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Effects of Lysosomotropic Weak Bases on Infection of BHK-21 Cells by Sindbis Virus

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The effects of the lysosomotropic weak bases chloroquine, ammonium chloride, and amantadine as well as dansylcadaverine (an inhibitor of receptor mediated endocytosis) on the replication of Sindbis virus in tissue-cultured cells was examined. Chloroquine had no effect on the expression of virus-induced homologous interference. None of these drugs significantly affected the ability of a complex of a cell and single virion to form an infectious center. Chloroquine and ammonium chloride were found to inhibit the synthesis of virus RNA in established infections when added early in infection. These drugs also inhibited the production of progeny virions when added any time after infection. These results suggest that the antiviral activity of these agents may not be due to an ability to prevent transport of the virus genome into the cell cytoplasm.

Experimental investigations of the events leading to the establishment of virus infections (i.e., attachment and penetration) have proven to be among the more difficult and controversial aspects of contemporary virology (6). Investigations of this important aspect of the virus life cycle have been extremely difficult to interpret because of two major technical difficulties in experimental design. (i) For a given population of animal viruses, it is not possible to experimentally demonstrate a one-to-one correspondence between the number of physical particles and the number of PFU. In the best of circumstances, less than 20% of a preparation of virions can be demonstrated to be infectious; in most circumstances, the percentage is much lower. (ii) It is unclear whether a potential host cell is capable of dealing with unlimited amounts of virus particles in an identical fashion (that is, a pathway leading to productive infection) (25). Limitations in the sensitivity of biochemical or morphological (electron microscopic) assays of events related to attachment and penetration of cells by viruses have forced investigators to employ large quantities of virus per cell to produce samples yielding quantities adequate for analysis by the techniques employed. These conditions are certainly to be considered artificial when compared with the situation that probably occurs in nature, that is, the interaction of a cell with a single infectious unit of virus. The consequences of laboratory studies employing large quantities of virus are that, in any morphological or biochemical assay of virus penetration, the majority of data are derived from virions that may not be capable of successfully infecting cells (problem number i, above). It is also not known whether cells discriminate between infectious and noninfectious particles at the point of uncoating, with infectious virus following one pathway and noninfectious virus following another (problem number ii, above). Therefore, it is possible, because of this latter limitation, that large numbers of infectious virions may engage in cell interactions that do not lead to the infection of the cell.

Two pathways for virus invasion of tissue-cultured cells have been proposed. One model suggests that viruses enter cells by a process morphologically similar to endocytosis (11, 17–20). After attachment to the cell surface, virions are incorporated into developing endocytotic vesicles and are internalized in these membrane-limited structures. Either virions may escape from the vesicles early after their formation, or the virus-containing endosome may fuse with lysosomal membranes. A second model suggests that viruses may enter cells by direct penetration of the plasma membrane. This hypothesis holds that membrane-containing viruses fuse with cell surface through the interaction of the virus envelope and the cell plasma membrane (5, 10, 14, 23).

Recent studies have focused again on the role that the cellular process of endocytosis may play in the invasion of tissue-cultured cells by animal viruses. This renewed interest has derived from two primary observations. First, it has been demonstrated that certain complex and simple weak basic compounds can accumulate in acidic compartments of cells and alter the pH of those compartments (21). This has led to a study of the effects that such weak basic compounds could have on the process of infection (12). These studies have led to the conclusion that an increase in the pH of intracellular compartments prevents cell penetration by a variety of enveloped viruses. Second, it has been discovered that a large number of enveloped virions can induce cell fusion if exposed to an acidic environment after attachment to the cell surface (9, 33–35). These two observations have formed the basis for a model that suggests that the route of virus entry is by endocytosis. According to this model, attached virions are incorporated into endocytotic vesicles; shortly after the separation of the endocytotic vesicle from the cell surface, a reduction in the pH of the vesicle (32) induces the fusion of the virus membrane with the membrane of the vesicle itself. This fusion event releases the virus genome into the cell cytoplasm, initiating the process of infection. Because of wide acceptance of this model, a large number of basic compounds (referred to as lysosomotropic weak bases) have been employed in studies examining the effects of these agents on the early events in virus infection. Compounds such as chloroquine, amantadine, ammonium chloride, and methylamine are now widely used to determine whether the acidic compartment is involved in the infection of a cell by a given virus (15, 20, 28, 31).

In this manuscript, we present experiments that review the effects of some lysosomotropic weak bases on the growth cycle of Sindbis virus (the prototype of the alphaviruses) in an attempt to determine whether explanations

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alternative to those previously provided can account for the antiviral activity of these compounds. Although a variety of agents are examined in this study, we have focused primarily on the drug chloroquine, since this compound has been most widely utilized in studies of this type.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK-21) cells were grown as described previously (22) in Eagle minimal essential medium (8). The heat-resistant strain of Sindbis virus (SVHR) and mutant *ts*-23 were propagated in BHK-21 cells as described previously (22). Virus titers were determined by plaque assay on BHK-21 cell monolayers (22).

Infectious center assay. The assay of the number of infected cells in a monolayer was determined essentially as described by Riedel and Brown (24). Monolayers of BHK-21 cells were infected with virus at a given multiplicity for a period of time at room temperature (25°C). Infection was stopped by washing the monolayers with a 1:100 dilution of anti-Sindbis virus serum (described below). The cells were then washed three times with minimal essential medium and lifted from the plastic substrate with a solution of 0.05% trypsin (GIBCO Laboratories; 1:250) and 0.02% EDTA in phosphate-buffered saline that was deficient in calcium and magnesium. The cells were gently pelleted and resuspended in cold complete medium with 1% fetal calf serum. The cell suspension was serially diluted in cold complete medium. A 1.0-ml sample of each dilution was mixed with 1.0 ml of freshly trypsinized and washed, uninfected BHK-21 cells (5×10^6 cells). This mixture was warmed to room temperature, and 1.0 ml of 2.1% low-gelling-temperature agarose (Sea Plaque, Rockland, Maine; FMC) in complete medium at 37°C was added to it. The suspension was carefully mixed and immediately transferred to petri dishes (60 by 15 mm) containing a preformed base of 1% agarose in complete Eagle medium. Plates stood at room temperature for 1 h and were then incubated at 32°C for 3 days. Plaques representing infected cells were counted after staining with neutral red. This assay procedure is 60% as efficient as a plaque assay.

Homologous interference assay. The ability of a Sindbis virus-infected BHK cell to exclude superinfecting homologous virus was assayed as described by Johnson et al. (13). Cells infected with Sindbis virus temperature-sensitive mutant *ts*-23 (interfering virus) were washed five times with minimal essential medium and were challenged with wild-type Sindbis virus (superinfecting virus) at the same multiplicity for an identical time period. Control experiments were conducted with only the interfering virus or the superinfecting virus. At the end of the infection, extracellular virus was inactivated with a 1:100 dilution of anti-Sindbis virus serum (described below). The monolayers were washed five times with complete minimal essential medium and incubated overnight (18 h) at 28°C (permissive temperature). Virus released was assayed at 28 and 37°C by plaque assay to determine temperature sensitivity. The yield of superinfecting virus was determined as a percentage of the total by dividing the titer at 37°C (nonpermissive temperature) by the titer at 28°C (permissive temperature).

Johnson et al. (13) have demonstrated that the interfering virus can maximally exclude superinfecting virus when applied to cells for 15 min at a multiplicity of 25 PFU/cell.

Uridine incorporation assay. The incorporation of [^{3}H]uridine into viral RNA was determined by a modification of the procedure described by Helenius et al. (12). Monolayers of BHK-21 cells were treated with actinomycin D (4 μg/ml) for 90 min before infection. We have found that 90 min is required to completely arrest host RNA synthesis (data not shown). Identical monolayers of cells were infected with 200 PFU/cell for 1 h (12). At the end of the infection period, the cells were washed with medium containing anti-Sindbis virus serum to arrest infection and 4 times with medium containing actinomycin D and placed in complete MEM plus actinomycin D. Cells were labeled with 10 μCi of [^{3}H]uridine per ml as indicated in the figure legends. The total label incorporated into RNA was determined by precipitation with ice-cold 15% trichloroacetic acid (TCA). Each value presented in the tables or as a point on a graph represents the average of four identical experiments.

Preparation of anti-Sindbis virus serum. SVHR produced by BHK-21 cells was purified by density gradient centrifugation, dialyzed against phosphate-buffered saline mixed with an equal volume of Freund complete adjuvant, and injected subscapularly into a rabbit. This injection was repeated 3 weeks later. One week before each bleeding, an additional injection of Sindbis virus was administered intravenously. A 1:100 dilution of the resulting serum inactivated 97% of

TABLE 1. Establishment of Sindbis virus mediated homologous interference in the presence and absence of chloroquine

Viruses[a]		Titer[b] (PFU/ml) determined at:		Titer at 37°C/titer at 28°C	Production of superinfecting (wild-type) virus (% of total)[c]
Interfering	Superinfecting	28°C	37°C		
None	SVHR	2×10^9	8×10^8	0.4	100
ts-23	None	5×10^9	1×10^5		
ts-23	SVHR	8×10^9	5×10^7	0.006	0.7
ts-23, chloroquine[d]	SVHR	4×10^9	4×10^7	0.01	1.4
None, chloroquine[d]	SVHR	2×10^9	8×10^8	0.04	100
ts-23, chloroquine	SVHR, chloroquine	9×10^8	7×10^7	0.07	6.0
SVHR, *ts*-23	None	2×10^9	7×10^8	0.35	49.0
SVHR, *ts*-23, chloroquine	None, chloroquine	1×10^9	3×10^8	0.3	42.0

[a] Cells were infected with 100 PFU of the virus indicated per cell for 30 min at room temperature. The cells were washed with phosphate-buffered saline at the end of each infection and either superinfected or placed in fresh medium and incubated overnight (18 h) at 28°C. The cells were treated with anti-Sindbis virus serum at the end of the second (or only) infection.

[b] Determined by plaque assay at the indicated temperature.

[c] Corrected for the efficiency of plating of wild-type virus at 37 and 28°C.

[d] Cells were pretreated with chloroquine (0.1 mM) for 30 min and infected in the presence of the drug at room temperature. At the end of the first (or only) infecting period, the cells were washed with media without chloroquine; the second infection (if applicable) was in the absence of chloroquine. At the end of the infection period, monolayers were washed with antiserum as described in footnote *a*.

Sindbis virus (2×10^9/ml) infectivity in 15 min at room temperature. The antiserum was employed at 1/100 concentration in the experiments presented above (see Table 2).

Chemicals. Amantadine (Sigma Chemical Co.) was dissolved in 140 mM NaCl–0.5 mM $MgCl_2$ buffered with 10 mM morpholine ethanesulfonic acid and was stored as a 0.5 M stock solution at 4°C. Ammonium chloride (1 M; J. T. Baker Chemical Co.) was dissolved in distilled water. Chloroquine (10 mM) was dissolved in phosphate-buffered saline that was deficient in calcium and magnesium, and both stocks were stored at 4°C. Dansylcadaverine (Sigma) was prepared fresh before each experiment as a 5 mM stock solution in phosphate-buffered saline acidified with 2 N HCl (28).

RESULTS

Lysosomotropic weak bases fail to inhibit the establishment of homologous interference and the formation of Sindbis virus-infected cell complexes. Studies designed to define the mechanism by which enveloped viruses enter cells have frequently employed agents capable of altering the pH of intracellular compartments (12). The presence of these compounds during infection prevented subsequent virus RNA synthesis and led to the conclusion that enveloped viruses enter cells via acidic compartments. The effects of these agents on late virus functions have not, however, been clearly defined. Therefore, we monitored the steps during Sindbis virus replication at which these agents exert their effect.

To define the effects of inhibitors on various stages in the replication of Sindbis virus it was necessary to determine the rate at which Sindbis virus penetrates cells and initiates gene expression. The rate at which Sindbis virus penetrates cells was defined as the rate at which virus-cell complexes become resistant to antiviral antiserum after virus adsorption. High multiplicities of infection (200 PFU/cell) were chosen for some of the studies presented below. This multiplicity was chosen to achieve a rapid and nearly synchronous infection of a cell monolayer to allow us to relate observations on rates of RNA and progeny virus synthesis to time of infection. In an infectious center assay, performed as described above we found that at this multiplicity of infection all cells were infected in 12 min (data not shown).

A homologous interference assay was employed to determine the onset of gene expression. It has been demonstrated that homologous interference can occur within 15 min after the addition of virus to cells and requires virus gene expression. It does not require RNA transcription or replication. The induction of homologous interference is one-fifth as sensitive to UV inactivation as infectivity (13). We have recently found that the induction of homologous interference prevents the expression of the superinfecting virus after the genome enters the cell cytoplasm (Adams and Brown, unpublished data). BHK-21 cells were infected with *ts*-23 (100 PFU/ cell), an RNA-positive Sindbis virus mutant that produces no mature virus at nonpermissive temperature (2, 30) and subsequently superinfected with wild-type Sindbis virus (SVHR). The virus produced was assayed for temperature sensitivity by a plaque assay at 28 and 37°C. More than 99% of the virus produced was temperature sensitive (Table 1). These results indicate that within 30 min after virus addition, the infecting virus has programmed the production of the viral function responsible for homologous interference.

TABLE 2. Effects of an inhibitor of endocytosis and some lysosomotropic weak bases on the formation of Sindbis virus BHK-21 cell infectious centers[a]

Drug tested	Concn (mM)	Infectious centers formed (% of control)
Chloroquine	0.1	71
Chloroquine	0.005	79
NH_4Cl	20	91
NH_4Cl	0.2	130
Dansylcadaverine	5	96
Amantadine	2	44
Nontreated cells[b]		2

[a] Infectious center assay was as described in the text. Monolayers of cells were infected at a multiplicity of 0.01 PFU/cell.

[b] Cells were infected at 4°C and treated with antiserum at 4°C. This experiment served as control to ensure that the antivirus serum would prevent infection of the cells by surface-associated virions after removal from the drug.

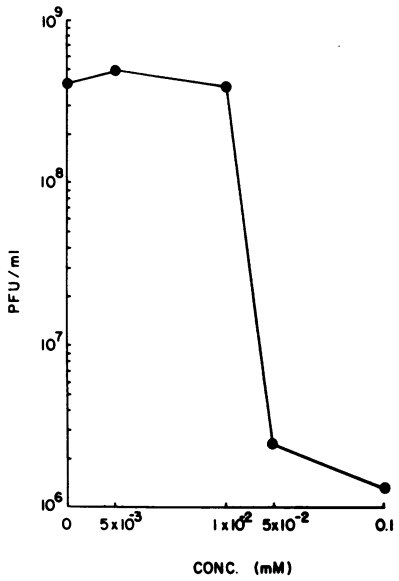

FIG. 1. Effect of varied concentration of chloroquine on the production of progeny Sindbis virus by infected BHK cells. Monolayers were infected with Sindbis virus as described in the text. Chloroquine was added at the indicated concentrations at 90 min after the addition of virus. Monolayers were incubated at 37°C overnight. The amount of virus produced was determined by plaque assay as described in the text.

We found that the inhibitory effects of chloroquine on both virus RNA synthesis and progeny virus production were reversible when the drugs were removed from the cultures within 60 min after the addition of virus (data not shown). The reversibility of the effects of chloroquine permitted us to assay for the expression of virus-induced homologous interference in the presence of chloroquine. BHK-21 cell cultures were treated with chloroquine for 30 min and infected with Sindbis virus *ts*-23 (100 PFU/cell) in the presence of chloroquine for 30 min. The cells were washed to remove the chloroquine and challenged with wild-type Sindbis virus (100 PFU/cell) for 30 min. The monolayers were then washed, treated with anti-Sindbis virus serum, and incubated overnight at 28°C. The virus produced was assayed at permissive and nonpermissive temperatures to determine the phenotype of the progeny virus. A similar experiment was conducted without drug treatment, and SVHR and *ts*-23 growth was assayed independently (nonmixed) (Table 1). In the presence of chloroquine, *ts*-23-infected cells established the ability to prevent expression of superinfecting, wild-type virus as efficiently as in nontreated cells. These results were not

significantly changed when exposure to the first virus in the presence of chloroquine was extended to 1 h, or when the multiplicities of infection of the interfering and superinfecting viruses were reduced to 25 (data not shown). Multiplicities less than 25 produced ambiguous results because, under the conditions employed in these experiments, lower multiplicities failed to infect all cells in the monolayer. The experiments could not be conducted with exposures to chloroquine longer than 1 h because cells treated with drug into 90 min postinfection irreversibly lose the ability to produce virus.

The data presented above may be explained by concluding that the interfering virus (*ts*-23) is retained in the endosomal compartment in the presence of chloroquine and rapidly infects the cell and establishes homologous interference once the drug is removed. The superinfecting virus, although added in the absence of drug, must go through a set of steps before it encounters the acidic compartment required for infection. (Helenius and co-workers [12] have shown that this event occurs within 5 min after attachment of the virus to the cell surface.) To test this possibility, the homologous interference experiment described above was carried out with both the interfering virus and the superinfecting virus added to cells for 30 min in the presence of chloroquine (Table 1). It was reasoned that if the interfering virus were held up in the endozyme waiting for pH to be restored to neutrality, the superinfecting virus would have an opportunity to reach an equivalent position in the cell and that both viruses would simultaneously infect the cell, producing a population of progeny viruses equivalent to a simultaneous infection with both viruses in the presence or absence of the drug. The first virus still efficiently interfered with the second virus (Table 1), once again suggesting that homologous interference was established in the presence of chloroquine.

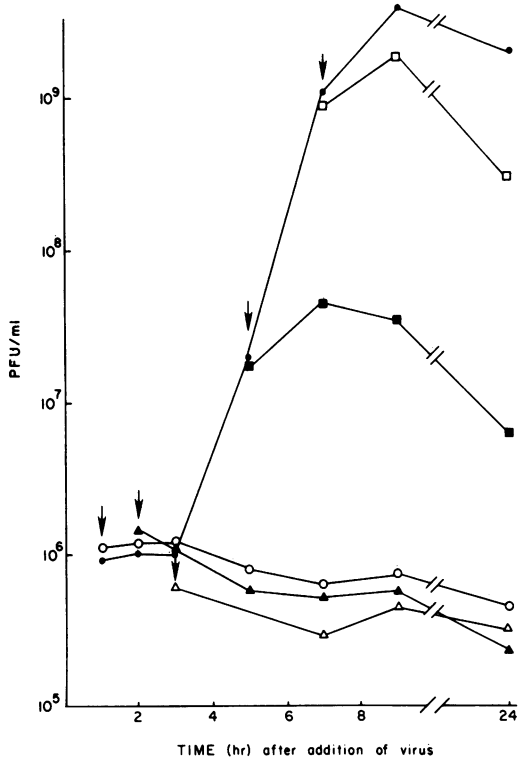

FIG. 2. Effect of chloroquine on the production of progeny virus when added at various times after infection of BHK-21 cell with Sindbis virus. Identical monolayers of Sindbis virus-infected BHK-21 cells were treated with 0.1 mM chloroquine at the times indicated (arrows) after the addition of infecting virus. The monolayers were incubated at 37°C, and the supernatants were assayed for total virus produced at the times indicated. Symbols: ●, no drug; ○, drug added at 1 h; ▲, drug added at 2 h; △, drug added at 3 h; ■, drug added at 5 h; □, drug added at 7 h.

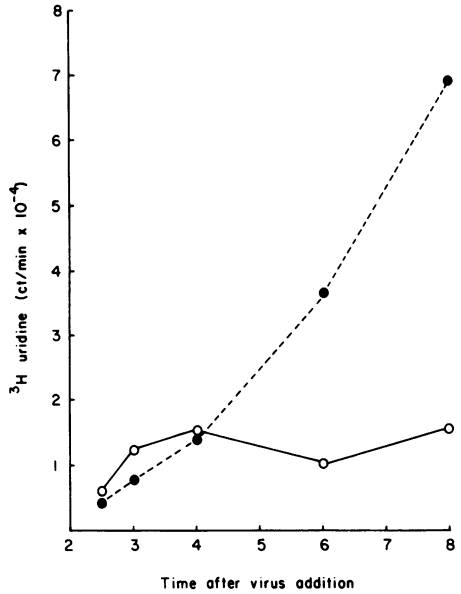

FIG. 3. Incorporation of [^{3}H]uridine into virus RNA in the presence and absence of chloroquine. Identical monolayers of BHK cells were infected with Sindbis virus as described in the text. At 90 min after the addition of virus, chloroquine was added to one set of monolayers to a final concentration of 0.1 mM. [^{3}H]uridine (10 μCi/ml) was added to all cultures at 2 h after the addition of viruses. Samples were assayed at the times indicated for total counts per minute incorporated into viral RNA as described in the text. Symbols: chloroquine-treated cells; ●, control (nontreated) cells.

Homologous interference has been demonstrated to be a virus-coded function (13). These data suggest that the expression of this function through translation of the infecting genome takes place in the presence of chloroquine.

To further define the effect of lysosomotropic weak bases on Sindbis virus penetration, we examined the formation of virus-infected cell complexes in the presence of these drugs. Four drugs were chosen for these experiments. Chloroquine and NH_4Cl were used at concentrations that have no effect on virus production (10) (this study; see below) or RNA synthesis and at concentrations utilized by others to raise the pH of intracellular compartments (21) and to demonstrate inhibitory effects on virus infection (12). Amantadine (a lysosomotropic weak base) and dansylcadaverine (an inhibitor of receptor-mediated endocytosis [4]) were used in concentrations previously employed to block virus infection (12, 18). The concentrations of these drugs (higher concentration in the case of NH_4Cl and chloroquine) are those commonly used to raise the pH of intracellular compartments (21). Experiments were carried out with dilutions of virus sufficient to ensure that no BHK-21 cell came in contact with more than a single virion (multiplicities of less than 1 virion per cell). Cell monolayers were exposed to

dilutions of virus in the presence or absence of the drugs for 1 h at room temperature. The monolayers were then washed in the presence of drug and treated with anti-Sindbis virus serum in the presence of drug. The cells were then removed from the monolayer, diluted, and mixed with uninfected, nontreated BHK-21 cells and plated in soft agar in the absence of drug. To ensure that further infection of the cells did not occur after the treatment with antiserum, an identically infected, nontreated culture was maintained throughout infection at 4°C and treated with antiserum (Table 2). This culture yielded only 2% of the number of infectious centers produced by cultures maintained at room temperature.

In this experiment, the virus-cell complexes were maintained in 0.1 mM chloroquin for a total of 80 min. Longer incubation times were not possible because of the nonreversible inhibitory effects of the drug on progeny virus production when present at times greater than 90 min postinfection. We have also found that uninfected cells exposed to 0.1 mM chloroquine for a period in excess of 90 min lost their ability to concentrate neutral red.

The results of these experiments are shown in Table 2. Chloroquine, ammonium chloride, and dansylcadaverine did not significantly interfere with the penetration of Sindbis virus into BHK-21 cells. Only amantadine significantly depressed the formation of infectious centers. This reduction may be the result of toxic effects of the drug (7), since we found that this concentration of drug reduced host cell RNA synthesis to 19% of control levels (data not shown). Chloroquine (0.1 mM), NH_4Cl (20.0 mM), and dansylcadaverine (5 mM) depressed the formation of infectious centers a maximum of 30%. This value was only achieved with 0.1 mM chloroquine. Chloroquine, at a concentration of 0.005 mM, is shown below to have no demonstrable inhibitory effects on either viral RNA synthesis (see Fig. 4) (12) or the production of progeny virions (Fig. 1), yet this concentration reduced the number of infectious centers formed nearly as much as did the higher concentration. This result suggests that most of the depression seen with 0.1 mM drug may result from an effect of the agent on the infected cell itself, increasing the fragility of the infected cell and reducing the number of infected cells that survive processing for the infectious center assay.

It can be argued that, in the presence of chloroquine, invading virus is retained in the prelysosomal compartment until the drug is removed, pH is restored to acid conditions, and then finally infection is established. It is important to point out, however, that although the pH of intracellular compartments is raised by these weak bases, the fusion of the endosome with the lysosome is not inhibited (11). Current research suggests that this fusion event occurs between 20 and 60 min after formation of the endosome (1, 3, 27). It seems unlikely that a single virion in an endosome of a single cell (a situation which would exist in the infectious center assay) could escape lysosomal contact for a period of 80 min. Such contact should have manifested itself as the loss of an infectious center, significantly reducing the number of cells registering as virus positive.

These experiments suggest that a single virion contacting a single cell can efficiently establish a productive infection in the presence of these drugs. The inability of danyslcadaverine to prevent the formation of infectious centers suggests that receptor-mediated endocytosis may not be essential for Sindbis virus infection of BHK cells.

Effect of chloroquine on the production of progeny virus. To determine what effect, if any, lysosomotropic agents could have on the outcome of virus infection, the effects of various concentrations of chloroquine on the production of infectious virus were determined (Fig. 1). Chloroquine was added to the cultures 30 min after a 1-h adsorption period (multiplicity of infections, 200). At low concentrations of chloroquine (5×10^{-3} and 1×10^{-2} mM), the amount of progeny virus produced was equivalent to control levels, whereas at higher concentrations (5×10^{-2} and 0.1 mM) virus was not produced. We also found that chloroquine-treated (5×10^{-2} and 0.1 mM), virus-infected cells did not release detectable levels of noninfectious virus particles (data not shown).

The effect of chloroquine (0.1 mM), added at various times postinfection, on the production of progeny virions is shown in Fig. 2. The inhibitory effects of chloroquine on virus production were dependent upon the time of addition. When added up to 3 h after the adsorption period, no progeny virus was produced in a 24-h period. When the drug was added at 4 or 5 h postinfection, virus titers increased slightly, and then virus production ceased. The increase in progeny virus produced after drug addition at later times (e.g., 5 h) suggests that the cells can produce virus from accumulated precursor components for up to 1 h after drug addition. Collectively, these results indicated that chloroquine can arrest the production of progeny virus even when added at times very late in infection.

The effects of chloroquine on the production of progeny viruses were found to be reversible only when the drug was removed before 90 min after the addition of virus. Infected cells exposed to 0.1 mM chloroquine for 30 min at 90 min postinfection produced only 4% the virus yield of nontreated cells in 18 h (data not shown).

Effects of lysosomotropic weak bases on the incorporation of [^{3}H]uridine into Sindbis virus RNA. Since lysosomotropic weak bases did not dramatically affect the rate of which Sindbis virus penetrated cells or established homologous interference, but did interfere with progeny virus production, we examined the effect of these drugs on the rate of incorporation of uridine into virus RNA in an attempt to elucidate the step in virus replication blocked by these compounds. Actinomycin D-treated, virus-infected cells

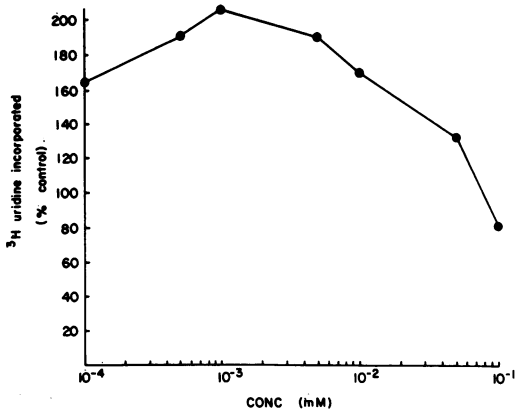

FIG. 4. Dose-response curve for the incorporation of [^{3}H]uridine into RNA in the presence of chloroquine. Identical monolayers of BHK cells were infected with Sindbis virus as described in the text. Cells were treated with the concentration of chloroquine indicated at 90 min after the addition of virus. [^{3}H]uridine (10 μCi/ml) was added to the cultures at 3 h, and the amount of label incorporated during a 30-min period was determined as described in the text. The amount incorporated is plotted as the percentage of that incorporated into untreated control cells.

TABLE 3. Effect of chloroquine on intracellular levels of [^{3}H]uridine

Expt	Treatment	Total cell-associated cpm (% of control)[a]	Total cpm in viral RNA[b] (% of total cpm)
a	Infected cells[c] labeled with [^{3}H]uridine (10 μCi/ml) for 30 min beginning 90 min after addition of virus (control)	4.5×10^5	4.7×10^2 (0.1)
b	Infected cells[c] treated with chloroquine (0.1 mM) and labeled with [^{3}H]uridine for 30 min at 90 min after addition of virus	6.9×10^5 (150)	1.7×10^3 (0.25)
c	As a above, except labeled 4 h after addition of virus (control)	4.7×10^5	7.0×10^2 (0.15)
d	As b above, except labeled 4 h after infection	6.0×10^5 (130)	3.7×10^3 (0.62)

[a] Determined by assaying total counts in dissolved monolayers after washing five times with phosphate-buffered saline.
[b] TCA-precipitable counts per minute from monolayer assayed as described in the text.
[c] Actinomycin D-treated BHK-21 cells infected as described in the text.

were treated with chloroquine (0.1 mM) at 90 min after the addition of virus. [^{3}H]uridine was added 30 min later, and the total incorporation of label was determined by TCA precipitation at various time intervals. Figure 3 shows that, initially, incorporation of label into RNA was higher in drug-treated cells than in nontreated control cells. With time, control cells continued to incorporate label into viral RNA, whereas no significant increase in the amounts of label in drug-treated cells was found during the time frame of this experiment.

The inhibitory effects of chloroquine on virus RNA synthesis were found to be reversible. Infected cells removed from drug began synthesizing RNA at typical kinetic rates within 1 h after removal of the drug (data not shown).

Dose-dependent chloroquine inhibition of Sindbis virus RNA synthesis. Helenius and co-workers (12) have investigated the dose response of viral RNA synthesis to lysosomotropic agents when the drugs were added to the cells during the period of infection. These investigators found a maximum inhibitory effect of chloroquine on incorporation of uridine into RNA when chloroquine was added at a concentration of 0.1 mM. Because our data (presented above) suggested an effect of these weak bases on RNA synthesis, we have performed a dose-response study of the effect of chloroquine on incorporation of uridine into TCA-insoluble material when the drug was added at 30 min after the 60-min infection period (the time at which levels of RNA synthesis in drug-treated cells were similar to those in controls [Fig. 3]). Chloroquine concentrations between 10^{-4} and 0.1 mM were investigated (Fig. 4). The results of this experiment were very surprising. At low drug concentrations, the amount of uridine incorporated into TCA-insoluble material was higher than control levels. At 10^{-4} mM, incorporation into drug-treated cells was 163% of control and increased with increasing chloroquine concentrations to 215% of control at 10^{-3} mM. At this point, the amount of label incorporated was reduced with increasing drug concentration and was suppressed to slightly below control levels at a concentration of 0.1 mM chloroquine. Similarly, we found that when cells were treated with dilutions of ammonium chloride at the same time after infection an enhanced incorporation of tritiated uridine into TCA-insoluble material was seen at low concentrations (162% of control at 0.2 mM), and levels roughly equivalent to control levels were seen at higher concentrations (110% of control at 20 mM).

The data presented in Table 3 and Figures 3 and 4 show that when chloroquine was added to infected cells an immediate increase of label into viral RNA in drug-treated cells relative to control cells was seen. Drug-treated cells ceased to synthesize RNA, whereas control cells surpassed drug-treated cells in incorporation of label. Chloroquine is therefore capable of (i) suppressing viral RNA synthesis with increasing concentration while (ii) actually increasing the relative amount of label incorporated into whatever amount of RNA is synthesized.

The effects of chloroquine on the incorporation of uridine into RNA when the drug was added at various times postinfection were also investigated (Fig. 5). In this experiment, equivalent monolayers of actinomycin D-treated cells were labeled with [^{3}H]uridine at 30 min after the initial 60-min infection period; this time was then considered as time 0. Chloroquine (0.1 mM final concentration) was added to

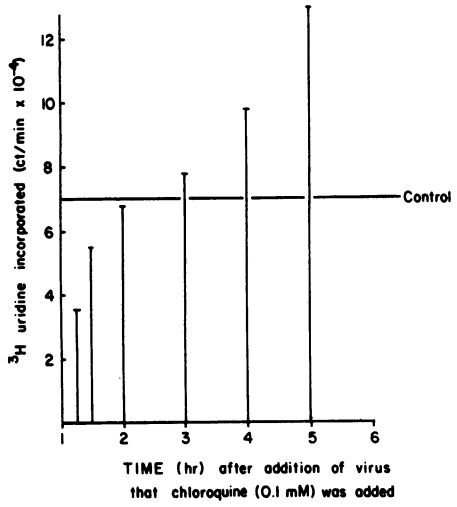

FIG. 5. Effect of chloroquine added to Sindbis virus-infected cell monolayers at various times after infection on the incorporation of [^{3}H]uridine into virus RNA. Identical monolayers of cells infected with Sindbis virus, as described in the text, were placed in medium containing [^{3}H]uridine at 60 min after the addition of virus. Chloroquine was added to monolayers at the times indicated, and the total label incorporated into each monolayer at 9 h after addition of virus was determined. Stated otherwise, the total counts per minute incorporated between 1 and 9 h postinfection was determined and compared with that of a nontreated control.

sample monolayers at 15, 30, 60, 120, 180, and 240 min after uridine addition. Total counts per minute incorporated into virus RNA were determined by TCA precipitation at 6 h after uridine addition. The effect of drug on incorporation of label into virus RNA (during the period from 90 min to 7.5 h after the addition of virus) was again dependent upon time of addition. When added early (15 to 30 min) the amount of label incorporated into RNA at the end of the 6-h incubation period was less than control levels. However, at later times the amount of label incorporated into RNA was equivalent to control (60 min) to much higher than control levels when added at 240 min. These data suggest that chloroquine can block incorporation of label into virus RNA when added early, whereas it can enhance incorporation when added late.

Effect of chloroquine on incorporation of tritiated uridine into Sindbis virus-infected BHK-21 cells. The enhanced incorporation of uridine into viral RNA when chloroquine was added at later times in infection suggested that chloroquine had some effect on the incorporation of uridine into RNA which was independent of its effect on the process of RNA synthesis itself. To explore the possibility that this effect might be on the transport of uridine into the cytoplasm of the infected cell, we examined the amount of cell-associated tritiated uridine in cells treated with chloroquine (0.1 mM) at either 30 min or at 3 h after the initial 60-min infection period (Table 3). Monolayers of cells that had been treated with chloroquine were found to have higher amounts of cell-associated label relative to untreated control monolayers. Similarly, the drug-treated monolayers incorporated more label into TCA-insoluble material (viral RNA) relative to nontreated controls. Thus, it seems that the enhanced incorporation of tritiated uridine into viral RNA is, in part, the result of an enhanced uptake of the label by the infected drug-treated monolayers.

The effects of chloroquine on virus RNA synthesis may be explained by proposing that the drug acts to prevent the formation of RNA replicating complexes. Such an inhibition would restrict the cell's RNA-synthesizing capacity to that existing at the time of drug addition. When drug is added late in infection, the formation of replicating complexes is complete (26), and RNA synthesis proceeds at a maximum rate. Incorporation of labeled uridine into RNA exceeds control levels because of the increased intracellular amounts of label and because progeny RNA is not matured into virions.

DISCUSSION

The experiments described in this report illustrate the difficulties inherent in experimentally elucidating the mechanism by which virus genomes pass from the cell exterior to the cytoplasm. The objective of our study was to determine the point at which lysosomotropic agents interrupted the Sindbis virus replicative cycle. In an effort to avoid the ambiguities associated with the use of high multiplicities of infection and the tracking of physical virus particles by morphological or biochemical means, we have followed the process of infection by measuring Sindbis virus gene expression. Four virus-specified activities were examined: homologous interference, infectious center formation, progeny virus production, and virus RNA synthesis.

The results presented above demonstrate that, whereas the earliest assayable events in the infection of cells by Sindbis virus, infectious center formation and homologous interference, are not significantly affected by any of the drugs tested in this study, virus RNA synthesis and the production of progeny virions are affected at any time drugs are added to the infected cells.

The conclusions generated by this study differ from those published elsewhere, primarily because of the differences in experimental design. We have utilized an infectious center assay to determine the effects of drugs on the ability of a single virion to infect a cell (Table 2). This assay shows that in the presence of drug for 1 h and 20 min, virions infect cells with an efficiency 80 to 90% of that of controls. We have utilized homologous interference as an assay for the translation of virus positive-strand RNA entering the cell cytoplasm. This event is not dependent upon replication or transcription of RNA (13) and is not inhibited by the presence of chloroquine (Table 1). We have utilized kinetic studies to determine the incorporation of uridine into virus RNA in the presence and absence of drug, and we have investigated the effects of varied drug concentration on incorporation. We found that chloroquine enhanced incorporation of label into RNA by increasing the amounts of intracellular label (Table 3) and that, when added early, the drug depressed virus RNA synthesis (Fig. 3 and 5). We have also found an inhibitory effect of chloroquine on virus maturation in cells that are still synthesizing RNA (Fig. 1 and 2).

In other experiments conducted as a part of the study presented herein, we found no significant effect of chloroquine or NH_4Cl on host cell RNA synthesis or on host or virus protein synthesis (data not shown). These observations are in agreement with results published previously (12) and suggest that these drugs may have some specific effects on cytoplasmic RNA synthesis.

The observations that chloroquine can inhibit virus specific RNA synthesis, increase intracellular levels of labeled uridine, and block virus maturation underscore the multiple effects that these drugs may have on biological processes not related to lysosomotropic characteristics. It has been previously demonstrated that, in addition to raising the pH of intracellular compartments, chloroquine inhibits protease and phospholipase activity and steroid and DNA synthesis. Chloroquine binds to cell surfaces and to membrane phospholipids and alters the fluidity of membranes (see reference 29 for review). During the course of these studies, we found that BHK cells exposed to 0.1 mM chloroquin for periods greater than 1 h lose their ability to concentrate neutral red (data not shown). The inhibitory effect of this drug on virus replication could be a direct or indirect result of any one of these effects or of effects currently unknown.

Implication of these experiments for understanding the route of virus entry into cells. Recently, wide support has developed for a model suggesting that the entry of enveloped viruses into cells is by endocytosis of adsorbed virus into vesicles that become acidic intracellular compartments. The acidic environment induces fusion of the virus membrane with the vesicle membrane to release the viral genome into the cell cytoplasm and initiate infection. This model has been derived primarily from two experimental observations. (i) Viruses such as Semliki Forest virus and Sindbis virus induce fusion from without when monolayers with large numbers of surface-associated virions are exposed to low pH (9, 33, 34). (ii) Lysosomotropic weak bases raise the pH of intracellular compartments (21) and, when present during infection, reduce incorporation of label into virus RNA (12). It was concluded that they do so by preventing the fusion of virions with vesicular membranes to release the viral RNA into the cell cytoplasm (17).

We believe that these observations do not support such a

model for the following reasons. (i) We have found (9, 16) and the protocols of others indicate (33, 34) that the fusion-from-without phenomenon does not occur when alphavirus cell complexes are exposed to acidic pH; rather, fusion occurs only when such monolayers are exposed to low pH for brief periods of time and then are subsequently returned to neutral pH. Monolayers may be maintained in low pH environments for hours without fusion occurring (16). Fusion occurs rapidly when monolayers are exposed to low pH for brief periods of time and then returned to neutrality; thus, the return to neutral pH seems to be essential for the acid-induced fusion event to occur (9, 16). There is no evidence suggesting that the pH of endocytotic-prelysosomal vesicles fluctuates from acid to neutral. (ii) The data presented above show that lysosomotropic weak bases have multiple effects on virus-infected cell monolayers when applied at concentrations reported to raise the pH of intracellular compartment (21). Many of these effects may be completely unrelated to their ability to alter the pH of intracellular vesicles.

The data presented in this paper do not support a model for infection of cells by enveloped viruses whereby viruses fuse directly with the cell plasma membrane. Furthermore, the data presented above do not argue against endocytosis as a route of virus infection. This data, and data we have published previously on the fusion of cell monolayers by Sindbis virus (9, 16), suggest that arguments currently employed to support acidic cell compartments as essential participants in alphavirus infection may not be valid.

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LITERATURE CITED

1. **Beguinot, I., R. M. Lyall, M. C. Willingham, and I. Pastan.** 1984. Down regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. Proc. Natl. Acad. Sci. U.S.A. **81**:2384–2388.
2. **Brown, D. T., and J. F. Smith.** 1975. Morphology of BHK-21 cells infected with Sindbis virus temperature-sensitive mutants in complementation groups D and E. J. Virol. **15**:1262–1266.
3. **Charalampous, F. C., N. K. Gonatas, and A. D. Melbourne.** 1973. Isolation and properties of the plasma membrane of KB cells. J. Virol. **59**:421–435.
4. **Cheng, S., F. Maxfield, J. Robbins, M. Willingham, and I. Palstan.** 1980. Receptor-mediated uptake of 3, 3, triiodo-L-thyronine by cultured fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **77**:3425–3429.
5. **Coombs, K., E. Mann, J. Edwards, and D. T. Brown.** 1981. Effects of chloroquine and cytochalasin B on the infection of cells by Sindbis virus and vesicular stomatitis virus. J. Virol. **37**:1060–1065.
6. **Dimmock, N. J.** 1982. Initial stages in infection with animal virus. J. Gen. Virol. **59**:1–22.
7. **Douglas, R. G.** 1979. Respiratory diseases, p. 385–459. *In*, G. J. Galasso, T. C. Merigan, and R. A. Buchanan, (ed.), Raven Press, New York.
8. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell cultures. Science **139**:432–437.
9. **Edwards, J., E. Mann, and D. T. Brown.** 1983. Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. J. Virol. **45**:1090–1097.
10. **Heine, J. W., and C. A. Schnaitman.** 1971. Entry of vesicular stomatitis virus into L cells. J. Virol. **8**:786–795.
11. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
12. **Helenius, A., M. Marsh, and J. White.** 1982. Inhibition of Semliki Forest virus penetration by lysosomotropic weak bases. J. Gen. Virol. **58**:47–61.
13. **Johnston, R., K. Wan, and H. Bose.** 1974. Homologous interference induced by Sindbis virus. J. Virol. **14**:1076–1082.
14. **Kato, N., and H. J. Eggers.** 1969. Inhibition of uncoating of fowl plague virus by 1-adamantanamine hydrochloride. Virology **37**:632–641.
15. **Koff, W. C., and V. Knight.** 1979. Inhibition of influenza virus uncoating by rimantadine hydrochloride. J. Virol. **31**:261–263.
16. **Mann, E., J. Edwards, and D. T. Brown.** 1983. Polycaryocyte formation mediated by Sindbis virus glycoproteins. J. Virol. **45**:1083–1089.
17. **Marsh, M., E. Bolzau, and A. Helenius.** 1983. Penetration of Semliki forest virus from acidic prelysosomal vacuoles. Cell **32**:931–940.
18. **Marsh, M., and A. Helenius.** 1980. Adsorptive endocytosis of Semliki Forest virus. J. Mol. Biol. **142**:439–454.
19. **Marsh, M., K. Matlin, K. Simons, H. Reggio, J. White, J. Kartenbeck, and A. Helenius.** 1982. Are lysosomes a site of enveloped-virus penetration? Cold Springs Harbor Symp. Quant. Biol. **46**:835–843.
20. **Matlin, K., H. Reggio, K. Simons, and A. Helenius.** 1982. The pathway of vesicular stomatitis virus leading to infection. J. Mol. Biol. **156**:609–631.
21. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. **75**:3327–3331.
22. **Renz, D., and D. T. Brown.** 1976. Characteristics of Sindbis virus temperature-sensitive mutants in cultured BHK-21 and *Aedes albopictus* (mosquito) cells by Sindbis virus. J. Virol. **23**:554–561.
23. **Richman, D. D., P. Yazaki, and K. Y. Hostetler.** 1981. The intracellular distribution and antiviral activity of amantadine. Virology **112**:81–90.
24. **Riedel, B., and D. T. Brown.** 1977. Role of extracellular virus in the maintenance of the persistent infection induced in *Aedes albopictus* (mosquito) cells by Sindbis virus. J. Virol. **23**:554–561.
25. **Rosenwirth, B., and H. J. Eggers.** 1979. Early processes of echovirus 12-infection: elution, penetration and uncoating under the influence of rhodanine. Virology **97**:241–255.
26. **Sawicki, D. L., S. G. Sawicki, S. Keranen, and L. Kaariainen.** 1981. Specific Sindbis virus-coded functions for minus-strand RNA synthesis. J. Virol. **39**:348–358.
27. **Scheefers-Borchel, U., H. Scheefers, J. Edwards, and D. T. Brown.** 1981. Sindbis virus maturation in cultured mosquito cells is sensitive to actinomycin D. Virology **110**:292–301.
28. **Schlegel, R., R. Dickson, M. Willingham, and I. Pastan.** 1982. Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of α_2-macroglobulin. Proc. Natl. Acad. Sci. U.S.A. **79**:2291–2295.
29. **Seglen, P. O.** 1983. Inhibitors of lysosomal function. Methods Enzymol. **96**:737–764.
30. **Smith, J. F., and D. T. Brown.** 1977. Envelopment of Sindbis virus: synthesis and organization of proteins in cells infected with wild-type and maturation-defective mutants. J. Virol. **22**:662–678.
31. **Talbot, P. J., and D. E. Vance.** 1980. Sindbis virus infects BHK-21 cells via a lysosomal route. Can. J. Biochem. **58**:1131–1137.
32. **Tycko, B., and F. R. Maxfield.** 1982. Rapid acidification of endocytic vesicles containing α_2-macroglobulin. Cell **28**:643–651.
33. **White, J., and A. Helenius.** 1980. pH dependent fusion between the Semliki Forest virus membrane and liposomes. Proc. Natl. Acad. Sci. U.S.A. **77**:3273–3277.
34. **White, J., J. Kartenbeck, and A. Helenius.** 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. J. Cell Biol. **87**:264–272.
35. **White, J., K. Matlin, and A. Helenius.** 1981. Cell fusion by Semliki Forest, influenza and vesicular stomatitis virus. J. Cell Biol. **89**:674–679.