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Effect of Glycosylation Inhibitors on the Release of Enveloped Vaccinia Virus

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Addition of 1 to 10 mM 2-deoxy-D-glucose (2-dg) or glucosamine (gln) to the growth medium of vaccinia virus-infected cells inhibited the release of extracellular enveloped vaccinia virus (EEV) without affecting the production of intracellular naked vaccinia virus (INV) particles. In contrast, INV infectivity (particles per PFU) was decreased sevenfold by 50 mM 2-dg. Treatment with 2-dg reduced but did not eliminate glycosylation of the INV 37,000-molecular-weight glycoprotein. The kinetics of sensitivity to inhibitor addition experiments and inhibitor reversal experiments indicated that EEV release was dependent on glycosylation before 8 h postinfection. This was supported by polyacrylamide gel electrophoretic analysis of the synthesis kinetics for cell membrane-associated vaccinia glycoproteins in 2-dg-inhibited infected cells. The dependence of vaccinia protein glycosylation before 8 h postinfection for efficient EEV release was observed in spite of the fact that the period of greatest glycoprotein synthesis was 8 to 12 h postinfection. The presence of 2-dg resulted in an incompletely glycosylated 89,000-molecular-weight glycoprotein, as indicated by a reduction in the apparent glycoprotein molecular weight. The morphological event affected by the inhibitors was the acquisition by INV of a double-membrane structure from the Golgi apparatus. This morphological intermediate is necessary for release of EEV.

Vaccinia virus infection of cells results in the production of two infectious forms. Large numbers of intracellular naked vaccinia (INV) amass inside the cell, but only 1 to 10% of these particles acquire an envelope and are released as extracellular enveloped vaccinia (EEV) (1). Although EEV is the less studied of the two virus forms, there has, nevertheless, accrued a considerable amount of information about EEV in recent years. The envelope of EEV contains one non-glycosylated protein and nine glycoproteins (16, 17). One of the glycoproteins has been identified as the vaccinia hemagglutinin (17, 18). The envelope is responsible for a much more rapid penetration of cells by EEV than the penetration observed for INV (21). The spread of virus infection in vitro is clearly caused by EEV, whereas EEV has been suggested to be involved in the in vivo dissemination of infection (1, 3, 18). The envelope has an antigenic composition different from that of INV (1, 3, 18, 22, 27), and antibodies specific for the envelope protect against both in vitro and in vivo infections (18).

Vaccinia virus acquires its envelope from infected cells by a unique mechanism (8, 15, 19). INV is not released by budding through the plasma membranes or by cell lysis (19). INV instead associates with cellular membranes morphologically similar to the Golgi apparatus and is wrapped by these membranes, thereby acquiring a double-membrane structure. Double-wrapped virions then migrate to the cell surface, where the outer membrane fuses with the plasma membrane, with the subsequent exgulfment of EEV composed of an internal virion enclosed in the inner Golgi membrane.

We have chosen to investigate the unique nature of vaccinia release by studying the requirements for virus release as revealed by the effects of inhibitors. N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) specifically inhibits release of EEV (12, 19) by blocking the formation of the double-membraned intracellular intermediate (18). The molecular point at which IMCBH affects the cell or the virus or both in blocking virus release has not been determined. In trying to elucidate the molecular events involved in the unique envelopment process, the effect of glucosamine (gln) and 2-deoxy-D-glucose (2-dg) on the release of EEV was investigated. These compounds are known to be inhibitors of protein glycosylation (reviewed in reference 23).

MATERIALS AND METHODS

Cells and virus. The rabbit kidney cell line RK-13 was cultivated in 25-cm^2 plastic flasks in minimum

essential medium–5% fetal calf serum. Cell monolayers were infected on day 3 or 4 postpassage with 0.3 ml of IHD-J vaccinia at a multiplicity of infection of 3 PFU per cell. After a 1-h incubation at room temperature, the unattached virus was removed, and the cells were washed twice with 2 ml of medium. Each flask was then further incubated (time zero) at 37°C with 5 ml of minimal essential medium–1% fetal calf serum. Glycoproteins were isotopically labeled, starting immediately after infection by the inclusion of 5 μCi of [^{3}H]gln per ml into medium, in which 90% of the glucose was replaced by fructose.

Compounds. The inhibitors gln and 2-dg (Sigma Chemical Co.) were dissolved in water at 1 M concentrations and stored frozen at −20°C.

Virus purification. INV was purified from Dounce-homogenized cells by ultracentrifugation on CsCl gradients as previously described (17).

The number of particles and the protein content in purified virus suspensions were determined by UV spectrophotometry (1 U of optical density at 260 nm = 1.2×10^{10} particles = 64 μg) (9).

Cell membrane purification. RK-13 cell membranes were purified by discontinuous sucrose ultracentrifugation of cells disrupted by Dounce homogenization (17). In experiments comparing membrane preparations in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), all preparations were adjusted to the same protein content.

Plaque assay. Extracellular virus released into the incubation medium during infection was assayed for infectivity after removal of free-floating cells by sedimentation at 1,500 rpm for 5 min. The cell sediments were pooled with cells scraped from the plastic flasks into 5 ml of phosphate-buffered saline. Cells were frozen, thawed, and sonicated to effect release of intracellular virus. Virus infectivity was assayed by plaque titration on RK-13 cells (20). Four petri dishes were used at each 10-fold dilution step.

PAGE. Sample preparation and conditions of PAGE in a slab gel apparatus (26) with a modified Laemmli discontinuous SDS buffer system (14) were as previously described (16). The stacking and separation gels were 4.4 and 15% acrylamide, respectively. The stacking gel also contained 0.6% agarose. Radioactively labeled proteins were detected by scintillation autofluorography (2).

Preparation of material for electron microscopy. Cells were fixed in situ with 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). Fixed cells were washed, postfixed in 1% osmium tetroxide, dehydrated in alcohol, and embedded in epoxy resin, as previously described (19). Thin sections were stained with lead citrate and uranyl acetate.

RESULTS

Effect on production of INV and EEV. RK-13 cells infected with IHD-J vaccinia were exposed to various concentrations of gln and 2-dg starting at time zero postinfection. The yield of infectious INV and EEV at 24 h postinfection (p.i.) compared with that of untreated vaccinia-infected cells was monitored. Both gln (Fig. 1a) and 2-dg (Fig. 1b) inhibited the production of infectious virus over the same concentration range

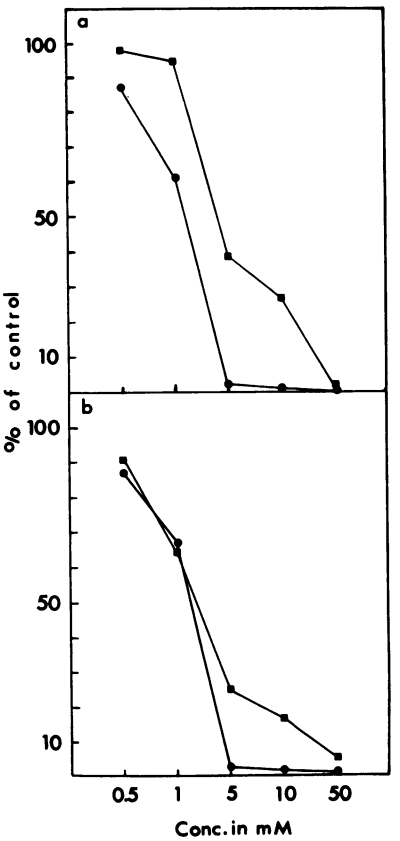

FIG. 1. Infected cells were treated with various concentrations of gln (a) or 2-dg (b), starting at time zero. Virus was harvested at 24 h p.i. and plaque assayed for INV (■) and EEV (●).

(0.5 to 50 mM). EEV yields were more affected than INV yields; a >90% reduction of EEV achieved by 5 mM concentrations of inhibitors was observed, whereas 50 mM concentrations were necessary to effect a >90% reduction in infectious INV.

Although Fig. 1 clearly demonstrates a dose-dependent effect on the production of infectious virus, it reveals no information on the production of virus particles. Figure 2 shows the distribution of [^{3}H]thymidine label in CsCl gradients of EEV and INV. EEV from untreated cultures (Fig. 2a) formed a peak of activity, whereas no [^{3}H]thymidine-labeled virus was detectable in CsCl gradients of extracellular material from gln- (Fig. 2b) or 2-dg (Fig. 2c)-treated cell cultures.

CsCl gradients of virus extracted from infected control cultures (Fig. 2d) revealed two peaks of [^{3}H]thymidine activity. These two peaks were identified by electron microscopy as INV and EEV. The presence of EEV in extracts of infected cells is not indicative of an intracellular

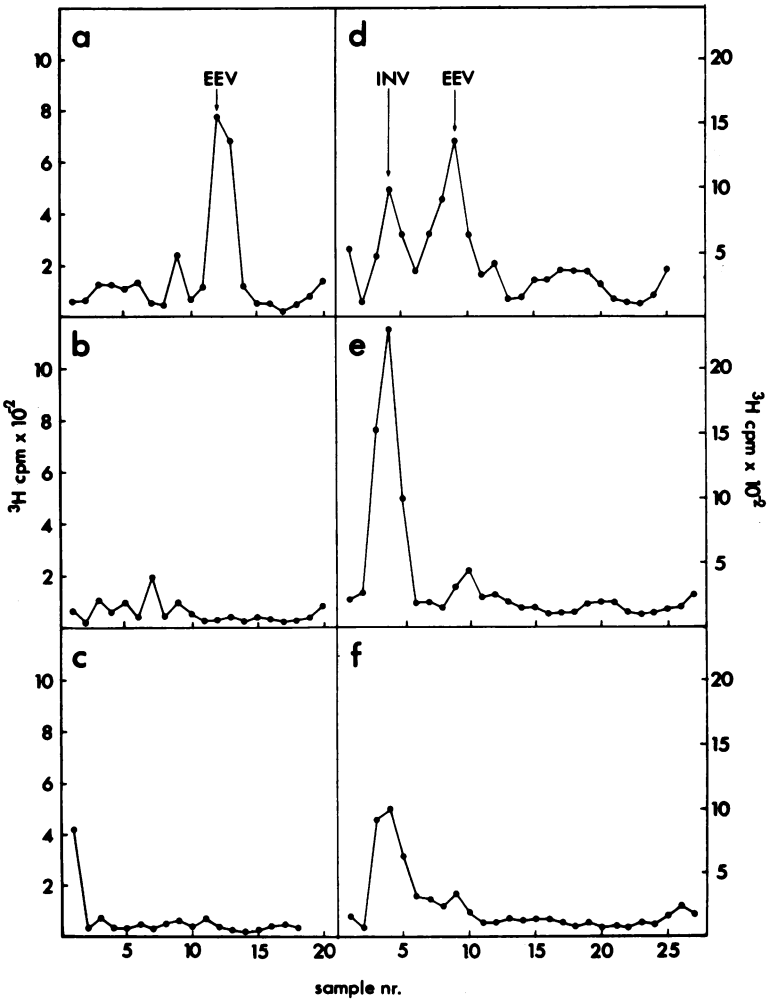

FIG. 2. Infected cells were exposed for 24 h to [^{3}H]thymidine in the absence of inhibitors (a, d) or in the presence of 5 mM gln (b, e) or 5 mM 2-dg (c, f). Extracellular virus (a, b, c) and cell-associated virus (d, e, f) were analyzed by CsCl gradient ultracentrifugation, as described in the text.

source for this virus; rather, it indicates that large numbers of EEV are seen associated with the cell surface (19) and are released in the process of INV extraction and are thereby detectable as an EEV peak in CsCl gradients. The EEV peak was absent from extracts of gln- (Fig. 2e) and 2-dg (Fig. 2f)-treated cells. INV, on the other hand, was not reduced by treatment with glycosylation inhibitors.

The inhibitor dose-dependent reduction in infectious INV yield (Fig. 1) and the lack of any reduction in the number of INV particles (Fig. 2) indicated that the inhibitor caused change in particle infectivity. This was confirmed by direct determinations of particle numbers measured by spectrophotometry and infectivities measured by plaque assay of CsCl density gradient-purified INV suspensions (Table 1). Very similar effects on particle PFU relationships were produced by gln and 2-dg. Inhibitor concentrations of 0.5 and 1 mM had no effect, whereas 5 and 10 mM concentrations of either inhibitor consistently resulted in a small decrease in particle infectivity. INV purified from cells exposed to 50 mM inhibitor concentrations had particle/PFU ratios of about 200. This is a significant decrease in particle infectivity, compared with a particle/PFU ratio of 30:1 for untreated INV. It is noteworthy that 50 mM gln reduced INV particle numbers by 50 to 75%, whereas 50 mM 2-dg had no such effect.

Kinetics of inhibitor-induced reduction in virus release. Addition of gln and 2-dg at 4-h intervals after infection had very similar effects on the 24-

TABLE 1. Effect of inhibitors on INV infectivity[a]

Treatment (in mM)	Number of particles x 10^{10}	Number of pfu x 10^{7}	Particle pfu
Control	2.4	80	30
Gln 50	o.6	3	200
Gln 10	2.4	41	59
Gln 5	2.1	52	40
Gln 1	2.1	72	29
Gln 0.5	2.1	90	23
2-dg 50	2.1	11	191
2-dg 10	2.2	32	69
2-dg 5	2.3	34	68
2-dg 1	2.3	76	30
2-dg 0.5	2.4	74	32

[a] Inhibitors were added at 0 h p.i., and infected cells were harvested at 24 h p.i. INV was purified and quantitated by spectrophotometry as described in the text.

h EEV yields (Fig. 3). Presence of the inhibitors, starting during the first 4 h p.i., allowed less than 10% of the EEV release seen in control cultures. Introduction of the inhibitors at later times resulted in a steady loss of an inhibitory effect. Inhibitor addition at 8 h p.i. permitted 45 to 50% of control culture EEV release, whereas addition at experimental time points of 12, 16, and 20 h p.i. resulted in virus yields greater than 70% of controls.

The reversibility of 2-dg and gln inhibition of EEV release was found to differ temporally (Fig. 4). 2-dg was reversible only up to 1 h p.i., whereafter a sharp decline in its reversibility occurred. 2-dg inhibition of EEV release was essentially irreversible after 3 to 4 h p.i. In contrast, gln inhibition was reversible until 3 h p.i. and became largely irreversible at 6 to 8 h p.i. The 2-dg block in EEV release was more effectively reversed by addition of a five-times excess of mannose than by simple removal of the inhibitor. The kinetics of 2-dg reversal were thus quite similar to that observed for gln reversal.

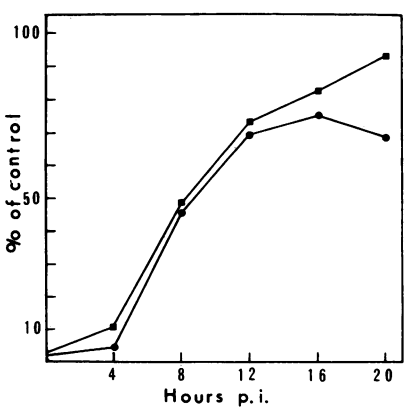

FIG. 3. Infected cell monolayers were exposed to 5 mM gln (●) or 5 mM 2-dg (■) at various times p.i. The 24-h EEV yield was determined by plaque assay and compared with the yield of untreated vaccinia-infected controls.

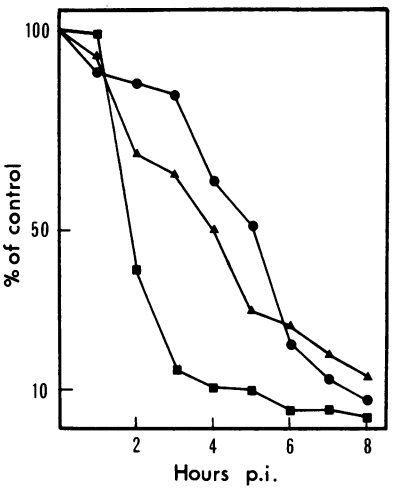

FIG. 4. Infected cell monolayers were exposed to 5 mM concentrations of inhibitors at time zero p.i. At various times p.i., the inhibitors were removed by washing 2 ml of the monolayers twice with inhibitor-free medium and then further incubating the monolayers with inhibitor-free medium. Mannose (25 mM) was added to one series of monolayers after removal of 2-dg. The 24-h EEV yields from cells after simple removal of gln (●) or 2-dg (■) or 2-dg removal followed by addition of 25 mM mannose (▲) were compared with EEV production from control cells.

Effect on glycosylation of vaccinia proteins. Purified INV was analyzed by PAGE to determine the effect of 2-dg at the polypeptide level. Treatment with 2-dg decreased but did not eliminate the glycosylation of the INV 37,000-molecular-weight (37K) glycoprotein at 1, 5, 10, and 50 mM concentrations (Fig. 5). There was no discernible effect on the Coomassie brilliant blue-stained polypeptide pattern at these inhibitor concentrations (data not shown).

[^{3}H]gln-labeled membranes from vaccinia-infected control and inhibitor-treated cells were analyzed by SDS-PAGE (Fig. 6). The dominant glycoprotein has a molecular weight of 89,000, whereas minor glycoprotein species have molecular weights of 42,000 and 20,000 to 23,000 (16, 17). Scintillation autofluorograms showed that treatment with 1 mM 2-dg did not significantly affect the amount of [^{3}H]gln incorporation, al-

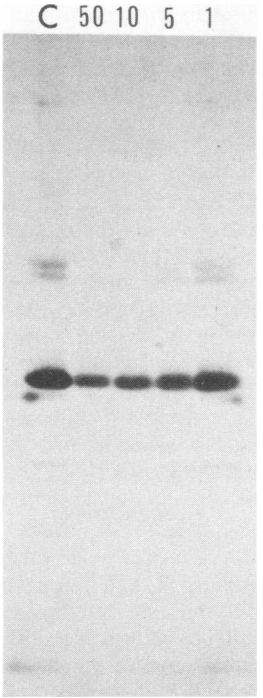

FIG. 5. INV was CsCl gradient-purified from cells exposed to [^{3}H]gln during a 24-h infection cycle. One hundred micrograms of INV grown in the absence of inhibitors (C) was compared by PAGE analysis with 100 μg of INV grown in the presence of 2-dg (1 to 50 mM). Numbers indicate millimolar concentrations of inhibitor.

though it was markedly reduced by treatment with 5 and 10 mM 2-dg concentrations. It is noteworthy that treatment with 5 and 10 mM 2-dg not only reduced isotope incorporation but caused a significant decrease in the molecular weight of the glycosylated product.

Kinetics of vaccinia cell membrane protein glycosylation. The experiments described above indicated that 2-dg and gln inhibited release of EEV and that the 2-dg block at least was in the inhibition of vaccinia cell membrane protein glycosylation. A proper understanding of the kinetics of glycosylation inhibition is dependent on knowledge of the normal glycosylation kinetics. Figure 7 shows the time-dependent appearance of vaccinia cell membrane glycoproteins. Infected cells were pulsed for 4-h intervals, harvested, and investigated by PAGE analysis. Vaccinia glycoproteins were barely detectable 0 to 4 h p.i., compared with the 4- to 8-h p.i. interval. The most intense glycosylation of vaccinia proteins occurred 8 to 12 h p.i. A dramatic decrease in glycosylation was observed 12 to 16 and 16 to 20 h p.i., and only a barely observable glycosylation was evident 20 to 24 h p.i.

Kinetics of 2-dg-induced inhibition of glycosylation. The effect of 2-dg addition on the glycosylation of vaccinia-specific cell membrane proteins was examined by PAGE analysis (Fig. 8). [^{3}H]gln labeling, starting at time zero in uninhibited cells (Fig. 8a), established the kinetics of glycosylation throughout the replication cycle. Glycosylation was barely detectable after 4 h p.i. but was extensive by 8 h p.i. Isotope incorporation was essentially complete by 12 h p.i. in uninhibited cultures. Introduction of both 5 mM 2-dg and [^{3}H]gln at time zero (Fig. 8b) resulted in very low levels of glycosylation at all times p.i., compared with the level of the uninhibited cultures. Eight times more protein was electrophoresed in the gel depicted in Fig. 8b than in the gel depicted in Fig. 8a. In this way, an interesting shift in the apparent molecular weight of the 89K glycoprotein was detectable with increasing times of inhibition. At 4 h p.i., the glycosylated protein pattern was undisturbed by the presence of 2-dg, which probably reflects a 2-dg rate of

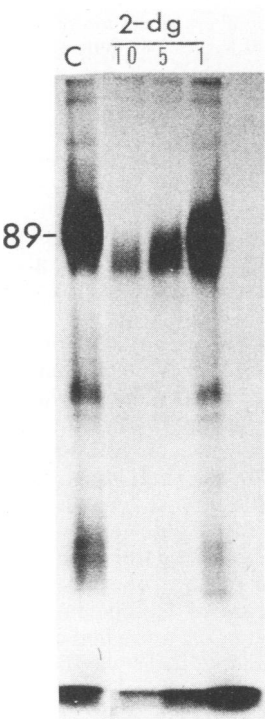

FIG. 6. Cell membranes were purified by discontinuous sucrose centrifugation from cells exposed to [^{3}H]gln during a 24-h infection cycle. Equal amounts of cell membrane protein from cells grown in the absence of inhibitors (C) and in the presence of 2-dg (1 to 10 mM) were compared by PAGE analysis. Numbers at top indicate millimolar concentrations of 2-dg. 89, 89K glycoprotein.

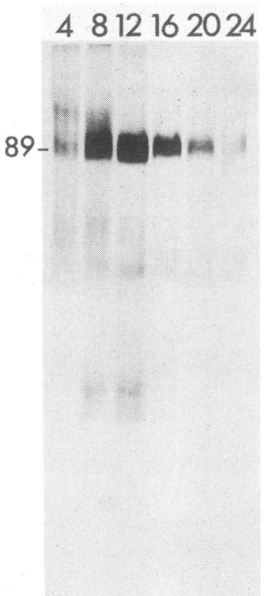

FIG. 7. Vaccinia-infected cells were pulse-labeled with [^{3}H]gln for 4-h periods before harvest. Cell membranes were purified after the pulse and analyzed by PAGE. The numbers at the top of the figure indicate the hour p.i. of harvest. 89, 89K glycoprotein.

inhibition slower than the rate of [^{3}H]gln incorporation. Inhibition by 2-dg became apparent at 8 and 12 h p.i. by a drastic reduction in the incorporation of [^{3}H]gln and by a shift of the 89K glycoprotein to a lower molecular weight. The disappearance of the 89K glycoprotein labeled from 0 to 4 h p.i. reflects a turnover of this glycoprotein.

The effect of 2-dg reversal on the glycosylation of vaccinia-specific cell membrane proteins was examined by PAGE analysis. Introduction of [^{3}H]gln, starting at 8 h p.i., established the kinetics of isotope incorporation late in infected but untreated cells (Fig. 9a). Glycosylation was first detectable after a 2-h pulse (10 h p.i.) and steadily increased with longer pulses. Infected cells treated with 2-dg from 0 to 8 h p.i. followed by a [^{3}H]gln pulse in the absence of inhibitor revealed a rate of isotope incorporation very similar to that of the untreated cells (Fig. 9b). On the other hand, if 2-dg was first added to the infected cells when the [^{3}H]gln was added to the cells at 8 h p.i. (Fig. 9c), then glycosylation between 8 and 12 h p.i. was greatly reduced. Furthermore, the 89K glycoprotein shifted to a lower molecular weight.

Effect on vaccinia morphogenesis. The conversion of INV to EEV is mediated by a double-membraned intracellular intermediate that is transferred to the plasma membrane, where the outer membrane fuses with the plasma membrane, resulting in the exgulfment of a single-membraned EEV particle (8, 15, 19). The effects of gln and 2-dg on this release process were examined electron microscopically and quantified by evaluation of thin sections under coded numbers (Table 2). A majority of intracellular virus was completely wrapped in a double-membraned structure at 12 h p.i. in control cells. This proportion was considerably reduced at 24 h p.i. In contrast, gln completely inhibited formation of wrapped virions at both 12 and 24 h p.i., resulting in all virions being in the naked form. Although causing a drastic reduction in the proportion of wrapped virions to controls, 2-dg was not as efficient as gln in eliminating the formation of the double-membraned intermediate at 12 h p.i. No wrapped virus particles were seen after treatment with 2-dg for 24 h.

DISCUSSION

The reproduction of a wide variety of enveloped RNA viruses (11) and herpesviruses (4) has been shown to be affected by gln and 2-dg, which inhibit glycosylation of the virus envelope glycoproteins (24). All of these viruses acquire an envelope by budding through the plasma or nuclear membranes. The inhibitors either block this release process or permit release of particles lacking their surface glycoproteins. The effects of gln and 2-dg on vaccinia virus-mediated activities have been studied (28). At the time of these studies, vaccinia virus was not generally considered to acquire an envelope; thus, there was a paucity of experiments designed to investigate the effect of glycosylation inhibitors on vaccinia release.

Vaccinia release was affected by gln and 2-dg at concentrations of 1 to 10 mM, as other viruses have been found to be affected. The decrease in infectious extracellular virus was greater than 90% at inhibitor concentrations of 5 mM. There was also a loss of INV infectivity, although the production of infectious intracellular virus was less sensitive to the presence of glycosylation inhibitors than was the production of infectious extracellular virus. The loss of extracellular virus infectivity was reflected in an absolute decrease in the yield of EEV particles released to the extracellular environment, whereas the yield of INV particles was not affected at inhibitor concentrations of 1 to 10 mM. Thus, gln and 2-dg preferentially depress the release of EEV.

INV contains a 37K glycoprotein containing only gln residues (5, 6). Addition of 2-dg reduced but did not eliminate incorporation of [^{3}H]gln into this glycoprotein. This is somewhat unexpected, since 2-dg is an analog of mannose, not gln. A dependence of INV infectivity on the 37K glycoprotein has been previously observed (7).

Very clearly, 2-dg demonstrated a dose-de-

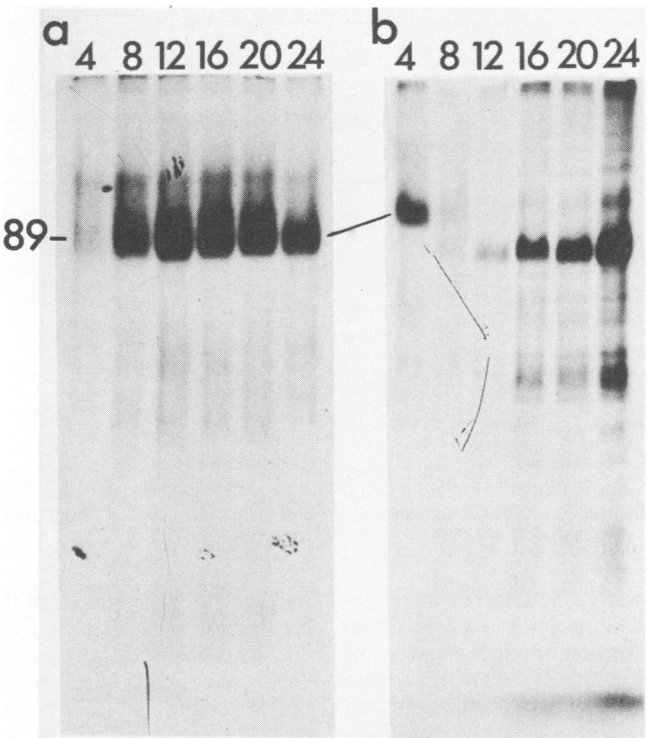

FIG. 8. Vaccinia-infected cells were labeled throughout the infection cycle by introducing [^{3}H]gln at time zero. Cells were harvested at the hour p.i. indicated at the top of the figure, and membranes were purified and compared by PAGE analysis. Membranes are from uninhibited cells (a) and from cells inhibited with 5 mM 2-dg, starting at time zero (b). Eight times more protein was electrophoresed in (b) than in (a). 89, 89K glycoprotein.

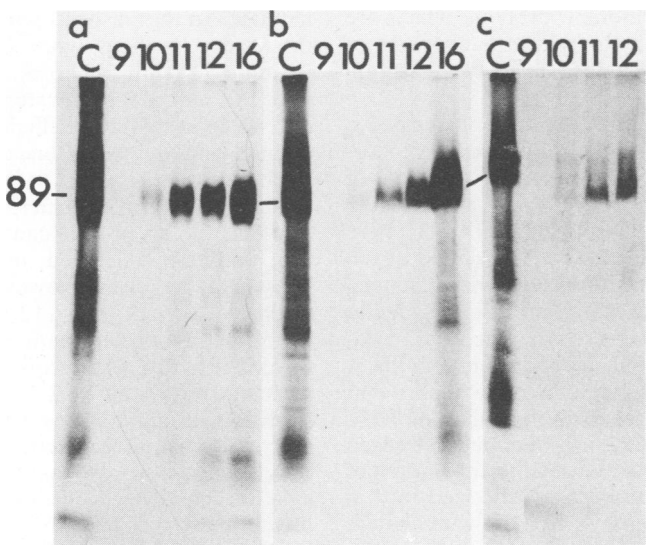

FIG. 9. Infected cells incubated in the absence of inhibitor (a) or the presence of 5 mM 2-dg from 0 to 8 h p.i. (b) or the presence of 5 mM 2-dg, starting at 8 h p.i. (c), were pulsed with [^{3}H]gln, starting at 8 h p.i. The removal of 2-dg at 8 h p.i. (b) and the addition of 25 mM mannose was performed as described in the legend to Fig. 5. Numbers at the top indicate the hour p.i. at which the pulse was ended and the cell membranes were purified by discontinuous sucrose gradient centrifugation. Equal amounts of protein were compared by PAGE with infected cell membranes labeled from 0 to 8 h p.i. (C) in the absence of inhibitor.

TABLE 2. Effect of inhibitors on wrapping of intracellular virus[a]

Treatment	Time (h p.i.)	% of total intracellular virus			Total no. of intracellular virions counted
		INV	Wrapped		
			Partially	Completely	
Control	12	31	15	54	424
Control	24	80	2	18	993
Gln	12	100	0	0	576
Gln	24	100	0	0	1160
2-dg	12	88	1	11	406
2-dg	24	99	1	0	959

[a] Inhibitors (0.005 M) were added at 0 h p.i., and the cells were harvested at the indicated times. Quantitation was by electron microscopic blind evaluation.

pendent effect on the glycosylation of vaccinia-specified cell membrane proteins. 2-dg only partially blocked glycosylation at concentrations of 1 to 10 mM. It is particularly noteworthy that 2-dg had the additional property of causing an apparent decrease in the molecular weight of the major 89K glycoprotein. A similar decrease has been observed for influenza (24) and herpes (13) glycoproteins synthesized in the presence of 2-dg and for vaccinia membrane glycoproteins synthesized in the presence of tunicamycin (25). It thus seems that an alteration in the glycosylation of vaccinia membrane proteins which are also the glycoproteins found in the envelope of EEV (16, 17) is sufficient to cause the cessation of EEV release.

The kinetics of inhibitor addition revealed a time-dependent relationship between glycoprotein synthesis and EEV release. Addition of inhibitor at 8 h p.i. could only cause a 50% reduction in EEV release. The lack of effective inhibition of EEV release by 2-dg added at 8 h p.i. was not the result of any virus-induced refractoriness to 2-dg inhibition. When added at 8 h p.i., 2-dg very effectively reduced glycosylation and shifted the 89K glycoprotein to a lower molecular weight. It should be remembered that at 8 h p.i., only 1% or less of the final total EEV yield from uninhibited cells has been released (17, 19). This suggests that a sufficient amount of the glycoproteins has been synthesized by 8 h p.i. to permit the release of 50% of the maximum EEV yield.

The reversibility of the glycosylation blockage on EEV release steadily declined with increasing exposure times and was essentially irreversible by 8 h p.i. This irreversibility was not due to a continued glycosylation inhibition after 2-dg removal, since synthesis of the 89K glycoprotein resumed at only slightly lower than normal levels. This indicates that glycoprotein synthesis during the first 8 h p.i. was not only sufficient but necessary for EEV release. It is interesting that the glycoproteins necessary for EEV release are synthesized largely before the most intense period of glycoprotein synthesis (8 to 12 h p.i.).

The block in EEV morphogenesis imposed by 2-dg and gln was determined to be an inhibition of the formation of the double-membraned intermediate. In this respect, gln was more effective than 2-dg. Previous work showed an effective inhibition by gln, whereas, 2-dg did not influence this morphogenetic step (28). This reported difference in inhibition efficiency remains unexplained and indeed is unexpected, since both inhibitors were reported to inhibit glycosylation.

The mechanism by which incompletely glycosylated vaccinia protein can inhibit formation of the double-membraned intermediate is not known. It is clear, however, that the most intensive period (8 to 12 h p.i.) of double-membraned virion formation (19) coincides with the highest rate of glycoprotein synthesis and with the beginning of 2-dg irreversibility. The synthesis, primary glycosylation and insertion of glycoproteins into membranes, occurs at the rough endoplasmic reticulum. Incomplete glycosylation could prevent formation of double-membraned virions at Golgi membranes by blocking the migration of the glycoproteins from the site of protein synthesis to the Golgi membranes or by preventing the glycoproteins that reach the Golgi membranes from taking part in the envelopment process. Blockage of these steps may result from the incompletely glycosy-

lated protein assuming a conformation not conducive to migration or envelopment. This hypothesis is not without some experimental support. The 89K vaccinia glycoprotein-mediated hemagglutinin activity (16) is greatly reduced by 2-dg (28). Furthermore, the glycoproteins of Semliki Forest virus have been shown to undergo a glycosylation-dependent conformational change necessary for their participation in the envelopment process (10).

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