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Antiviral Activity of Tunicamycin on Herpes Simplex Virus

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Tunicamycin (0.5 μg/ml) significantly lowers (2 to 3 $\log_{10}$) the infectious yield of herpes simplex virus type 1 grown in chicken embryo fibroblasts and in BSC1 cells. Although virus particles are formed and the synthesis of the viral deoxyribonucleic acid is only partially affected by the antibiotic, the glycosylation of herpesvirus glycopeptides is almost completely inhibited. The morphology of virus particles made in the presence of tunicamycin is similar to that of intact virus particles, as demonstrated by electron microscopy. This suggests that the absence of the carbohydrate side-chain from the viral glycopeptides does not affect the overall integrity of the virion but decreases very significantly the infectivity of these particles.

Herpes simplex type 1 virus (HSV) is an enveloped deoxyribonucleic acid (DNA)-containing virus that assembles by budding from the cell nuclear membranes during its maturation process (13, 14). The virion contains more than 30 structural polypeptides, to several of which carbohydrate side chains are covalently linked (7, 21). The role of these glycopeptides in the overall integrity and stability of the virions is yet to be clarified.

Tunicamycin is a glucosamine-containing antibiotic (23, 24) that specifically inhibits glycosylation of proteins (2, 18, 22). It blocks the transfer of *N*-acetylglucosamine-1-phosphate from uridine 5'-diphosphate-*N*-acetylglucosamine to the dolichyl monophosphate that serves as a lipid carrier (12, 25) by inhibition of the transferase involved (6). Changes in the cell morphology and in the membrane properties of normal and virus-transformed fibroblasts occur in the presence of tunicamycin (3, 4). Tunicamycin significantly lowers the yield of several enveloped ribonucleic acid (RNA)-containing viruses (10, 15, 17, 20). Whereas formation of virions of Semliki Forest, fowl plague (20), Sindbis, and vesicular stomatitis viruses (10) is greatly inhibited by tunicamycin, formation of Rous sarcoma and influenza virions occurs in the presence of the antibiotic; however, these virus particles show reduced infectivity and undetectable amounts of glycoproteins (15, 20).

In the present study, we examine the effect of tunicamycin on formation, structure, and infectivity of HSV particles to determine the involvement of the carbohydrate side chains of the glycopeptides in these viral processes and functions.

MATERIALS AND METHODS

Cells. BSC1 cells were grown as monolayer cultures in medium 199 (M199) supplemented with 10% calf serum, and chicken embryo fibroblasts were grown in M199 containing 5% calf serum. Vero cells were cultivated in RPMI medium containing 10% calf serum.

Viruses. Stocks of HSV type 1 HF strain and poliomyelitis type 1 viruses were prepared in BSC1 cells, and Semliki Forest virus was prepared in chicken embryo fibroblasts.

Infection procedure. Monolayers of cells in 5-cm-diameter plastic petri dishes (Sterilin, Teddington, England) containing approximately 5×10^6 cells were infected with 0.3 ml of virus suspension at a multiplicity of 5 plaque-forming units per cell. After 30 min at 37°C, the cultures were washed and further incubated with 4 ml of M199 supplemented with 2% calf serum.

Plaque assay. Confluent monolayers of cells in 5-cm-diameter plastic petri dishes were infected with 0.3 ml of virus dilutions. After adsorption for 1 h at 37°C, the cultures were overlaid with minimal Eagle medium (MEM) supplemented with 1% Noble agar (Difco Laboratories, Detroit, Mich.) and 5% calf serum. When visible plaques appeared (poliomyelitis and Semliki Forest on day 3 and HSV on day 5), the cultures were fixed with 20% Formalin in buffered saline and stained with crystal violet (0.1%).

CsCl gradient analysis of DNA. Infected cells were labeled with [^{3}H]thymidine (3 μCi/ml) starting 2 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 sulfate (SDS) and pronase solution (Calbiochem, Lucerne, Switzerland, grade B, preheated at 37°C for 1 h) were added at a final concentration of 1% and 0.3 mg/ml, respectively. After incubation for 4 h at 37°C, the DNA was centrifuged in a CsCl density gradient in buffer containing 0.001 M ethylenediaminetetraacetic acid and 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, in a Ti 50 rotor at 35,000 rpm at 20°C

TABLE 1. *Growth of several animal viruses in the presence of tunicamycin*

Virus	Cells	Virus titer (PFU/ml)[a]			Inhibition (%)
		0 h	22 h		
			Control	+Tunicamycin	
HSV	Chicken fibroblasts	1.7×10^4	1.2×10^6	1.3×10^3	99.89
HSV	BSC1	4.3×10^4	1.4×10^9	2.2×10^7	98.43
Poliomyelitis 1	BSC1	5.0×10^3	1.8×10^7	3.0×10^7	0
Semliki Forest	Chicken fibroblasts	1.2×10^4	2.7×10^8	7.3×10^4	99.97
Semliki Forest	Vero	7.0×10^2	2.7×10^7	2.0×10^6	92.60

[a] Monolayer cultures were infected with the viruses at a multiplicity of 1 plaque-forming unit (PFU) per cell. After adsorption for 1 h at 37°C, one culture was immediately harvested (0 h). The other cultures were incubated for an additional 22 h in the absence or presence of tunicamycin (0.5 μg/ml). Virus titer was determined as described in Materials and Methods.

for 72 h. Fractions (10 drops) were collected from the bottom of the tube. The density of the fractions and radioactivity were determined after trichloroacetic acid precipitation.

Isolation of virus particles. Cultures which were labeled with [^{3}H]thymidine, [^{35}S]methionine, or [^{3}H]-glucosamine were harvested at 22 h postinfection, suspended in 0.6 ml of RSB (0.01 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris, pH 7.7), and sonicated for 1 min in a Bransonic model 12 ultrasonic cleaner. The broken cells were layered on a 12 to 52% (wt/wt) sucrose gradient in Tris-buffered saline (0.85% 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 after trichloroacetic acid precipitation.

Polyacrylamide gel analysis. A discontinuous buffer system (9) was used for SDS-polyacrylamide gel electrophoresis. The separating 13-cm-long gel contained 9% acrylamide, 0.18% *N,N'*-methylene-bis-acrylamide, 0.375 M Tris (pH 8.8), and 0.1% SDS. The samples for electrophoresis were solubilized by incubation in 2% SDS and 1% mercaptoethanol at 100°C for 1 min. Electrophoresis took place at a constant current of 2 mA per gel overnight, until the dye front was close to the bottom of the gel. After staining for 5 h with 0.1% Coomassie brilliant blue in 10% trichloroacetic acid, the gels were destained with 7.5% acetic acid. Either gels were sliced into 1-mm fractions and dissolved in H_2O_2, and radioactivity was determined, or they were sliced longitudinally, dried, and then placed in contact with X-ray film.

Electron microscopy. HSV-infected BSC1 cells in the absence or presence of tunicamycin (0.5 μg/ml) were harvested at 22 h postinfection. The cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at room temperature. The fixed pellets were then washed in the same buffer and post-fixed in 1% osmium tetroxide in the above buffer for an additional hour at 4°C. Osmium was rinsed from the cells with several washes of 0.1% aqueous 2% uranyl acetate before a 1-h period of staining en bloc at 4°C. Dehydration was carried out in a graded series of alcohol, embedded in Spurr, and allowed to polymerize for 16 h in a 70°C oven. Thin sections were stained with uranyl acetate and lead citrate and studied with a Philips 300 M transmission electron microscope.

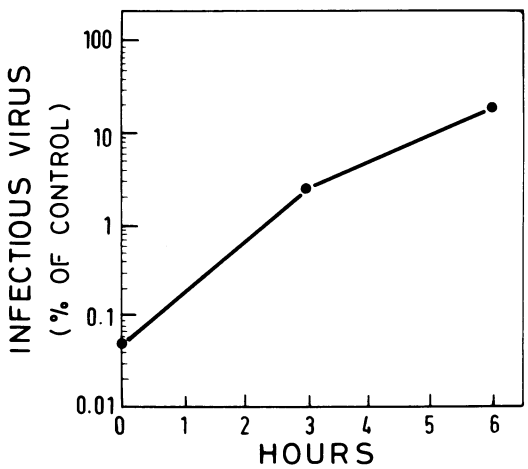

FIG. 1. *Effect of tunicamycin added to cells at various intervals after infection. Tunicamycin (0.5 μg/ml) was added to infected cultures either at the end of a 30-min adsorption period or at 3 or 6 h. Cultures were harvested at 22 h postinfection for determination of virus titer.*

Chemicals and isotopes. Tunicamycin was kindly donated by R. L. Hamill, Lilly Research Laboratories, Indianapolis, Ind. Cytosine arabinoside was purchased from Sigma Chemical Co., St. Louis, Mo. [^{3}H]thymidine (38.4 Ci/mmol) was obtained from Nuclear Research Centre, Negev, Israel; D-[^{3}H]glucosamine (18.8 Ci/mmol), D-[^{14}C]glucosamine (56 mCi/mmol), and L-[^{35}S]methionine (545 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Effect of tunicamycin on the growth of HSV. In the present study, the effect of tunica-

mycin on the growth of HSV, an enveloped virus which buds through the cell nuclear membrane, was studied. Semliki Forest, an enveloped virus which grows in the cytoplasm of the cell, and poliomyelitis type 1, an envelope-deficient virus, served as positive and negative controls for the inhibition caused by tunicamycin. The results presented in Table 1 indicate that the growth of Semliki Forest virus in chicken fibroblasts is highly inhibited by tunicamycin. A similar rate of inhibition was previously obtained by this virus-cell system (20), indicating that our tunicamycin preparation is of a comparable potency. When the effect of tunicamycin on the growth of Semliki Forest virus was examined in Vero cells, a significantly lower rate of inhibition was observed. The growth in BSC1 cells of poliomyelitis, which is a nonenvelope virus, was not affected by tunicamycin. This result indicates that the inhibitory effect of tunicamycin on HSV grown in BSC1 cells does not result from its effect on the host cell and that this inhibitory effect is specific to the virus. When the growth of HSV in chicken fibroblasts was observed in the presence of tunicamycin, a 99.89% inhibition was obtained. The rate of inhibition was somewhat lower when the host cells were BSC1 cells. Since extensive biochemical studies on the growth of HSV in animal cells were previously carried out in several human and

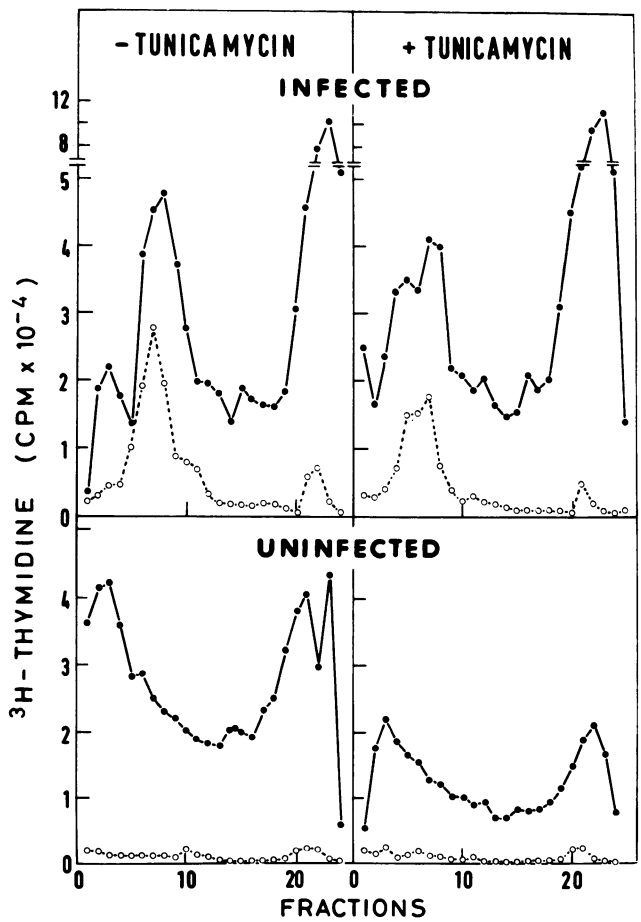

FIG. 2. *Formation of virus particles. Infected cells were labeled with [^{3}H]thymidine (5 μCi/ml) starting at 2 h postinfection. The cultures were harvested after an additional 20 h, and virus particles were isolated as described in Materials and Methods. One sample (50 μl) of each fraction was directly precipitated with trichloroacetic acid, and another was digested first with deoxyribonuclease (50 μg/ml, Worthington Biochemicals Corp. Freehold, N.J.) in the presence of 10 mM $MgCl_2$, for 30 min at 37°C. ●, Total; ○, deoxyribonuclease resistant.*

monkey origin cell lines (1), we chose to study the effect of tunicamycin on HSV in such cells (BSC1).

The importance of the time of addition of tunicamycin to HSV-infected cells for improving the inhibition of virus growth was determined. BSC1 cells were infected with HSV, tunicamycin was added either at the end of a 30-min adsorption period or 3 or 6 h later, and the cultures were harvested at 22 h postinfection. The results presented in Fig. 1 show that the earlier tunicamycin is added, the higher the inhibition of virus growth. Therefore, in all subsequent experiments, tunicamycin was added to the infected cultures at the end of a 30-min adsorption period.

Formation of virus particles in the presence of tunicamycin. Radioactive labeling with [^{3}H]thymidine was used to observe the formation of virions of HSV in the presence of tunicamycin. The extracts of the cells were examined for the presence of labeled DNA, coated with proteins, which acquire resistance to deoxyribonuclease. Infected and uninfected cultures, treated with tunicamycin (0.5 μg/ml), were labeled for 20 h with [^{3}H]thymidine. The cells were then analyzed for virus particles by sucrose gradient centrifugation, as described in Materials and Methods, and then deoxyribonuclease digested. In the infected cultures a [^{3}H]-thymidine-labeled peak, which is resistant to deoxyribonuclease, was observed (Fig. 2). The position of this peak in the sucrose gradient is similar to that of purified HSV particles. Such a peak in this area of the sucrose gradient was not found in uninfected cells. Tunicamycin inhibits by approximately 30% the formation of these particles (Fig. 2). The decrease observed here is smaller than that found for virus infectivity (>99%), suggesting that the ratio of infectivity to virus particles is lower for virus particles made in the presence of tunicamycin.

Viral DNA synthesis. The effect of tunicamycin on HSV DNA synthesis was studied to determine whether the observed reduction in the yield of HSV particles caused by tunicamycin was a result of inhibition of viral DNA synthesis. Cells which were labeled with [^{3}H]thymidine were harvested 22 h postinfection and treated with pronase and SDS at 37°C, as described in Materials and Methods. A sample was mixed with CsCl solution, and after centrifugation, both the viral and cellular DNA were examined. The DNA profiles of the infected cells in the absence and presence of tunicamycin are presented in Fig. 3. The distinguished densities of the viral and the cellular DNA (1.712 and 1.695 g/ml, respectively) (19) enabled us to observe the effect of tunicamycin on each of the DNA species separately. Although tunicamycin did not affect significantly the synthesis of the cellular DNA, the level of the newly synthesized viral DNA decreased to approximately 40% as compared with that of the untreated control (Fig. 3).

The decrease in viral DNA synthesis caused by tunicamycin is similar to the reduction in virus particles formed in the presence of the antibiotic. When cytosine arabinoside (50 μg/ml), an efficient inhibitor of HSV DNA synthesis, was used in the same system, viral and cellular DNA syntheses were completely inhibited (Fig. 3).

Synthesis of HSV polypeptides and glycopeptides. Radioactive labeling with [^{14}C]glucosamine was used to observe the synthesis of HSV glycopeptides in the presence of tunicamycin. BSC1 cultures in MEM containing 50% of the regular concentration of glucose and 2% dialyzed calf serum were labeled with [^{14}C]glucosamine (1 μCi/ml) starting at 3 h postinfection. The cultures were harvested 20 h later, and the glycopeptides in the cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis. The results presented in Fig. 4 indicate that the

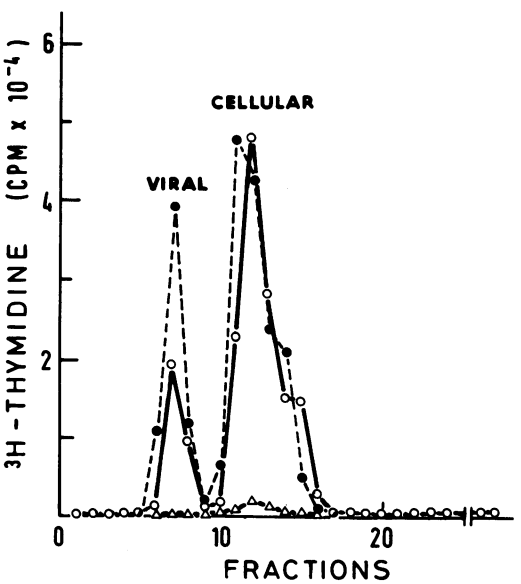

FIG. 3. *CsCl gradient analysis of DNA. Infected cells labeled with [^{3}H]thymidine were harvested at 22 h postinfection. The DNA was sedimented in CsCl gradients. The results obtained from three gradients are superimposed. ●, Infected untreated cells; ○, infected cells treated with tunicamycin (0.5 μg/ml); △, infected cells treated with cytosine arabinoside (50 μg/ml).*

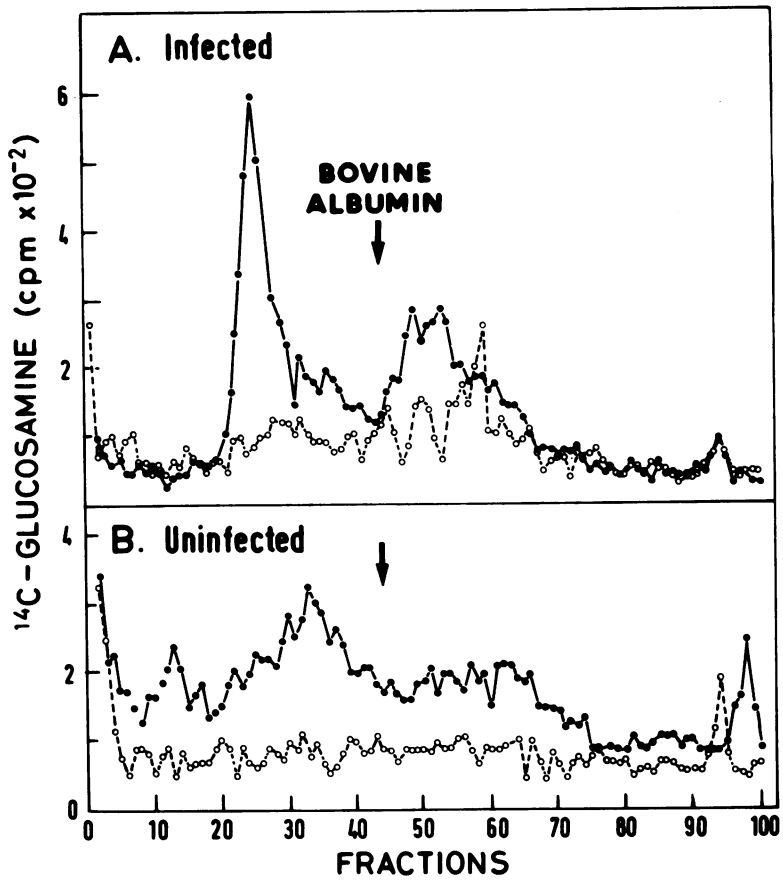

FIG. 4. *SDS-polyacrylamide gel electrophoresis of ^{14}C-labeled glycopeptides from BSC1 cultures infected with HSV. Radioactive labeling started at 3 h postinfection, and the cultures were harvested 20 h later for analysis. ●, Control cultures; ○, cultures treated with tunicamycin (0.5 μg/ml).*

profile of the glycopeptides obtained from infected cells is very different from that derived from uninfected cells; in the first, the main peaks corresponding to HSV glycopeptides (7, 21) are clearly seen. When infected cells were treated with tunicamycin and labeled as described above, the main viral structural glycopeptides were almost undetectable (Fig. 4). This observation indicates the lack of glycosylation of the corresponding glycopeptides in the presence of tunicamycin. To determine whether any glycosylated polypeptides are assembled into particles, control and HSV-infected cells were labeled with [^{3}H]glucosamine in the absence or presence of tunicamycin, harvested, and then separated on sucrose density gradients under conditions suitable for HSV purification as described in Materials and Methods. In control HSV-infected cells, several peaks containing high levels of radioactive glucosamine were observed (Fig. 5). When the labeled glycopeptides of the three main peaks (1, 2, and 3) were examined by polyacrylamide gel electrophoresis, a profile similar to that shown in Fig. 6 (for peak 3), which is characteristic of the profile of the glycopeptides of HSV (21), was observed. Very little [^{3}H]glucosamine labeling was associated with particles made in cells treated with tunicamycin (Fig. 5).

To examine the effect of tunicamycin on the biosynthesis of the viral polypeptides, to BSC1 cultures in MEM containing 10% of the regular concentration of methionine and supplemented with 2% dialyzed calf serum, 20 μCi of [^{35}S]-methionine per ml was added at 3 h postinfection. The cultures were harvested 20 h later, and the labeled polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. The autoradiographs are shown in Fig. 7. Several differ-

ences in the electrophoretic migration of several polypeptides are observed when polypeptides from control and tunicamycin-treated infected cells are compared. These differences may be attributed to interference of glycosylation of those viral polypeptides which are normally being glycosylated. Consequently, their molecular weight is lower and they migrate faster in the gel. Determination of the correlation between the low-molecular-weight polypeptides, which appear in the presence of tunicamycin, and the corresponding glycopeptides needs a detailed additional analysis.

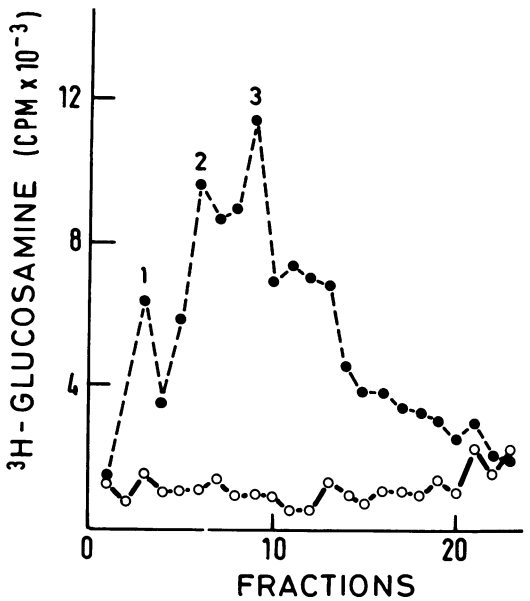

FIG. 5. *Glycopeptide labeling of virus particles. Infected cells were labeled with [^{3}H]glucosamine (5 μCi/ml) starting at 3 h postinfection. The cultures were harvested at 22 h postinfection, and virus particles were sedimented in a sucrose gradient as described in Materials and Methods. ●, Control cultures; ○, cultures treated with tunicamycin (0.5 μg/ml).*

Electron microscopy. The virion of HSV is composed of four major subvirion structural components: core (which contains the viral DNA), capsid, tegument, and a three-layered envelope. The viral glycopeptides are confined to the envelope structure of the virion (16). In thin sections of HSV-infected cells, many viral structures were seen by electron microscopy (Fig. 8A). No changes in the morphology of HSV particles were observed as a result of the treatment with the antibiotic (Fig. 8B). This observation further demonstrates that the incorporation of nonglycosylated polypeptide chains into the virus particles does not affect their structural integrity.

DISCUSSION

The role of the carbohydrate residue in viral glycoproteins can be studied by using inhibitors of glycosylation, of which tunicamycin was found to be one of the most specific. The effect of tunicamycin on cells infected with various RNA-containing enveloped viruses is essentially similar for all the viruses tested so far. There is a reduction in the infectivity of the virus and interference in the synthesis of the glycopeptides (10, 15, 17, 20). Whereas in a few systems noninfectious virus particles are made (Rous sar-

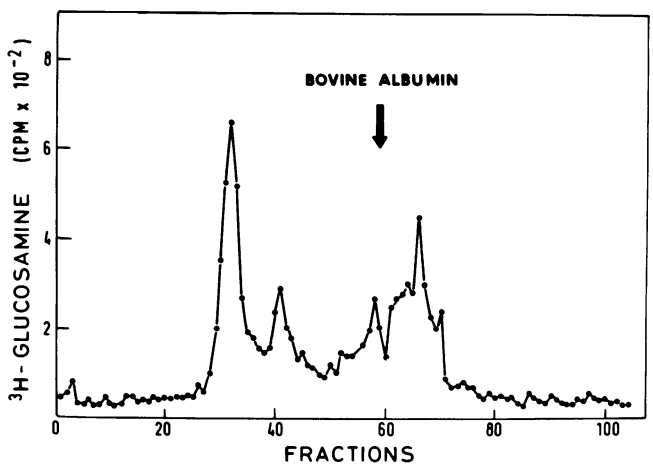

FIG. 6. *Polyacrylamide gel electrophoresis analysis of HSV structural glycopeptides. Sample from peak 3 region of Fig. 5 was analyzed. Bovine albumin serves as a molecular weight (68,000) marker.*

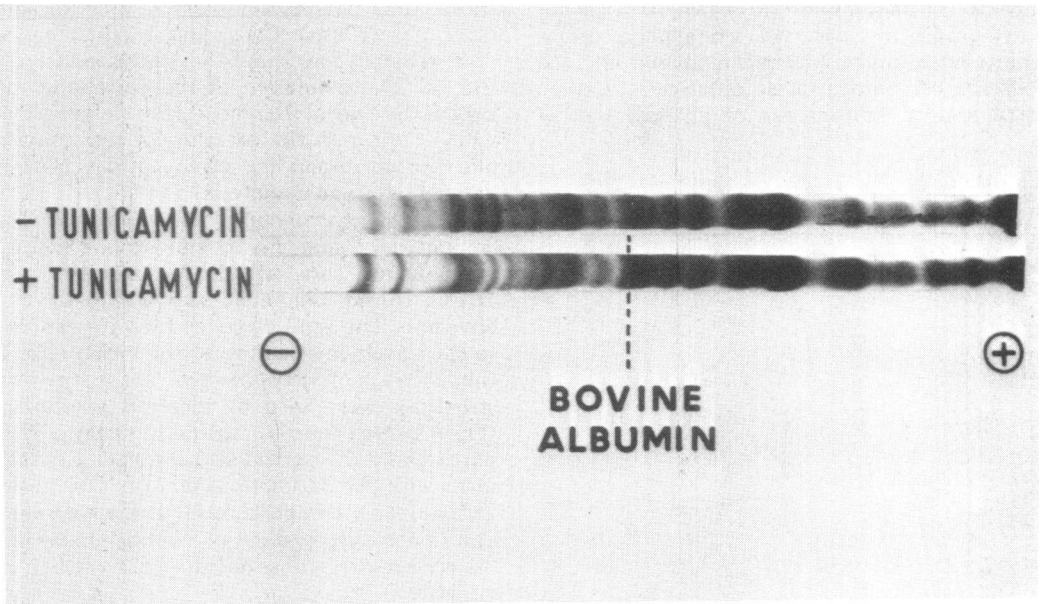

FIG. 7. *Polyacrylamide gel electrophoresis analysis of viral polypeptides. The infected cells were labeled with [^{35}S]methionine and harvested at 23 h postinfection. Radioautographs of the gels are shown.*

coma and influenza) (15, 20), in others (Sindbis, Semliki Forest, vesicular stomatitis, and fowl plague viruses), viral particles are almost undetectable (10, 20). With regard to vesicular stomatitis virus, it was mainly attributed to the change in the conformation and solubility of protein G (10, 11). More recent studies have demonstrated that even in the absence of glycosylation, a small fraction of the virus migrated to the plasma membrane and eventually was able to function as an infectious virus (5). In fowl plague virus-infected cells, the polypeptides that failed to become glycosylated were unstable and degraded soon after their synthesis (20).

A previous study (1) using 2-deoxy-D-glucose as a glycosylation inhibitor suggested that the oligosaccharide units of the viral glycoproteins are needed for the synthesis of infectious HSV. However, since this compound acts at multiple sites in the cell metabolism, by serving as a mannose analog in glycoprotein biosynthesis (8) or lowering the cellular pool of adenosine 5'-triphosphate (26), it was difficult to determine the specific effect of the compound on the virus.

In the present study, we observed the inhibition of HSV growth in BSC1 cells by tunicamycin. HSV differs from the viruses studied so far with regard to their growth inhibition by tunicamycin, in that its nucleic acid is DNA and most of its maturation and membrane envelopment processes are carried out in the nucleus of the cell. We observed the effect of tunicamycin on the biosynthesis of HSV. The newly made DNA was only partially inhibited, whereas the synthesis of the host cell DNA was not affected by tunicamycin. The overall synthesis of the viral polypeptides proceeded without a marked interruption in the presence of tunicamycin. The antibiotic caused only a 23% decrease between 3 and 7 h postinfection in the trichloroacetic acid-precipitable [^{35}S]methionine-labeled polypeptides (E. Katz, unpublished data). The viral DNA made in the presence of tunicamycin is encapsidated with proteins, thus acquiring resistance to deoxyribonuclease. The most prominent effect of tunicamycin in HSV-treated cells is the inhibition of glycosylation of the viral glycopeptides. In spite of the lack of the carbohydrate moiety from the viral glycopeptides, the polypeptide components are available and participate in virus assembly. Together with the other viral polypeptides and the viral DNA, they are capable of forming membrane-surrounded virus structures which show similar morphology to intact virus particles, as demonstrated by electron microscopy. The glycosylated component of the glycopeptides of HSV seems to play an essential role in processes involved in virus infectivity. Among such processes are attachment, penetration into the host cell, and uncoating of the viral particles. Experiments that will further clarify the effect of the failure in glycosylation on virus infectivity are currently under way in our laboratory.

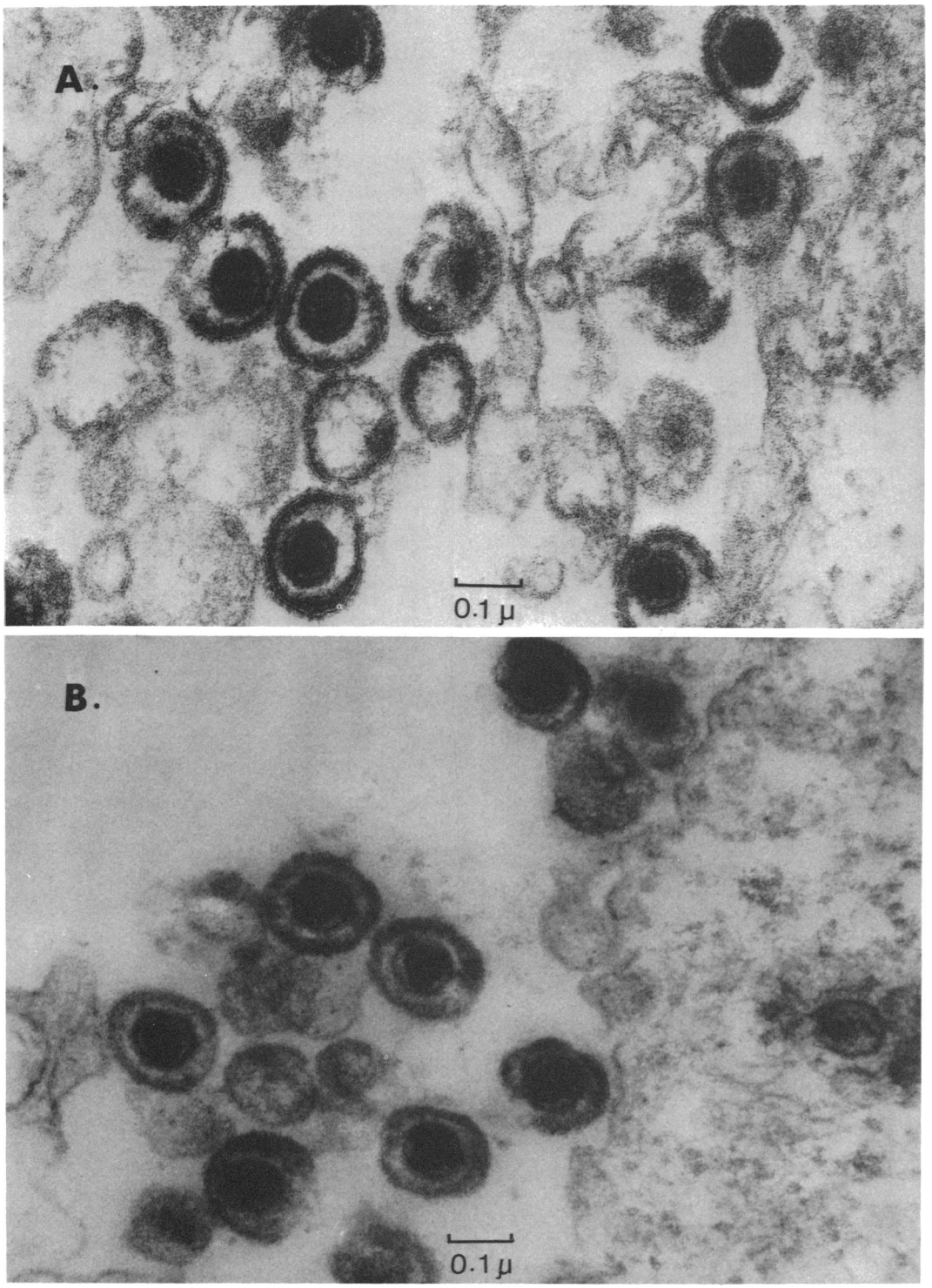

FIG. 8. *Electron micrographs of infected cells untreated (A) and treated with tunicamycin (0.5 μg/ml) (B).*

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