

## Tunicamycin Inhibits Glycosylation and Multiplication of Sindbis and Vesicular Stomatitis Viruses

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Tunicamycin (TM), an antibiotic that inhibits the formation of *N*-acetylglucosamine-lipid intermediates, thereby preventing the glycosylation of newly synthesized glycoproteins, inhibits the growth of Sindbis virus and vesicular stomatitis virus in BHK cells. At 0.5  $\mu\text{g}$  of TM per ml, the replication of both viruses is inhibited 99.9%. Noninfectious particles were not detected. All the viral proteins were synthesized in the presence of TM, but the glycoproteins were selectively altered in that they migrated faster than normal viral glycoproteins when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggesting defective glycosylation. Within 1 h after TM addition, [ $^{14}\text{C}$ ]glucosamine incorporation into glycoproteins was inhibited 20%, whereas [ $^{35}\text{S}$ ]methionine incorporation was unaffected. By 2 to 3 h after TM addition, glucosamine incorporation had fallen to 15% of control value, with methionine incorporation being 60% of normal. TM did not affect the growth of the nonenveloped encephalomyocarditis virus in BHK cells, demonstrating that TM is not a general inhibitor of protein synthesis. These data demonstrate that TM specifically inhibits the glycosylation of viral glycoproteins and that glycosylation may be essential for the normal assembly of enveloped viral particles.

Sindbis virus and vesicular stomatitis virus (VSV) are enveloped RNA viruses that are assembled by budding from cellular membranes during virus maturation. Sindbis virus contains three proteins: two envelope glycoproteins (E1, E2) and a nucleocapsid protein (C) (21, 29). During cellular maturation of Sindbis, another virus-specified glycoprotein (PE2), a precursor to E2, can be detected (23). VSV contains five viral polypeptides (35): a membrane glycoprotein (G), a nonglycosylated membrane protein (M), a nucleocapsid protein (N), the viral transcriptase (L), and a nonstructural protein (NS). The latter three proteins are nonmembrane components. The M protein is thought to line the inner surface of the virion envelope, whereas G, like E1 and E2 of Sindbis, is found on the outer surface of the virus, presumably comprising the spikes seen under the electron microscope (5, 6, 18). No additional viral proteins are detected in VSV-infected cells, and there is no evidence for the existence of a precursor for the glycoprotein (35).

Previous studies using 2-deoxy-D-glucose and D-glucosamine, compounds that inhibit glycosylation of glycoproteins, have suggested that the oligosaccharide units of the viral glycoproteins are necessary for the formation of infectious virus (8, 11-13, 25-27). However, since both of these compounds affect cellular processes other

than glycosylation, the data obtained do not allow unequivocal interpretations (2, 3, 26, 36).

Tunicamycin (TM) is a glucosamine-containing antibiotic with potent antiviral activity (30, 32, 33). In studies with Newcastle disease virus (NDV), Takatuski and Tamura found TM to preferentially interfere with glycoprotein biosynthesis (33). In addition, TM inhibits glycoprotein synthesis in mammalian (33) and microbial cells (4, 14, 30, 32, 33). Recently, TM has been shown to inhibit the formation of *N*-acetylglucosamine-lipid intermediates in calf liver and chicken embryo microsomes (31, 34) and in *Bacillus subtilis* (4). Since these lipid intermediates serve as sugar donors for the synthesis of the core regions of the oligosaccharide units of glycoproteins (16), this finding provides an explanation for the selective inhibition of glycosylation of newly synthesized glycoproteins.

We have used TM to study the role of the oligosaccharide units of Sindbis virus and VSV glycoproteins in viral replication and particle formation. Our results demonstrate that the carbohydrate moieties of viral glycoproteins are necessary for virion formation.

### MATERIALS AND METHODS

**Materials.** [ $^3\text{H}$ ]glucosamine (48 mCi/mmol) was purchased from New England Nuclear Corp., Bos-

ton Mass. [<sup>35</sup>S]methionine (270 Ci/mmol) and NCS tissue solubilizer were purchased from Amersham/Searle Corp., Arlington Heights, Ill. Na<sup>125</sup>I (100 mCi/ml), reagent grade, was obtained from Mallinckrodt, Inc., St. Louis, Mo. Pronase was purchased from Calbiochem, Los Angeles, Calif. Minimal essential medium (MEM) and fetal calf serum were purchased from K. C. Biological, Inc., Lenexa, Kan., and alpha MEM was obtained from Flow Laboratories, Rockville, Md. Glutamine, penicillin, and streptomycin were purchased from Sigma Chemical Co., St. Louis, Mo. TM was a gift of G. Tamura of the University of Tokyo, Tokyo, Japan. Encephalomyocarditis (EMC) virus was a gift of R. Thach and Peter Yau of Washington University, St. Louis, Mo.

**Cells.** BHK cells were grown as monolayers in MEM supplemented with 6% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml.

Chinese hamster ovary (CHO) cells were grown as monolayers in alpha MEM supplemented with 10% fetal calf serum, 50 U of penicillin per ml, and 50 µg of streptomycin per ml.

**Growth of virus.** Nearly confluent monolayers of cells growing on 35-mm plastic tissue culture petri dishes were infected with Sindbis virus at a multiplicity of infection of 200 to 400 PFU per cell. The virus was allowed to adsorb to the cells for 1 h at 37°C in a volume of 0.2 ml, followed by removal of the unadsorbed inoculum and the addition of 2 ml of fresh medium. Virus was harvested 12 to 16 h after infection. For VSV growth, the same procedure was followed except that the multiplicity of infection was 10 to 40 PFU/cell.

For EMC growth, we used the same procedure with the following exceptions: virus at a multiplicity of infection of 20 PFU/cell was added to the monolayer and allowed to adsorb for 1 h at room temperature in room air. The dishes were then placed in a 37°C incubator under a 5% CO<sub>2</sub>-95% air atmosphere. In these experiments, the time of the shift-up to 37°C is referred to as "hours postinfection." The unattached virus was removed at 1.5 h postinfection, and 2 ml of fresh medium was added. Virus was harvested at the times indicated in Table 3.

**Time of addition of TM to infected cells.** Unless indicated otherwise, TM at 0.5 µg/ml was added to Sindbis virus-infected cells at 2 h after infection, to VSV-infected cells at 1 h, and to EMC virus-infected cells at 1.5 h.

**Radioactive labeling of virus.** To label Sindbis virus with [<sup>35</sup>S]methionine the MEM added after removal of unadsorbed virus lacked amino acids. At 5 h after infection, 5 to 10 µCi of [<sup>35</sup>S]methionine or 25 µCi of [<sup>3</sup>H]glucosamine was added per plate. To label VSV, the same procedure was followed except that the label was added 4 h after infection.

**Radioactive labeling of infected cells.** Infected cells were incubated with complete medium containing 5 to 10 µCi of [<sup>35</sup>S]methionine or 25 µCi of [<sup>3</sup>H]glucosamine. The time of addition of label is indicated for each experiment. For the double-label experiments, 25 µCi of [<sup>3</sup>H]glucosamine and 10 µCi of [<sup>35</sup>S]methionine were added per plate at the indicated times.

**Virus purification.** Viruses were purified by centrifugation to equilibrium in a sucrose gradient (22). A 2-ml portion of medium was layered onto the following gradient: 1 ml of 60% sucrose, 5 ml of 30 to 50% sucrose (continuous gradient), and 4 ml of 15% sucrose. The sucrose solutions contained 0.1% bovine serum albumin. The gradients were spun at 100,000 × *g* for 2 h in an International centrifuge using an SB283 rotor at 4°C.

**Determination of PFU.** Harvested media were diluted and applied onto appropriate monolayers. Sindbis virus was titered on chicken embryo fibroblasts and VSV on mouse L cells. In most cases, samples were titered within 12 h of the experiment and were not frozen.

**Hemagglutination.** EMC virus hemagglutinating activity is proportional to the concentration of infectious virus. Medium to be assayed was removed from the monolayer at the indicated times, and the hemagglutinating activity of a 50-µl sample was determined as described by Martin et al. (19). Human erythrocytes were used for the assay. The hemagglutination titer is expressed as the reciprocal of the final dilution scored as positive.

**SDS-polyacrylamide electrophoresis.** Purified Sindbis virus, VSV, and VSV-infected cells were prepared for electrophoresis by reduction and boiling in 1% sodium dodecyl sulfate (SDS) (10), whereas Sindbis-infected cells were reduced and alkylated prior to electrophoresis (23). Samples were subjected to electrophoresis on discontinuous slab gels (23) or cylindrical gels (10). The slab gels were subjected to autoradiography, and the cylindrical gels were sliced and counted. Strips of autoradiograms were scanned at 600 nm in a Gilford spectrophotometer equipped with a linear transport device. Samples labeled with <sup>125</sup>I were counted directly in a gamma counter, but samples labeled with <sup>35</sup>S were solubilized as described by Ames (1) prior to counting in a liquid scintillation counter.

**Iodination of infected cells.** Cells were iodinated using a slight modification of the lactoperoxidase method, which has been described previously (28).

**Protein determination.** Protein was determined by the method of Lowry et al. (17).

## RESULTS

**Inhibition of VSV and Sindbis virus multiplication by TM.** TM is a potent inhibitor of Sindbis virus and VSV multiplication, producing 99.9% inhibition in the yield of virus at 0.5 µg/ml (Tables 1 and 2). At this concentration of TM, very little radioactivity is released into the medium and virtually none of it sediments to the density of virions when samples of the medium are subjected to sucrose gradient centrifugation. Figure 1 shows these data for VSV; identical results have been obtained with Sindbis virus. Lower concentrations of TM also produce a significant inhibition of both PFU and radioactivity released into the medium.

Particles released in the presence of low doses of TM sedimented in the same region of the sucrose gradient as virus purified from control cells (data not shown). The inhibition of infectious particle formation (PFU) correlates closely with the inhibition of particle formation, indicating that noninfectious particles are not formed in the presence of TM.

**Time of addition of TM to infected cells.** Addition of TM to Sindbis-infected BHK cells within the first 2 h postinfection appears to inhibit virus production maximally (Fig. 2). However, TM added as late as 4 h after infection still causes 80% inhibition. Takatsuki and Tamura have shown that TM does not affect adsorption of NDV to host cells (32). Our data support this observation, since we see no significant difference in inhibition when the antibiotic is added along with the infecting virus or 2 h postinfection. Based on this experiment, TM was added routinely to Sindbis-infected cells 2 h postinfection. In experiments performed with VSV, a virus that replicates more

TABLE 1. Inhibition of the multiplication of Sindbis virus as a function of the concentration of TM

TM ( $\mu\text{g}/\text{ml}$ )	PFU/ml ( $\times 10^{-9}$ )	% Inhibition of PFU	cpm/0.025 ml <sup>a</sup>	% Inhibition of cpm	cpm/PFU ( $\times 10^5$ )
0	9.0		16,092		7.1
0.03	3.6	60	5,733	64	6.4
0.05	1.8	81	4,423	72	9.8
0.075	1.7	82	1,903	88	4.4
0.15	0.49	95	1,090	93	8.8
0.3	0.15	98	572	96	15.3
0.5	0.018	99.8	0	100	

<sup>a</sup> To determine counts per minute of [<sup>35</sup>S]methionine, a 0.025-ml sample of the medium was precipitated in trichloroacetic acid, filtered, and counted. A correction for background counts was made by subtracting the counts (1,384) obtained from the plate containing 0.5  $\mu\text{g}$  of TM per ml. This is valid, since virtually none of the counts in this sample sedimented in the virus region on equilibrium sucrose gradient centrifugation.

TABLE 2. Inhibition of multiplication of VSV as a function of the concentration of TM

TM ( $\mu\text{g}/\text{ml}$ )	PFU/ml ( $\times 10^{-8}$ )	% Inhibition of PFU	cpm/ml <sup>a</sup>	% Inhibition of cpm	cpm/PFU ( $\times 10^4$ )
0	8.0	0	84,668	0	1.1
0.05	0.75	90.6	10,627	87	1.4
0.1	0.12	98.5	1,298	97.5	1.1
0.5	0.006	99.9	0	100	

<sup>a</sup> The determination of counts per minute of [<sup>35</sup>S]methionine and the correction for background counts were performed as described in footnote a, Table 1. In the presence of 0.5  $\mu\text{g}$  of TM per ml, the acid-insoluble cpm/ml was 7,900. Essentially none of these counts sedimented in the virus region on equilibrium sucrose gradient centrifugation (see Fig. 1).

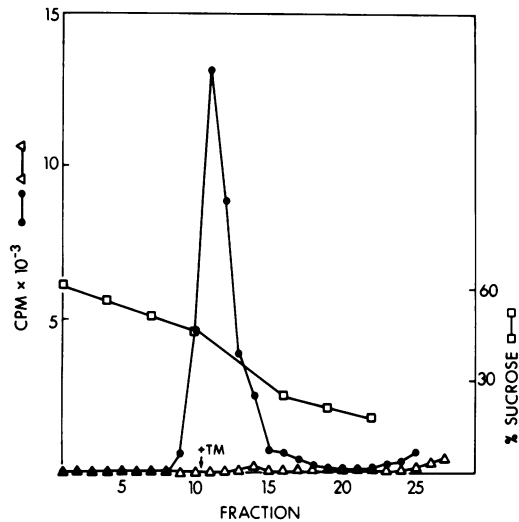


FIG. 1. Purification of [<sup>35</sup>S]methionine-labeled particles released from VSV-infected cells by centrifugation in a sucrose gradient. TM (0.5  $\mu\text{g}/\text{ml}$ ) was added to cells 1 h postinfection. [<sup>35</sup>S]methionine was added to cells 4 h postinfection, and the medium was harvested at 16 h postinfection. A portion of the medium was taken to determine PFU, and the remainder was sedimented in a sucrose gradient. A portion from each fraction was precipitated with trichloroacetic acid and counted. Symbols: ●, control media from untreated, infected cells; △, media from TM-treated, infected cells.

rapidly than Sindbis, TM was added 1 h postinfection.

**Inhibition of virus yield after removal of TM.** To determine if the effect of TM could be reversed, virus-infected cultures were exposed to TM for various periods of time, then the cells were washed and incubated in medium free of TM, and virus yield was determined at 13 h. Sindbis virus-infected cultures exposed to TM for only 30 min showed 68% inhibition of infectious virus formation, whereas a 60-min exposure to TM gave 96% inhibition (Fig. 3). Cultures exposed to TM for 2 h or longer were inhibited greater than 99.9%, and this effect was not reversible. The same results were obtained with VSV-infected cells. These results are in agreement with the finding of Kuo and Lampen (15), who reported that inhibition of glycoprotein synthesis in yeast protoplasts is not eliminated by washing out the TM.

**Synthesis of viral-specific proteins in infected cells.** Since TM at a concentration of 0.5  $\mu\text{g}/\text{ml}$  completely inhibited the release of particles from infected cells, we next analyzed the proteins synthesized in the infected cells to see if abnormal viral proteins were being made. Cells were infected with either Sindbis or VSV

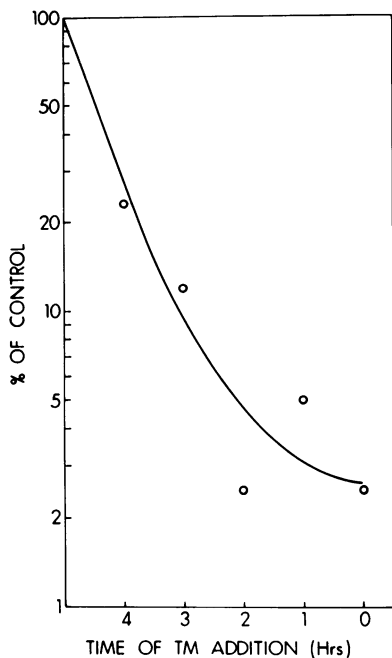


FIG. 2. Inhibition of the formation of Sindbis virus as a function of the time of addition of TM. Cells were infected, and TM (0.5  $\mu\text{g/ml}$ ) was added at various times after infection. At 17 h postinfection, the medium was harvested and titered to determine the yield of infectious virus.

and labeled with [ $^{35}\text{S}$ ]methionine in the presence and absence of TM. At appropriate times after infection, the cell monolayer was washed with phosphate-buffered saline, and the cells were solubilized in 2% SDS. Newly synthesized proteins were analyzed by electrophoresis on polyacrylamide slab gels in the presence of SDS followed by autoradiography. In Sindbis virus-infected cells, four viral proteins can be seen, corresponding to PE2, E1, E2, and C (Fig. 4). In contrast, Sindbis virus-infected, TM-treated cells show three major bands. One band corresponds to the viral capsid, whereas the other two bands migrate faster than PE2 and E1 and are presumably nonglycosylated forms of the viral glycoproteins. These rapidly migrating envelope proteins are similar to those seen by Duda and Schlesinger in Sindbis-infected cells treated with glucosamine (8). These workers found that the faster-moving bands had tryptic peptide maps almost identical to those of the normal viral envelope proteins. The proteins formed in the presence of TM appear to be stable, since chases of up to 1 h did not alter the gel pattern (data not shown). Little or no conversion of PE2 to E2 was seen in TM-treated

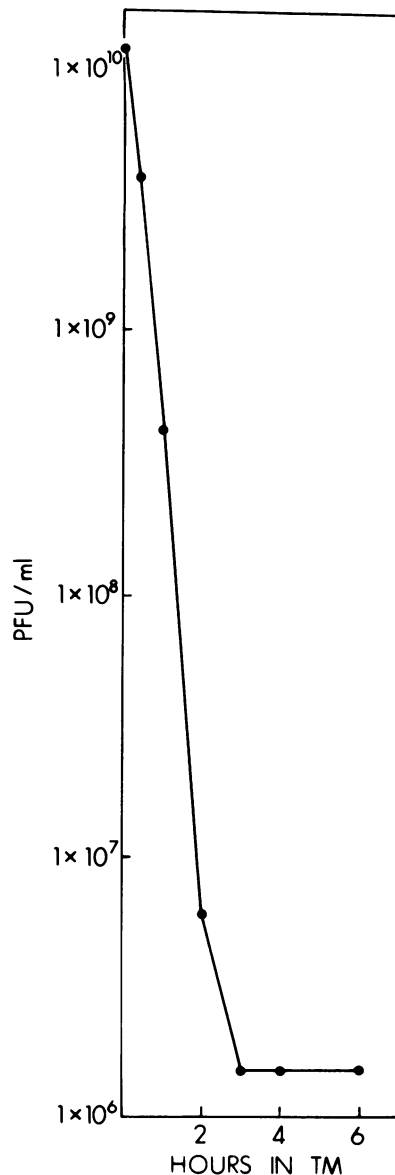


FIG. 3. Effect of removing TM on the yield of Sindbis virus. TM (0.5  $\mu\text{g/ml}$ ) was added to Sindbis virus-infected cells 2 h postinfection. At the indicated times, the drug-containing medium was removed from the plate, the monolayers were washed two times with warm medium, and 2 ml of fresh medium was added. The medium was harvested at 13 h postinfection and titered to determine the yield of infectious virus.

cells, and no movement of labeled bands toward higher-molecular-weight species was observed during chases after removal of TM. Identical results were obtained with both BHK and CHO cells. The remaining experiments were per-

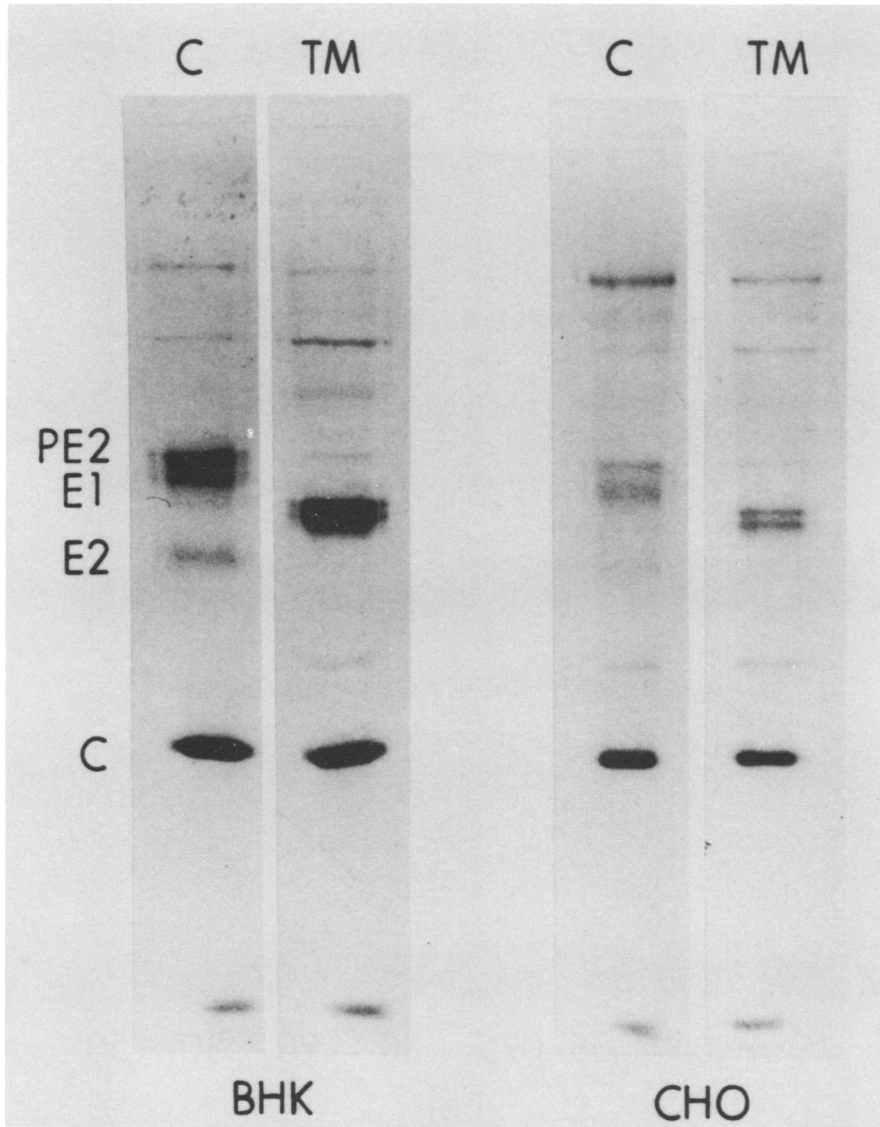


FIG. 4. Autoradiograms of SDS-polyacrylamide gels of [ $^{35}\text{S}$ ]methionine labeled, Sindbis-infected BHK cells and CHO cells in the presence and absence of TM. C represents control infected cells, and TM indicates that TM (0.5  $\mu\text{g}/\text{ml}$ ) was added 4 h postinfection. [ $^{35}\text{S}$ ]Methionine was added 6 h postinfection, and the cells were harvested 7 h postinfection.

formed with BHK cells because the virally induced inhibition of host protein synthesis was usually more pronounced in these cells than in CHO cells.

In VSV-infected cells, bands corresponding to L, G, N, NS, and M were seen (Fig. 5). The pattern was identical in cells treated with TM, except for the disappearance of band G and the appearance of a faster-migrating band. This is similar to the pattern seen by Printz and Wag-

ner (20) with their temperature-sensitive VSV mutant *ts52* at the nonpermissive temperature. The glycoprotein of *ts52* is thought to be nonglycosylated at the nonpermissive temperature, although the primary defect is not known.

**[ $^3\text{H}$ ]glucosamine uptake and incorporation into protein in infected cells.** To obtain more direct evidence that TM was selectively blocking glycosylation of the viral glycoproteins, we studied the effect of TM on the uptake and

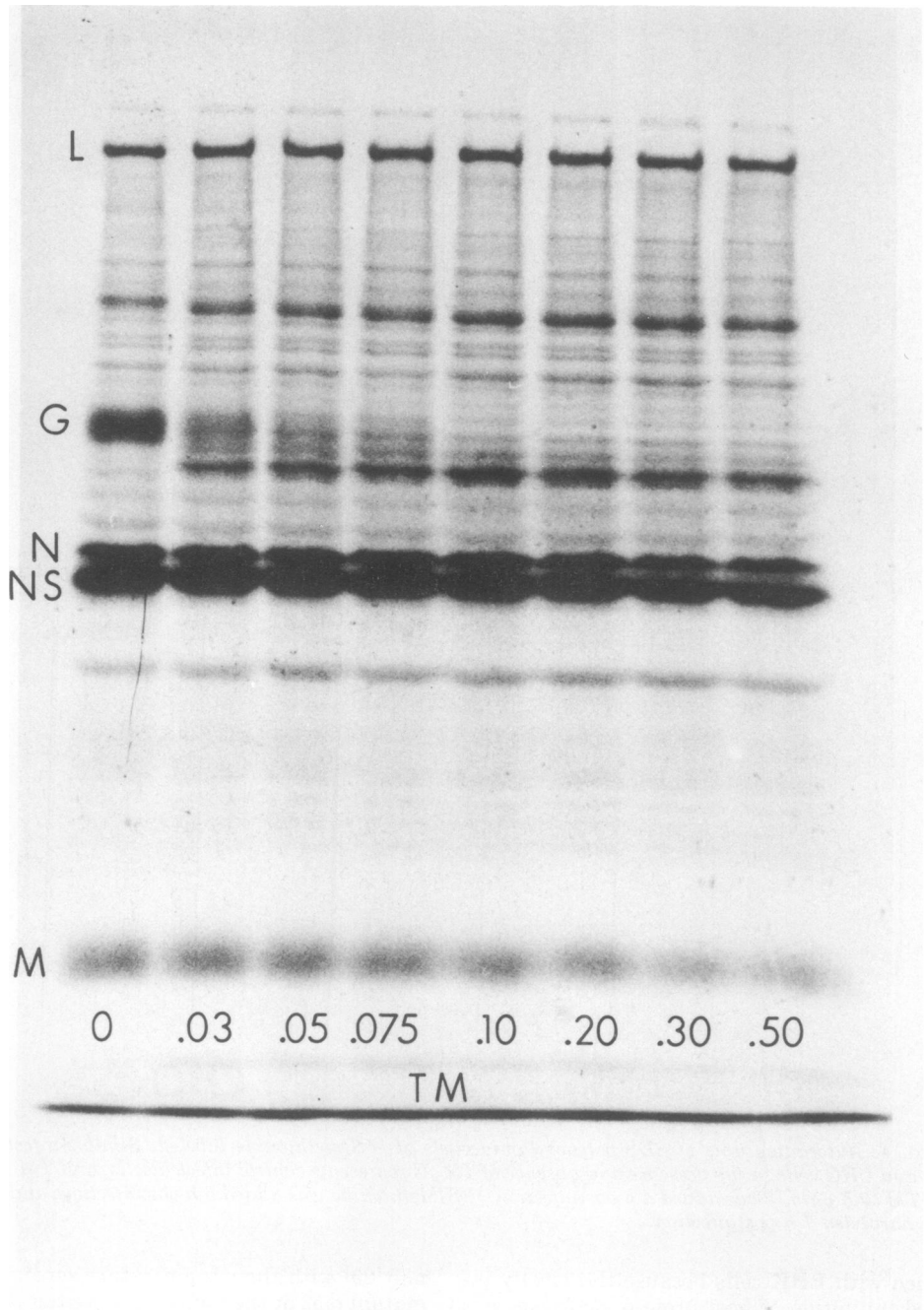


FIG. 5. Autoradiograms of SDS-polyacrylamide gels of [ $^{35}\text{S}$ ]methionine-labeled BHK cells infected with VSV. TM was added 1 h postinfection. [ $^{35}\text{S}$ ]methionine was added 4 h postinfection, and cells were harvested 7 h postinfection. The amount of TM added is expressed as micrograms per milliliter.

incorporation of [ $^3\text{H}$ ]glucosamine and [ $^{35}\text{S}$ ]methionine in infected cells. With both VSV- and Sindbis virus-infected cells, 0.5  $\mu\text{g}$  of TM per ml inhibited [ $^3\text{H}$ ]glucosamine incorpo-

ration into trichloroacetic acid-precipitable material by approximately 85% after 2 to 4 h (Fig. 6). TM had no effect on the total uptake of [ $^3\text{H}$ ]glucosamine, indicating that TM was not

blocking glucosamine transport into the cells (data not shown). To determine if the inhibition of [ $^3\text{H}$ ]glucosamine incorporation into the viral glycoproteins could be explained by a block in the formation of UDP-*N*-acetylglucosamine, the extracts of [ $^3\text{H}$ ]glucosamine-labeled cells were analyzed by paper chromatography (Fig. 7). The size of the UDP-*N*-acetylglucosamine pool was identical in treated and untreated Sindbis virus-infected cells. In each case, approximately 70% of the label was present as UDP-*N*-acetylglucosamine. Kuo and Lampen have made a similar observation in yeast protoplasts treated with TM (15).

We have also observed a decrease in [ $^{35}\text{S}$ ]methionine incorporation into trichloroacetic acid-precipitable material in TM-treated cells (Fig. 6). However, the inhibition of protein synthesis occurs after the decrease in glucosamine incorporation, and the former effect is not as marked as the latter (Fig. 6). In Sindbis-infected cells, the rate of incorporation of [ $^{35}\text{S}$ ]methionine is normal during the first hour after TM addition, whereas glucosamine incorporation is significantly decreased over this interval. This suggests that the observed decrease in protein synthesis is secondary to the derangement in glycoprotein synthesis. In other experiments we found that TM does not inhibit uridine incorporation into RNA (data not shown); this is supported by the following EMC virus experiments.

**Effect of TM on the multiplication of non-enveloped virus.** The effect of TM on both the multiplication of EMC virus and on the incorporation of [ $^{35}\text{S}$ ]methionine into infected cells was tested to rule out the possibility that a primary site of TM action is inhibition of pro-

tein synthesis or inhibition of viral RNA replication. EMC is a single-stranded RNA virus, like Sindbis virus and VSV, but it has no outer envelope and contains no viral glycoproteins. BHK cell monolayers were infected with EMC as described above, and TM was added after adsorption of the virus. The cells were labeled with [ $^{35}\text{S}$ ]methionine during the indicated time periods, and virus formation was determined at 11.5 h postinfection using a hemagglutination

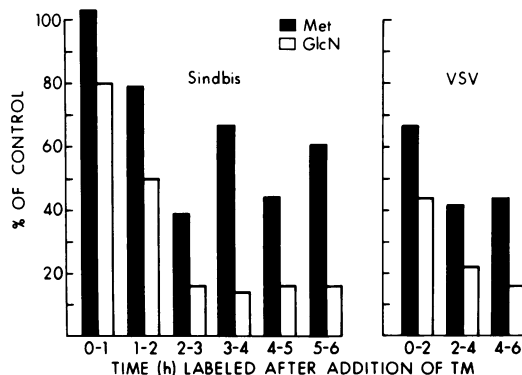


FIG. 6. Rate of glucosamine and methionine incorporation into virus-infected cells in the presence of TM. Cells were infected with either Sindbis virus or VSV, and TM ( $0.5 \mu\text{g/ml}$ ) was added at 2 or 1 h postinfection, respectively. Single plates were double labeled with [ $^{35}\text{S}$ ]methionine and [ $^3\text{H}$ ]glucosamine for the indicated time intervals. At the end of the labeling period, monolayers were solubilized in 2% SDS, and total radioactivity, trichloroacetic acid-precipitable radioactivity, and total protein were determined. The radioactivity incorporated was expressed as a function of total protein to correct for differences between plates.

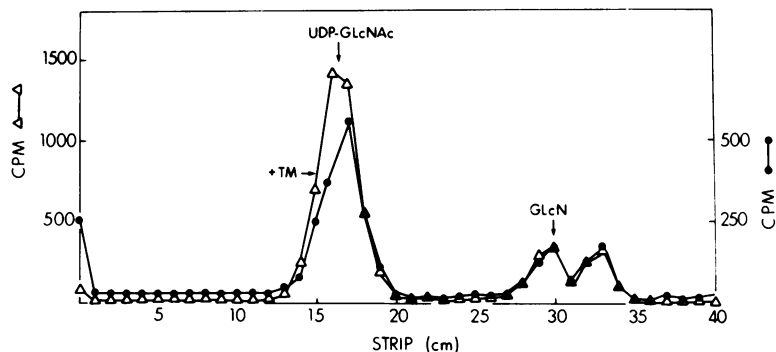


FIG. 7. Formation of UDP-*N*-acetylglucosamine in the presence of TM. Cells were infected with Sindbis virus and treated with TM ( $0.5 \mu\text{g/ml}$ ) 2 h later. [ $^3\text{H}$ ]glucosamine was added at 6 h postinfection. At 8 h, the cells were solubilized in 2% SDS. Samples were concentrated and applied to Whatman 3MM chromatography paper. After descending chromatography in 1 M ammonium acetate-95% ethanol (3:7 by volume) for 18 h, the paper was dried, cut into 1-cm strips, and counted. Symbols: ●, control: infected cells; Δ, infected cells treated with TM.

assay. Both virus yield and the rate of protein synthesis in infected cells were unaffected by doses of TM up to 0.5  $\mu\text{g/ml}$  (Table 3). From these data we concluded that TM has no effect on the multiplication of EMC virus.

**Synthesis of viral proteins in infected cells at low doses of TM.** We next analyzed the proteins synthesized in infected cells treated with low doses of TM to determine if the inhibition of virus formation could be correlated with the appearance of nonglycosylated viral glycoproteins. Concentrations of TM ranging from 0.03 to 0.1  $\mu\text{g/ml}$  caused a progressive decrease in the amount of fully glycosylated G and the appearance of increasing amounts of nonglycosylated G in VSV-infected BHK cells (Fig. 5). At TM doses greater than 0.1  $\mu\text{g/ml}$ , predominantly nonglycosylated G was detected. The progressive decrease in the formation of glycosylated G correlated well with the inhibition of virus formation (Fig. 8). In contrast, the observed inhibition of protein synthesis did not correlate with the inhibition of virus formation. The level of inhibition of protein synthesis observed at 0.03  $\mu\text{g}$  of TM per ml was not significantly increased at higher concentrations of the drug, whereas the yield of PFU decreased nearly 100-fold between 0.03 and 0.5  $\mu\text{g}$  of TM per ml (Fig. 8). Similar results have been obtained with Sindbis virus-infected cells (data not shown).

**Labeling of infected cells by lactoperoxidase.** Lactoperoxidase iodination of intact cells has been used to identify viral glycoproteins on the infected cell surface (28). We used this method to determine if nonglycosylated viral glycoproteins were inserted into the plasma membrane in a normal fashion. The nonglycosylated glycoproteins of both Sindbis virus and VSV were inaccessible to lactoperoxidase labeling, whereas the normal viral glycoproteins were heavily labeled, being the dominant membrane protein iodinated by the enzyme (Fig. 9).

TABLE 3. Effect of TM on EMC multiplication

TM ( $\mu\text{g/ml}$ )	$^{35}\text{S}$ Met incorporation: cpm/mg of protein <sup>a</sup> ( $\times 10^{-5}$ )		Hemagglutination per 0.05 ml of medium
	5-7 <sup>b</sup>	9-11.5	
0	6.1	4.3	4,096
0.1	6.6	5.2	4,096
0.5	6.2	5.6	4,096

<sup>a</sup>  $^{35}\text{S}$ Methionine ( $^{35}\text{S}$ Met) was added either at 5 or 9 h postinfection for a 2- or 2.5-h pulse, respectively.

<sup>b</sup> Hour postinfection.

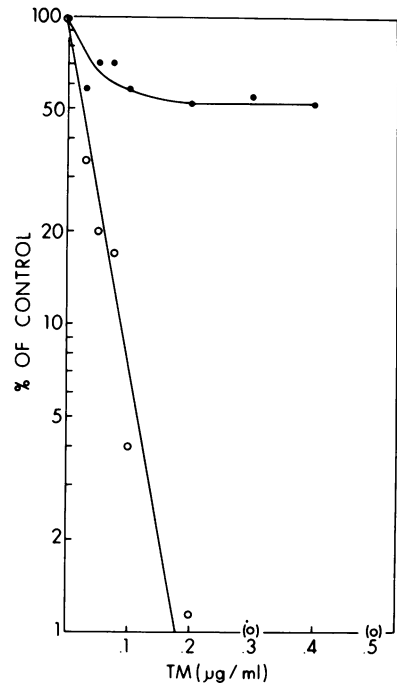


FIG. 8. Rate of protein synthesis and inhibition of infectious VSV at various concentrations of TM. BHK cells were infected with VSV, and TM was added 1 h later.  $^{35}\text{S}$ methionine was added 4 h postinfection, and the cells and medium were harvested 3 h later. Media were titered to determine the yield of infectious virus. A portion of the solubilized cells was precipitated with trichloroacetic acid and the radioactivity was determined. The rate of protein synthesis is expressed as counts per minute per milligram of protein. Symbols: ●, radioactivity incorporated; ○, PFU/ml.

Cells labeled as late as 13 h post-infection gave identical patterns, indicating that the nonglycosylated glycoproteins fail to reach the outside of the plasma membrane rather than just being delayed. We cannot, however, exclude the possibility that these proteins are on the cell surface but fail to label because of a conformational change caused by the lack of carbohydrate.

## DISCUSSION

The role of the carbohydrate portion of viral envelope glycoproteins has been studied by two different approaches. The first is to grow virus in a host cell that restricts oligosaccharide synthesis due to a defined host cell defect. Sindbis virus and VSV grown in a line of CHO cells deficient in a specific UDP-*N*-acetylglucosamine-glycoprotein *N*-acetylglucosaminyl trans-



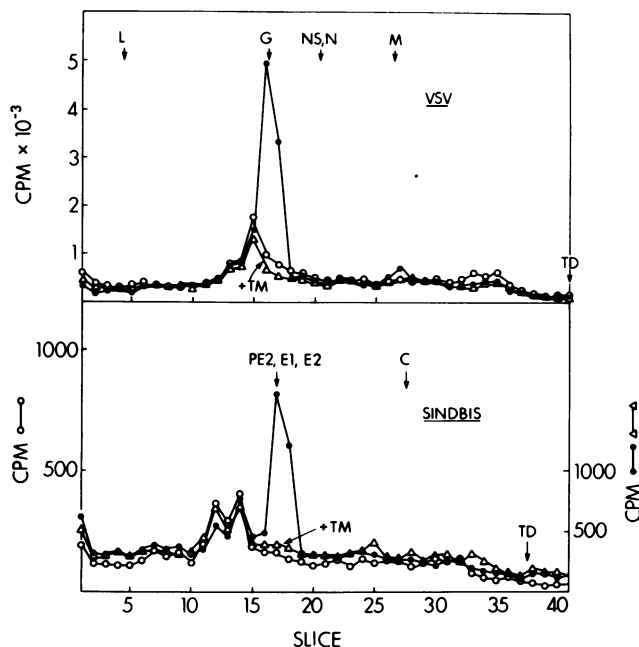


FIG. 9. Lactoperoxidase labeling of infected cells. Cells were infected with Sindbis virus or VSV, and TM ( $0.5 \mu\text{g/ml}$ ) was added 2 or 1 h postinfection, respectively. Sindbis virus-infected cells were iodinated at 8 h postinfection, and VSV-infected cells were iodinated at 6 h. Symbols: ●, control infected cells not treated with TM; △, infected cells treated with TM; ○, uninfected cells.

ferase (22) produce normal amounts of infectious virus although the viral glycoproteins contained incomplete oligosaccharide chains (22). These data demonstrated that the outer sugars of the viral glycoprotein oligosaccharide units are not essential for normal virus production. Schloemer and Wagner showed that VSV grown in mosquito cells is deficient in sialic acid and has reduced infectivity, presumably due to a defect in adsorption (24).

The second approach is to use inhibitors of glycosylation in virus-infected cells. This approach has been used by many groups, who have found that 2-deoxy-D-glucose and D-glucosamine inhibit glycosylation of viral glycoproteins and virus production (8, 11-13, 25-27). The problem with these two inhibitors is that they act at multiple sites within the cell, affecting various metabolic and biosynthetic processes. Thus, glucosamine, in addition to interfering with the glycosylation of glycoproteins, depletes the UTP pool of the host cell and affects viral RNA synthesis (2, 3). 2-Deoxy-D-glucose is incorporated into viral glycoproteins as a mannose analogue (11, 13), making it probable that under-glycosylated glycoproteins are being synthesized. In addition, 2-deoxy-glucose decreases the ATP pool of the cells (36). Therefore, it is difficult to determine if the inhibition

of virus production is due to impaired glycoprotein synthesis or the result of some other effect of the inhibitor.

The work of Tamura et al. (30, 32, 33) as well as the findings reported in this paper indicate that TM is an excellent agent for studying the function of the viral glycoprotein oligosaccharide units. TM appears to primarily affect the glycosylation of proteins. Furthermore, inhibition of the transfer of the linkage sugar *N*-acetylglucosamine to nascent proteins causes a complete block in glycosylation so that no ambiguities due to underglycosylation are encountered.

Previous work by Tamura and co-workers have demonstrated that TM has potent antiviral activity and that it preferentially interferes with glucosamine incorporation into glycoproteins in NDV-infected cells (33). Our data demonstrate that TM is also a potent inhibitor of VSV and Sindbis virus production. The polyacrylamide gel electrophoretic analysis of infected cells reveals that all the viral proteins are synthesized in the presence of TM but that the glycoproteins are selectively altered in that they migrate more rapidly than their counterparts from infected cells without TM. Given the known site of action of TM, this finding is most compatible with the formation of nonglycosyl-

ated glycoproteins, which are known to migrate as lower-molecular-weight forms on SDS-polyacrylamide gels (8). This interpretation is supported by our finding that the rapidly migrating glycoprotein band contained no [ $^3\text{H}$ ]glucosamine, demonstrating that it is devoid of carbohydrate (data not shown).

Although a significant amount of viral protein is synthesized in the presence of 0.5  $\mu\text{g}$  of TM per ml, we could not demonstrate the assembly of any viral particles, either infectious or noninfectious. At lower doses of TM (0.03 to 0.05  $\mu\text{g}/\text{ml}$ ), the viral particles that formed were fully infective and contained a normal amount of completely glycosylated glycoprotein along with a trace of protein that migrated in the region of the nonglycosylated glycoprotein (Leavitt, Schlesinger, and Kornfeld, unpublished observation). We cannot exclude the possibility that the normal virus is produced by a small population of TM-resistant cells. However, the presence of nonglycosylated glycoprotein in these particles argues against this point. It is possible that under certain conditions particles can be formed in the absence of glycoprotein. Deutsch has reported a temperature-sensitive mutant of VSV that appears to make noninfectious particles that contain no detectable G protein (7). On the other hand, Printz and Wagner have studied a temperature-sensitive mutant of G that makes no detectable particles at the nonpermissive temperature (20).

The modest inhibition of protein synthesis that occurs in Sindbis- and VSV-infected cells treated with TM is similar to that observed when TM is added to NDV-infected chicken embryo fibroblasts (33) and most likely is secondary to the inhibition of glycosylation. This interpretation is supported by two lines of evidence. First, in Sindbis-infected cells, TM addition results in the inhibition of glucosamine incorporation prior to the inhibition of [ $^{35}\text{S}$ ]methionine incorporation (Fig. 6). Second, TM does not inhibit the rate of protein synthesis in EMC-infected BHK cells. Although we cannot exclude the possibility that TM affects a step in protein synthesis unique to enveloped viruses, it seems more likely that the block in glycoprotein glycosylation is somehow affecting protein synthesis. The initial glycosylation is believed to occur while the nascent protein is still on the polysome, and impaired glycosylation might inhibit the release of the newly synthesized glycoprotein. If these polysomes were to accumulate it could cause a decrease in the number of available ribosomes, and this might lead to a general inhibition of protein synthesis. Regardless of the mechanism, it is clear

that the observed inhibition of protein synthesis cannot account for the striking inhibition of virus formation that is induced by TM.

The reason why the failure to glycosylate the viral glycoproteins results in impaired viral assembly is not clear. Since the proportion of newly synthesized viral proteins associated with the 100,000  $\times g$  pellet is essentially the same in both TM-treated and control infected cells, it is unlikely that there is a major alteration in the subcellular location of the various proteins (Leavitt et al., unpublished observation). However, the lactoperoxidase experiments demonstrate that the nonglycosylated glycoproteins of both Sindbis virus and VSV fail to reach the outside of the plasma membrane. It is likely, therefore, that the lack of carbohydrate is affecting the movement of the glycoproteins at some step between their site of synthesis on membrane-bound polyribosomes and their ultimate appearance in the plasma membrane. Studies are in progress to determine the subcellular location of the nonglycosylated glycoprotein molecules.

We conclude that although the synthesis of completed oligosaccharide units of VSV and Sindbis virus glycoproteins is not necessary for normal virus production (22), there may be some minimal amount of carbohydrate that is essential for the normal assembly of viral particles.

It is of interest that TM also inhibits the secretion of the glycoprotein enzyme invertase by yeast (14) and the secretion of immunoglobulin by various plasma cell tumors (S. Hickman, A. Kulczycki, and S. Kornfeld, unpublished observation). These data, together with the present findings, suggest that there may be an essential role for glycosylation in the normal processing of glycoproteins destined to become plasma membrane components or to be secreted. A number of possible mechanisms should be considered to explain this phenomenon. The nascent glycoproteins may bind to glycosyltransferases in the rough endoplasmic reticulum or the Golgi regions and not be released from these enzymes because the core sugars are not transferred to the protein. Alternatively, the lack of carbohydrate may alter the solubility of these proteins, causing aggregation and impaired mobility through the intracellular membranous system. Finally, the core sugars of the glycoproteins may have a positive role in the intracellular movements of these proteins, as originally postulated by Eylar (9). The subcellular fractionation studies now in progress should help to distinguish between these alterations.

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