuclei, control; △, nuclei, tilorone hydrochloride added.*

fection of 5. The radioactivity of the labeled virus, introduced to each culture, was 12,500 counts/min. At various time intervals the cells were scraped into the culture media, washed, and suspended in RSB. The nonionic detergent NP-40 was then added to a final concentration of 0.1%. After centrifugation at 1,000 × *g* for 2 min, the supernatant consisted of the cytoplasmic fraction of the cells, whereas the pellet contained the nuclei. Radioactivity in the cytoplasmic and nuclear fractions was determined after trichloroacetic acid precipitation. The results presented in Fig. 3 show that radioactivity of the input virus DNA reached its maximum level in the cytoplasm and in the nuclear fraction within 1 h after infection and remained at this level during the next 3 h. The amount of radioactivity in the cells that were precipitated by trichloroacetic acid represents approximately 20% of the radioactivity of the infecting virus, and the radioactivity that accumulated in the nuclei was two to three times higher than that of the cytoplasm. The results presented in Fig. 3 do not show any significant inhibition in the rate of penetration of the input virus DNA into the cytoplasm or the nuclei, during the first 4-h period after infection, due to the presence of tilorone hydrochloride.

Formation of virus particles. Since the yield of infectious virus was very low in the presence of tilorone hydrochloride, we asked whether noninfectious virus is formed under these conditions. It is possible to detect herpesvirus particles in sucrose gradient as a definite band after high-speed centrifugation. When a radioactively labeled virus, either its nucleic acid or protein capsid, is used, it is possible to determine the location of the virus by a radioactive peak in the sucrose gradient. BSC1 cells were infected with herpes simplex virus at a multi-

plicity of infection of 0.5 in Eagle medium containing 5% of the regular concentration of methionine and 2% calf serum. Three hours after infection, 2 μCi of [^{35}S]methionine was added. The cultures were harvested 22 h after infection, and virus was purified as described above. To one culture, tilorone hydrochloride (10 μg/ml) was added at the time of infection, and to another was added cytosine arabinoside (50 μg/ml), an inhibitor of DNA synthesis in animal cells (13) and also of herpesvirus particle synthesis (15). The results show that the formation of [^{35}S]methionine-labeled virus particles was greatly inhibited by either tilorone hydrochloride or cytosine arabinoside (Fig. 4).

Synthesis of viral DNA. The failure of herpesvirus to form virions in the presence of tilorone hydrochloride might result from inhibition of viral DNA synthesis. BSC1 cells were infected with herpesvirus at a multiplicity of 0.5 in the presence of tilorone hydrochloride (10 μg/ml) or cytosine arabinoside (50 μg/ml). Three hours after infection, [^{3}H]thymidine (2 μCi/ml) was added. The cells were harvested 22 h after infection, and the DNA was analyzed in CsCl gradient as described above. It is possible to follow both cellular and viral DNA under these conditions, since the density of herpesvirus DNA is higher than that of cellular DNA (20). The results presented in Fig. 5 show that in untreated infected cells, we can detect both viral DNA as a very prominent peak at a density of 1.712 g/ml, and cellular DNA, which has a density of 1.695 g/ml. In the presence of tilorone hydrochloride, the viral peak is missing, whereas we can detect the cellular DNA, which is somewhat inhibited. In the presence of cytosine arabinoside, both types of labeled DNA are almost non-detectable. This indicates

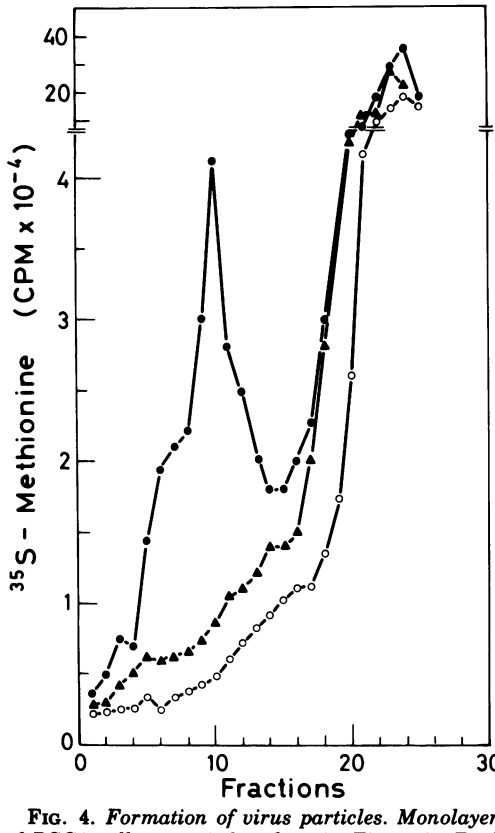

FIG. 4. *Formation of virus particles. Monolayers of BSC1 cells were infected as in Fig. 1 in Eagle medium containing 5% of the regular concentration of methionine and 2% calf serum in the presence of tilorone hydrochloride (10 μg/ml) or cytosine arabinoside (50 μg/ml). Three hours after infection, 2 μCi of [^{35}S]methionine was added to each culture. The cultures were harvested 22 h after infection, and the virus was purified in sucrose gradient. The results obtained from the three gradients (●, control; ▲, cytosine arabinoside added; ○, tilorone hydrochloride added) are superimposed in this figure.*

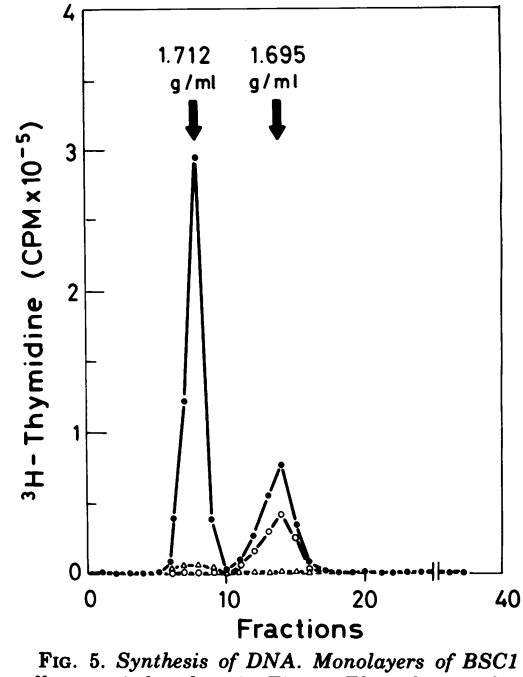

FIG. 5. *Synthesis of DNA. Monolayers of BSC1 cells were infected as in Fig. 1. Three hours after infection [^{3}H]thymidine (2 μCi/ml) was added. The cultures were harvested 22 h after infection and the DNA was analyzed in CsCl gradients. The density of the fractions was measured by weighing 100-μl samples. The results obtained from the three gradients (●, control; ○, tilorone hydrochloride added [10 μg/ml]; and △, cytosine arabinoside added [50 μg/ml]) are superimposed in this figure.*

that cytosine arabinoside is highly toxic to the host cell, as compared with tilorone hydrochloride, at the drug concentrations we used.

Synthesis of viral polypeptides. Herpesvirus has a relatively large genome and should, in principle, be able to code for about 100 polypeptides. The use of SDS-polyacrylamide gels to separate the various herpesviral proteins provides a useful tool to identify and characterize the major proteins (18, 21). We examined the polypeptides that were synthesized in the presence of tilorone hydrochloride and compared them with those found in the presence of cytosine arabinoside. BSC1 cells were infected with herpes simplex virus at a multiplicity of infection of 0.5 in Eagle medium containing 5% of the regular concentration of methionine, supplemented with 2% calf serum. At 3 h postinfection 2 μCi of [^{35}S]methionine was added, and the cultures were harvested 19 h later. The cells were washed and suspended in RSB, and NP-40 was added to a final concentration of 0.1%. The supernatant, after centrifugation at 1,000 $\times$ g for 2 min, consisted of the cytoplasmic fraction of the cells. It was found that cytosine arabinoside inhibited the incorporation of [^{35}S]methionine into the trichloroacetic acid-precipitable fraction of the cell cytoplasm by 17% and tilorone hydrochloride by 39%. The cytoplasmic fraction was boiled in the presence of SDS and mercaptoethanol, and the polypeptides were analyzed by polyacrylamide gel electrophoresis, as described above. The results, presented in Fig. 6, show that although many viral polypeptides were synthesized, both tilorone hydrochloride (10 μg/ml) and cytosine arabinoside (50 μg/ml) caused significant quantitative inhibition of synthesis of a few of the viral polypeptides. This is in agreement with the findings of Geder et al. (9), Nii et al. (17), Frenkel and Roizman (8), and Becker and Olshevsky (4), who demonstrated that synthesis of herpesvirus ribonucleic acid, antigens, and capsid proteins occurs also when viral DNA synthesis is inhibited.

Herpesvirus growth in tilorone-pretreated cells. We wished to find out whether the resistance of tilorone-treated cells to herpesvirus infection is retained after the removal of the drug. BSC1 cells were treated with tilorone hydrochloride (10 μg/ml) 24 h before infection. One set of cultures was washed at the time of infection, and medium without the drug was added. Herpesvirus was infecting the cultures at a multiplicity of 0.3. At various time intervals the cultures were harvested and virus infectivity was determined. The results shown in Fig. 7 indicate that tilorone-pretreated cells are not protected from herpesvirus infection after the removal of the drug and that the virus growth curve obtained is similar to that of virus grown in cells that were not treated at all with tilorone hydrochloride. We can therefore conclude that continuous presence of the drug in the infected culture is necessary for the inhibition of herpes virus growth.

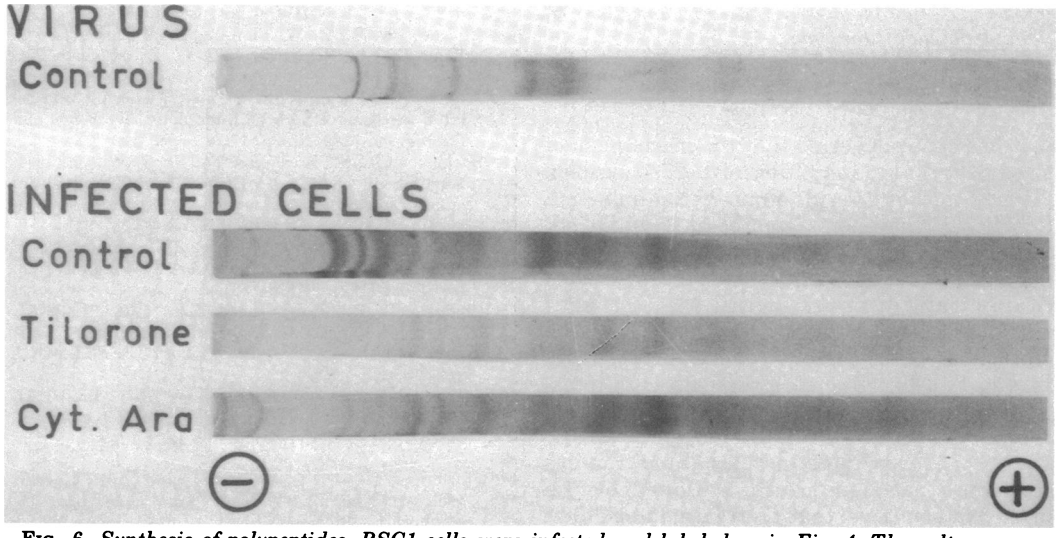

FIG. 6. *Synthesis of polypeptides. BSC1 cells were infected and labeled as in Fig. 4. The cultures were harvested 22 h after infection, the proteins of the cytoplasm were disrupted by SDS and mercaptoethanol, and the polypeptides were analyzed by polyacrylamide gel electrophoresis. A preparation of [^{35}S]methionine-labeled purified herpesvirus was similarly disrupted by SDS and mercaptoethanol and analyzed by polyacrylamide gel electrophoresis. A photograph of the X-ray film is presented.*

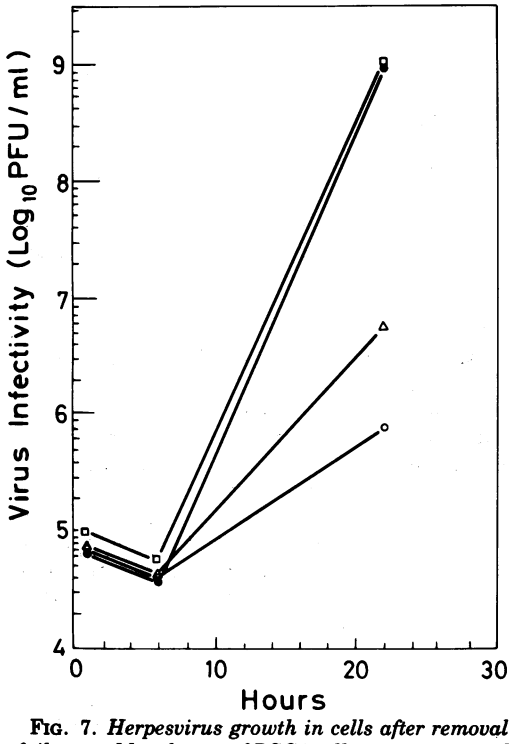

FIG. 7. *Herpesvirus growth in cells after removal of tilorone. Monolayers of BSC1 cells were pretreated with tilorone hydrochloride (10 μg/ml) 24 h before infection with herpesvirus at a multiplicity of infection of 0.3. At time intervals the cultures were harvested, and virus infectivity was determined by plaque assay in BSC1 cells. Symbols: □, tilorone removed just before infection; ○, tilorone present all the time; △, tilorone present only after infection; ●, tilorone absent all the time.*

DISCUSSION

It has been suggested that the mechanism of the intracellular inhibition of herpes simplex virus in mice is a result of interferon induction (6, 14, 22). However, Giron et al. (10) did not find a correlation between interferon induction and viral protection in mice. Our recent studies (Katz et al., in press) showed that the antiviral activity of tilorone hydrochloride in tissue culture is of a narrow range; furthermore, viruses known to be more susceptible to interferon than herpesvirus, such as western equine encephalomyelitis (R. M. Brown, M.S. thesis, Univ. of Texas, Austin, 1966) and Sindbis (11), were not significantly inhibited by the drug. We now show that pretreatment of BSC1 with tilorone hydrochloride for 24 h does not result in resistance of the cells to infection with herpesvirus when the infection is done in the absence of the drug. This finding indicates that even if a factor that inhibits herpesvirus growth is induced by tilorone hydrochloride, it is unlikely that it is interferon, because of its rapid inactivation. Tilorone hydrochoride has to be present in the culture continuously to maintain the cell resistance to infection with herpesvirus.

In the present study we have shown that herpesvirus adsorption and penetration of viral DNA into the cytoplasm and into the nuclei of BSC1 cells take place normally in the presence of tilorone hydrochloride. However, the synthesis of viral DNA is completely inhibited by the drug. Under these conditions, the rate of viral protein synthesis is reduced and virus particles are not formed. These findings suggest similarities between the effects of cytosine arabinoside and tilorone hydrochloride on the macromolecular synthesis of herpesvirus. The question of whether tilorone hydrochloride acts by a mechanism similar to that of cytosine arabinoside needs further elucidation, since in contrast to cytosine arabinoside, the antiviral activity of tilorone hydrochloride against herpesvirus is dependent upon a low multiplicity of infection (Katz et al., in press).

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