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Antihistaminics, local anesthetics, and other amines as antiviral agents

(vesicular stomatitis virus/Sendai virus/Semliki Forest virus/influenza/lysosomes)

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ABSTRACT **A number of lipophilic amines of diverse chemical structure, including antihistaminics, local anesthetics, and chloroquine, were found to exhibit similar kinetics in inhibiting the infection of BHK cells by vesicular stomatitis virus. The inhibition occurred prior to both primary and secondary RNA transcription but following transfer from the cell surface to an intracellular site, presumed to be the lysosomes. A similar inhibition, by these agents, of infection by Sendai, influenza strain WSN, and Semliki Forest viruses suggested a lysosomal involvement in infection by these viruses as well.**

The mechanism of entry of vesicular stomatitis virus (VSV) into cells and removal of its viral envelope has been the subject of considerable controversy, with microscopic evidence supporting penetration both by endocytosis (1) and by fusion with the cell plasma membrane (2). From comparison of the microscopic techniques used by Simpson *et al.* (1) and Heine and Schnaitman (2), Dahlberg (3) found that almost no VSV virions enter cells by fusion with the plasma membrane under the conditions generally used to infect cells. Entry of VSV occurred by adsorption to cellular coated pits, internalization into coated vesicles within a few minutes, and intracellular accumulation of virus into larger vesicles, some of which were lysosomes. In support of these observations, Fan and Sefton (4) found that cells infected with VSV are not susceptible to complement-mediated, antibody-dependent cell lysis during the penetration phase of viral infection.

Semliki Forest virus (SFV) was also endocytosed quickly through coated vesicles and then moved into larger intracellular vacuoles and lysosomes (5). Removal of the SFV viral envelope occurred in the lysosome where the low internal pH was assumed to catalyze the fusion of the viral membrane with the lysosomal membrane, thus releasing the nucleocapsid into the cytoplasm. This process was inhibited by chloroquine and other amines (5). Lipophilic amines concentrate rapidly within lysosomes, raising the lysosomal pH and inhibiting lysosomal hydrolysis (6, 7). The infection of BHK cells by VSV was similarly found to be inhibited intracellularly by chloroquine early in its infection cycle (8).

Many diverse pharmacologically active substances, including antiinflammatory drugs, antihistaminics, local anesthetics, and aliphatic amines, have been suggested to have lysosomal effects (9–12). All these amines might be expected to diffuse through cell membranes in their uncharged form and to accumulate in lysosomes where, like chloroquine, they would raise the pH. Several such compounds have been shown to do this (6), and some also induce lipid storage in lysosomes (13).

We have investigated the effect of various lipophilic amines in order to substantiate a common intracellular inhibition of VSV infection. All substances tested inhibited viral infection with the same time dependence, prior to the onset of primary RNA transcription. Experiments with Sendai virus (three strains), influenza, and SFV all showed similar inhibition with these agents. Thus, all of these viruses appear to have in common an intracellular step that can be inhibited by lipophilic amines of diverse structure.

MATERIALS AND METHODS

Virus Stocks. Stock VSV, Indiana serotype (Birmingham strain), was grown in confluent BHK-21F cells as described (8). The Z, RU, and Obayashi strains of Sendai were a gift of Frank Landsberger (Rockefeller University). Stock solutions were grown for 2 days in the allantoic cavity of 10-day embryonic chicken eggs and clarified. All three strains had titers of about 2000 hemagglutination units (HU)/ml; 1 unit was the reciprocal of the greatest viral dilution that produced agglutination of an equal volume of a 0.5% suspension of fresh human erythrocytes (type AB+) in phosphate-buffered saline. Influenza (WSN 1541) and SFV were gifts of Robert Simpson (Rutgers University) and Victor Stoller (Rutgers Medical School), respectively. All stock virus was diluted to 10^9 plaque-forming units (pfu)/ml, stored at −70°C, and used only once after thawing.

Virus Inhibition Assays. For secondary VSV transcription, confluent BHK cells were infected in one of two ways: (*i*) 4 pfu per cell (4×10^6 pfu per 35-mm dish) for 1 hr at 37°C in 1 ml of serum-free minimal essential medium containing 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid at pH 7.0, 100 μg of bovine serum albumin per ml, and 10 μg of actinomycin D per ml; or (*ii*) 10–20 pfu per cell for 1 hr at 5°C in 1 ml of the above medium. The virus medium was removed, and the cells were washed once with phosphate-buffered saline and then incubated with 1 ml of the above virus-free medium containing 5 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]uridine (Amersham; 5 Ci/mmol) per ml at 37°C for either 4 hr (for cells originally incubated with virus at 37°C) or 5 hr (for cells incubated with virus at 5°C). After this incubation, viral RNA was precipitated and assayed for radioactivity as described (14).

To measure primary VSV transcription, identical conditions of viral adsorption and subsequent incubation with [^{3}H]uridine were used with the following exceptions: (*i*) all incubation media contained cycloheximide at 100 μg/ml (15); (*ii*) unless otherwise noted, adsorptions were for 1 hr at either 5 or 37°C at a multiplicity of infection (moi) of 1000 in 1 ml of medium containing also 10 μg of DEAE-dextran (500,000 molecular weight; Pharmacia) per ml; and (*iii*) 10 μCi of [^{3}H]uridine (29 Ci/mmol) per ml was used.

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Abbreviations: VSV, vesicular stomatitis virus; SFV, Semliki Forest virus; moi, multiplicity of infection; HU, hemagglutination unit(s); pfu, plaque-forming unit(s).

Identical conditions (at 5°C) were used with the other viruses except for the following: SFV, moi of 1; Sendai, moi of 50 HU/10^6 cells, incubated at 12–15 hr at 37°C; influenza, moi of 2 without actinomycin D, incubated 20 hr at 37°C in 2% fetal calf serum and assayed for HU.

The effect of various inhibitors was tested by adding 50 μl of a 20-fold concentrated solution of the amines in minimal essential medium to plates at the indicated times. Once added, the amines were maintained with the cells continuously until harvest. The amount of inhibition of viral infection was determined by comparison of the total radioactivity in the presence of inhibitor to a standard curve of serially decreasing concentrations of infectious virus (14). All reagents were from Sigma except: pyrilamine maleate, Merck; promethazine·HCl, Wyeth; lidocaine (Xylocaine), Astra; chlorpheniramine, Schering; propylamine, Eastman; aminopyrine, Aldrich; tetracaine·HCl, Cooper.

RESULTS

Inhibition of VSV RNA Production by Amines. All the amines tested in the VSV RNA assay completely inhibited VSV infection when added to the cells prior to infection. Inhibition increased with increasing concentrations of each drug. The concentrations producing 50% inhibition are listed in Table 1. Chloroquine was effective at the lowest concentration of any of the compounds tested; dibucaine, which contains a similar quinoline ring structure, was nearly as effective. Aliphatic amines were only 1% as effective.

Binding and internalization of [^{35}S]methionine-labeled VSV was not affected by the presence of chloroquine, pyrilamine maleate, ethylenediamine, or methylamine at concentrations that gave complete inhibition of viral transcription (not shown). The compounds shown in Table 1 did not act by inducing irreversible changes in the cells; preincubation for 0.5–2 hr with concentrations of chloroquine, dibucaine, lidocaine, pyrilamine maleate, or methylamine sufficient to give 100% inhibition of viral transcription, followed by removal of the drug and subsequent viral infection with VSV, produced no lasting inhibitory effect.

The reversibility of viral infection in the presence of the amines was observed in an experiment in which cells were exposed to VSV for 1 hr at 5°C, washed, and then incubated at 37°C in the presence of 25 mM methylamine (Fig. 1). At various times, the cells were washed and medium containing [^{3}H]uridine without methylamine was added. In cells treated with methylamine either before or after adsorption of virus ("preincubation"), the onset of secondary transcription occurred *ca.* 1 hr later than in control cells never treated with methylamine. The total amount of RNA synthesized, however, was the same as in the control cells. After the lag period, the amine-treated cells synthesized RNA more rapidly than the control cells. Thus, the amines did not prevent binding of virus to the cells; instead, they seemed to inhibit reversibly the infectious virus in the cells in such a way that, when the inhibition was released, there was a synchronized burst of viral transcription. Essentially identical results were obtained with chloroquine (100 μM) and pyrilamine maleate (1.25 mM) except that the lag in RNA synthesis after removal of the drug was *ca.* 1 hr longer than that found with methylamine (not shown). These results suggest that all these compounds have a similar reversible action on VSV infection.

Table 1. Inhibitors of VSV transcription

Group	Drug	Concentration for 50% inhibition, mM
Antimalarial	Chloroquine	0.02
Local anesthetic	Dibucaine	0.025
	Tetracaine	0.20
	Lidocaine	0.22
	Procaine	3.3
Antihistaminic	Pyrilamine maleate	0.05
	Chlorpheniramine	0.4
	Promethazine·HCl	0.5
Antipyretic	Aminopyrine	2.3
Miscellaneous amines	Dansylcadaverine	0.4
	Ethylenediamine	1.5
	1-Propylamine	4
	Imidazole	4
	Methylamine	5.5

Infection with a moi of 4 at 37°C.

Effects of Chloroquine and Pyrilamine Maleate on the Rate of Primary and Secondary RNA Transcription. If the amines in Table 1 act to prevent uncoating of the input virions, then

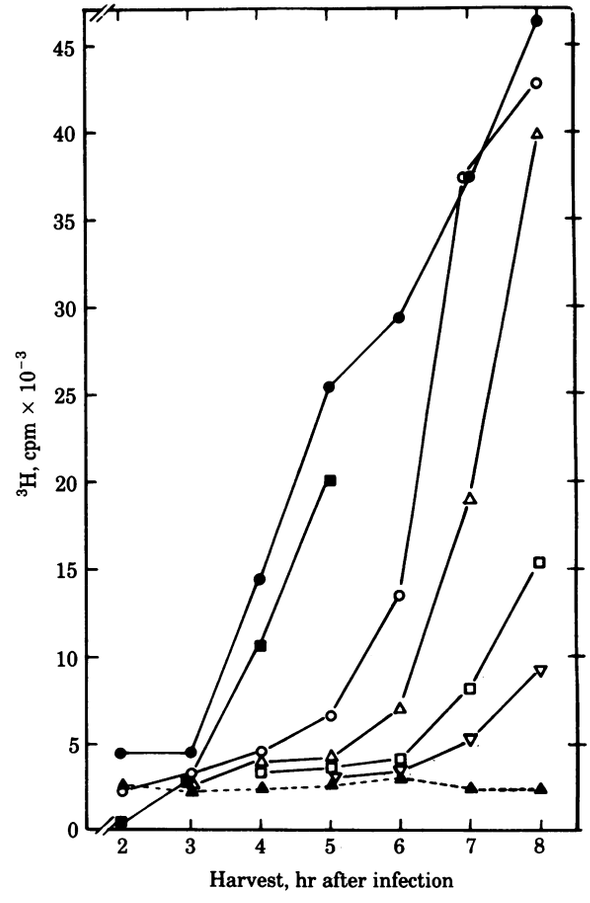

FIG. 1. Time course of synthesis of secondary VSV RNA after removal of 25 mM methylamine. Infection was initiated by incubation at a moi of 15 for 1 hr at 5°C. ■, Cells preincubated with actinomycin D and methylamine for 2 hr at 37°C and then methylamine removed prior to infection. Preincubation with actinomycin D alone had no effect on the rate or amount of viral RNA synthesis (not shown) compared with cells neither preincubated nor exposed to methylamine (●). At various washout times, methylamine-containing medium was replaced by identical medium lacking methylamine: ○, 1 hr; △, 2 hr; □, 3 hr; ▽, 4 hr. ▲, Cells incubated with methylamine continuously after infection.

inhibition of either primary or secondary RNA production should be observed only if the agent is added early relative to RNA production. In contrast, if the agents have an effect directly on the transcription process, inhibition should occur even if the amine is added at a time when RNA production is already well advanced. In order to distinguish between these alternatives, chloroquine or pyrilamine maleate was added at different times after infection and the rates of primary and secondary transcription were measured.

In agreement with the results of Flamand and Bishop (16), we found that the accumulation of primary transcripts proceeded approximately linearly for at least 5 hr after infection, although a slight early lag in production was a reproducible feature (Fig. 2 *Lower*). For secondary RNA synthesis there was a marked lag, with little synthesis occurring prior to about 3 hr after infection (Fig. 2 *Upper*). It is clear that, to be maximally effective at inhibiting either primary or secondary RNA production, chloroquine must be added well before the time that most of the RNA is produced. Addition of the drug between 3 and 4 hr after infection, when much of both primary and secondary RNA was being formed, had little inhibitory effect on the overall rate of transcription. Identical results were obtained with pyrilamine maleate (not shown).

In an experiment in which both chloroquine and [^{3}H]uridine were added to the cells at different times after infection, it was shown that chloroquine did not act by limiting the accessibility of the radiolabel to the nucleotide pool used for RNA synthesis. Such a limitation would be expressed in this experiment as an increase in apparent inhibition upon addition of [^{3}H]uridine at later times after infection. Although the total radioactivity incorporated was decreased when [^{3}H]uridine was added at later times, the amount of inhibition due to chloroquine added at any specific time after infection remained unchanged (not shown). Similar results were obtained when [^{3}H]uridine was present as a pulse for only 1 hr immediately after infection. Thus, the decrease in [^{3}H]uridine incorporation into RNA induced by chloroquine was independent of when the radiolabel was added to the cells or how long it was present.

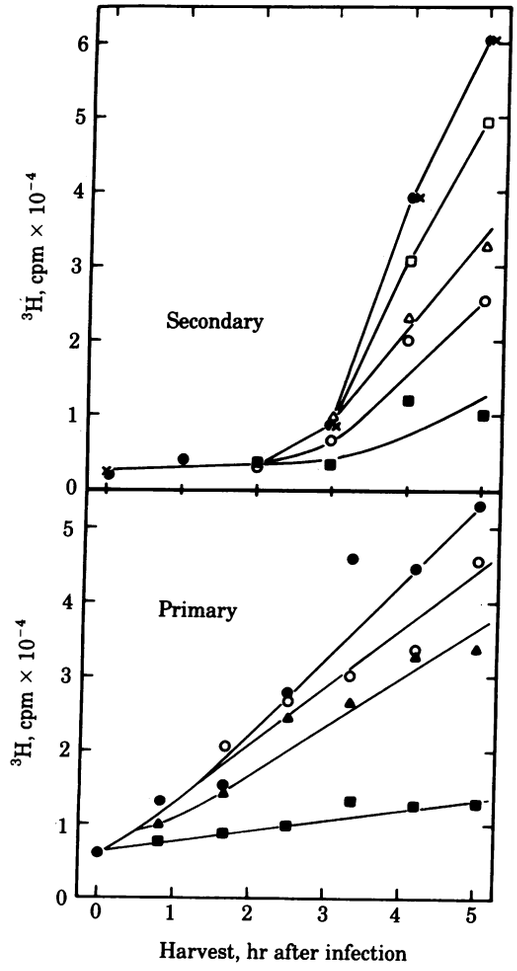

FIG. 2. Inhibition of VSV RNA transcription by chloroquine added at various times after infection in assays measuring secondary (*Upper*) and primary (*Lower*) RNA synthesis. VSV was adsorbed for 1 hr at 5°C at moi of 15. Inhibitor added at: ■, 0 hr; ▲, 0.5; ○, 1; △, 2; □, 3; ×, 4; ●, no addition.

Time of Inhibition by Amines. Results from the previous experiments suggested that the inhibition of VSV infection by the agents listed in Table 1 occurred prior to transcription. Consequently, the drugs were added to primary and secondary RNA assays at increasing times after infection to determine their inhibition times more exactly. Both chloroquine and pyrilamine maleate showed a fall-off of inhibition of primary transcription as a function of length of time after infection; 50% of inhibition was lost by about 30 min after infection (Fig. 3). Similar results were found with ethylenediamine and methylamine (not shown).

It was somewhat surprising, however, to find that, by comparison, inhibition of secondary transcription fell off much more slowly; a 50% loss of inhibition by the five compounds tested occurred at about 2 hr after infection (Fig. 3). Cycloheximide, which had a direct effect on secondary transcription itself, showed a much longer-lasting inhibition, closely mirroring the time course of secondary RNA production (cf. Fig. 2 *Upper*). This is a further indication that the effect of the amines is not on secondary transcription itself but on some earlier stage in viral infection.

The different time courses for loss of inhibition of primary and secondary transcription by the drugs were related to the moi used in the two experiments (Fig. 4). The half-time for loss of inhibitory potency of pyrilamine maleate [or chloroquine (not shown)] in secondary transcription varied from about 2 hr after

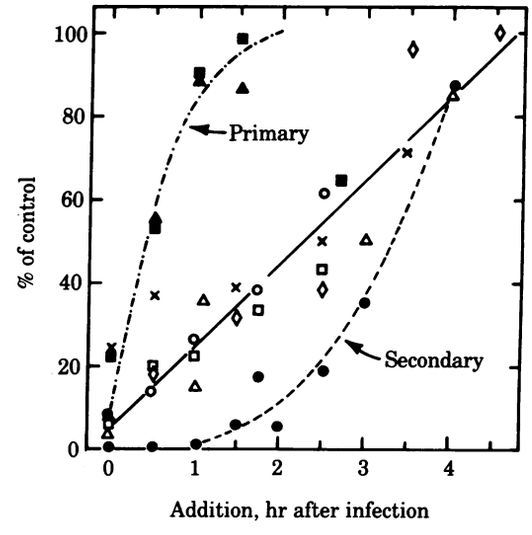

FIG. 3. Inhibition of VSV primary (▲, ■) and secondary (open symbols and ●) RNA transcription by amines as a function of time of addition. VSV was incubated with cells for 1 hr at 5°C. Except as indicated, all drugs were tested in secondary transcription assays. △, ▲, 100 μM chloroquine; □, ■, 1.2 mM pyrilamine maleate; ○, 12 mM ethylenediamine; ×, 15 mM lidocaine; ◇, 25 mM methylamine; ●, 36 nM cycloheximide.

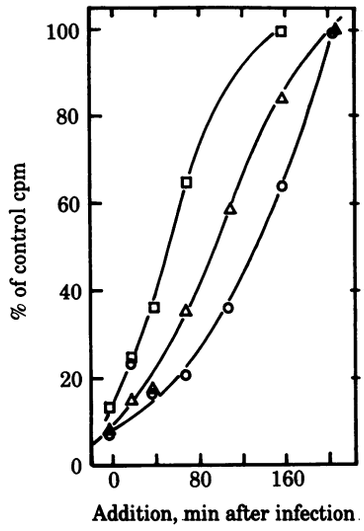

FIG. 4. Effect of moi on inhibition by pyrilamine maleate. Secondary VSV RNA transcription was measured after addition of 1.25 mM pyrilamine maleate at various times after infection. VSV was added for 1 hr at 5°C at: ○, 10 pfu per cell; △, 100; □, 1000.

infection at a moi of 10 pfu per cell to less than 1 hr after infection at a moi of 1000 pfu per cell. Similar effects of moi on the rate of RNA synthesis by VSV have been reported (17). Thus, the time course for loss of inhibition of secondary transcription by all of the compounds tested approached that seen for primary transcription at the high moi values needed to measure primary transcription. These results are consistent with a single inhibitory action of all the drugs tested, occurring prior to the time of primary VSV transcription.

In addition to the much higher moi values in the primary compared to the secondary transcription assays, an additional difference between the assays was the presence of DEAE-dextran in the former. Binding studies with highly radiolabeled infectious VSV added to cells at 10 pfu per cell showed that DEAE-dextran induced a 4-fold increase in virion binding, with no effect on either the proportion of total bound virus internalized or the rate of internalization (data not shown; cf. ref. 8). In parallel with the increased binding, the presence of DEAE-dextran increased total primary RNA production also by a factor of about 4, without any change in the time of onset or the rate of production of either primary or secondary RNA (data not shown). The increase in the amount of bound and internalized virions induced by DEAE-dextran was therefore sufficient to account for the entire increase in RNA production observed. This finding provides a further indication that specific cell surface receptors are not required for VSV to initiate infection (8).

Concentrations of amines that gave less than 100% inhibition when added immediately after infection gave even less inhibition when added at later times; the rate of loss of inhibition was parallel to that shown in Fig. 3 (not shown). At concentrations greater than those required to cause 100% inhibition, the drugs did not inhibit RNA formation for longer times after infection; the loss of inhibition occurred with a similar time course at all concentrations tested, up to concentrations that were toxic to the cells. These kinetics suggest that the drugs arrive essentially immediately at their site of inhibitory action, consistent with the previously determined rapid rate of accumulation of several of these drugs in lysosomes (7).

Inhibition of SFV, Sendai Virus, and Influenza Virus Infections. The addition of 100 μM chloroquine to BHK cells infected with several other enveloped viruses produced a similar inhibition: 100% with SFV and influenza virus; 92%, 95%, and 98% inhibition with Z (high fusion), RU (medium fusion), and Obayashi (low fusion) strains of Sendai virus, respectively. A 50% loss of inhibitory potency was observed when the amines were added at *ca.* 2.5 hr after infection for SFV (Fig. 5 *Upper*) or 4.5 hr after infection for Sendai virus (Fig. 5 *Lower*). The relationship between cycloheximide inhibition of Sendai virus RNA synthesis and inhibition caused by the amines is similar to that found for VSV (Fig. 3), although RNA synthesis occurred more slowly in Sendai virus-infected cells. As expected, the cycloheximide inhibition curve followed closely the rate of secondary RNA produced in the Sendai virus-infected cells (data not shown) in which significant RNA was formed after about 8 hr with a maximum found in the cells at 12–14 hr.

DISCUSSION

In this study, various basic lipophilic compounds inhibited infection by VSV and several other enveloped viruses. The similar dependences on time and moi displayed by these compounds in viral inhibition (Figs. 3 and 5) suggest that they all act at the same point in the virus infectious cycle. That point of inhibition has been shown to precede both primary and secondary transcription (Fig. 2). Furthermore, neither cell surface binding nor internalization of labeled VSV is affected by these drugs; the drugs are effective if added at a time—e.g., 1 hr after infection—at which internalization is already complete (8). This is in contrast to recent reports concerning receptor-mediated endocytosis, in which certain primary amines were found to inhibit a surface function related to internalization (18). In the present experiments, both primary and secondary transcription were affected with similar kinetics (Fig. 3).

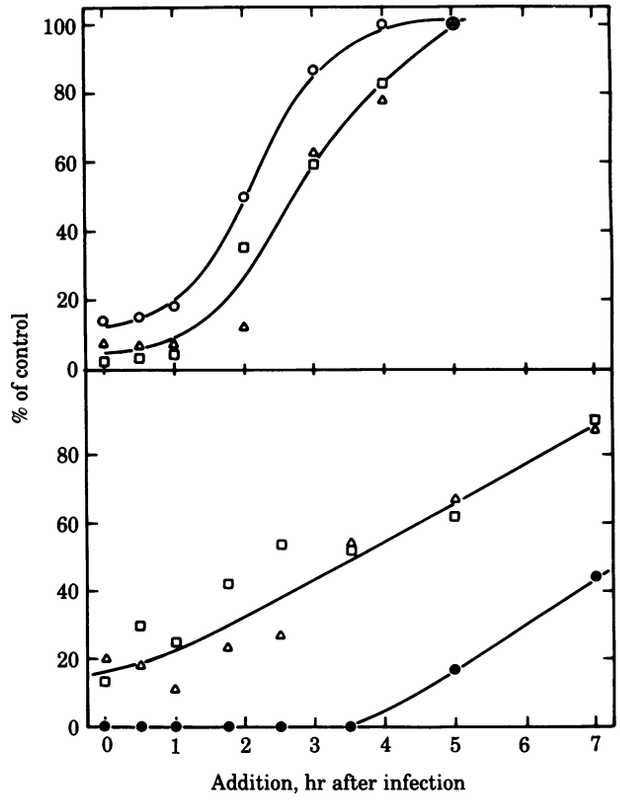

FIG. 5. Inhibition of RNA production in *Upper* and Z strain Sendai virus (*Lower*) by amines and cycloheximide as a function of time of addition. Inhibitors were added at the indicated times after removal of virus (incubated for 1 hr at 5°C); ○, 100 μM chloroquine; △, 1.25 mM pyrilamine maleate; □, 12.5 mM ethylenediamine; ●, cycloheximide at 1 μg/ml.

Several of the compounds in Table 1 have been directly demonstrated to accumulate in lysosomes and to inactivate lysosomal functions (7), and others have been suggested to have a similar action (9–12). Ohkuma and Poole (6) found that, whereas lysosomal pH decreased sharply after washout of amines that were effective in increasing lysosomal pH, many minutes were required for complete return to the original pH. The lag period observed between removal of the amines and the onset of RNA synthesis (Fig. 1) may represent the time required for a complete recovery of lysosomal function. The fact that this lag was longer for chloroquine than for methylamine is consistent with the finding by Ohkuma and Poole (6) that lysosomes recovered more slowly from chloroquine treatment than from methylamine treatment.

Some endocytic vesicles have been observed to undergo saltatory motion in the cytoplasm without fusing with lysosomes for several hours following internalization (19). If a similar event occurred with VSV-containing endocytic particles, this could account for the *ca.* 2-hr half-life with which VSV at moi < 1 pfu per cell passes through the chloroquine-sensitive step (Fig. 3). Based on other observations, endocytic vesicles bearing VSV would be expected to fuse with lysosomes even when the amines were present (20–22). The burst of transcription noted in Fig. 1 after removal of the amines could then result from a synchronized uncoating of virus that has accumulated in the lysosomes.

Extensive evidence has been obtained that uncoating of SFV occurs in the lysosomes (5, 23). Earlier reports indicated that the amine amantadine, an active lysosomotropic agent (6), inhibits the uncoating of influenza and fowl plague viruses (24–26). Amantadine and isoquinoline derivatives have also been shown to inhibit infection by paramyxoviruses (25, 27). Previous investigations with adenovirus, hepatitis virus, vaccinia, polyoma virus, and reovirus have suggested a necessary role of lysosomes in uncoating of the virus (9, 28). The present findings are most readily interpreted in terms of an obligatory lysosomal step in the uncoating of all the enveloped viruses studied and suggest that there may be a general pathway for viral uncoating.

Abundant evidence exists that fusion of the Sendai virus envelope is necessary for infection (12, 29). Our own observations are consistent with this: the amount of Sendai virus RNA synthesized is directly proportional to the fusogenic capacity of the virus, whereas the time course of RNA synthesis for these different strains remains the same (unpublished data). The similarity between Sendai virus and the other viruses studied in the present experiments implies, however, either (*a*) it is not fusion with the plasma membrane but rather endocytosis followed later by intracellular fusion that initiates Sendai virus infection, or (*b*) there is a separate intracellular, and probably lysosomal, step necessary to complete the uncoating of Sendai virus after fusion with the plasma membrane. Neither of these two possibilities has been eliminated. Infectious Sendai virions have variable capability (depending upon the strain) to fuse with membranes and synthetic bilayers not only at neutral pH but also at pH 5.5; Sendai virus-induced hemolysis occurs at pH 5.5 with an efficiency of about 70% of that at pH 7.0 (30). Furthermore, the Sendai virus nucleocapsids have been found to remain firmly attached to the inner surface of erythrocytes after fusion and can be released only by a separate action (cf. ref. 31), suggesting the possibility that an additional intracellular step may be needed before uncoating is complete. The much slower kinetics of Sendai virus compared with the other viruses studied (Figs. 2 and 5) suggests that this intracellular step in Sendai virus infection could be different in character from that found for the other viruses and could provide an explanation for the relatively long eclipse period that characterizes this virus.

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