



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Attenuation of Murine Coronavirus Infection by Ammonium Chloride

LEE MIZZEN,¹ ANNE HILTON, STEVE CHELEY,² AND ROBERT ANDERSON^{1,3}

*Department of Microbiology and Immunology, University of Western Ontario,
London, Ontario N6A 5C1, Canada*

Received August 13, 1984; accepted December 4, 1984

Ammonium chloride at a concentration of 20 mM delayed by 4-5 hr the production of virus progeny in mouse L-2 cells infected at high multiplicity with mouse hepatitis virus (MHV). This delay was seen in the production of both intracellular and extracellular virus. However, the final titers were similar to those produced by MHV-infected cells maintained in normal medium. The manifestation of virus-induced cell fusion was similarly found to be delayed, but not otherwise decreased in severity, when ammonium chloride was present in the culture medium. Ammonium chloride caused similar delays in production of virus-specific, positive-sense RNAs and of viral polypeptides. The relative proportions and apparent molecular weights of viral RNAs and polypeptides were similar to those found in MHV-infected cells cultured in normal medium. *In vitro* translation of endogenously produced viral RNAs in cell extracts, prepared from MHV-infected cells, was not inhibited by ammonium chloride. Thus, ammonium chloride has no specific, inhibitory effect on viral protein synthesis. Ammonium chloride did not reduce the number of virus-infected cells in culture, as monitored by infectious center assay. Analysis of early events in MHV infection showed that ammonium chloride did not affect adsorption or internalization of MHV by L-2 cells. However, the subsequent eclipse phase, as monitored by decline in infectivity of internalized virus inoculum proceeded less efficiently in the presence of ammonium chloride. On the basis of the known inhibitory effects of ammonium chloride on lysosomal/endosomal functions, the results suggest an endosomal mechanism of MHV uncoating. Thus the primary effect of ammonium chloride on MHV infection of L-2 cells is to attenuate virus uncoating, thereby chronologically displacing all subsequent virus-encoded functions. © 1985 Academic Press, Inc.

INTRODUCTION

The earliest events involved in virus infection of susceptible cells remain to be fully characterized. While a seemingly limited number of viruses such as Sendai virus effect entry into the cell by a direct fusion process of viral envelope with host plasma membrane (Choppin and Compans, 1975), it now appears that most lipid-enveloped viruses are taken up by an

endocytic mechanism (Dales, 1973; Fan and Sefton, 1978; Marsh and Helenius, 1980; Simpson *et al.*, 1969). In the case of the rhabdovirus, vesicular stomatitis virus (Schlegel *et al.*, 1982), and the togavirus, Semliki Forest virus (Helenius *et al.*, 1980; Marsh *et al.*, 1983), the endocytic process has been shown to involve uptake into clathrin-coated pits similar to those implicated in the receptor-dependent uptake of hormones and other proteins (Dickson *et al.*, 1981; Goldstein *et al.*, 1979). Once inside the coated vesicle and subsequent endosome (Helenius *et al.*, 1980) or receptorosome (Willingham and Pastan, 1980), however, a low pH-dependent fusion event mediates final entry of the viral genome into the cytosol (Helenius *et al.*, 1980; Maeda *et al.*, 1981; White *et al.*, 1981).

¹ Present address: Department of Microbiology & Infectious Diseases, University of Calgary, Health Sciences Centre, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada.

² Present address: Cold Spring Harbor Laboratories, Box 100, Cold Spring Harbor, N. Y. 11724.

³ Author to whom requests for reprints should be addressed.

Lysosomotropic agents such as ammonium chloride and a variety of amines have been demonstrated to inhibit the replication of viruses from the toga- (Helenius *et al.*, 1982), rhabdo- (Schlegel *et al.*, 1982), orthomyxo- (Kato and Eggers, 1969), retro- (Pazmino *et al.*, 1974), and herpes- (Shimizu *et al.*, 1972) virus families. A common characteristic of these agents is that they are weak bases and accumulate within acidic intracellular compartments, notably lysosomes, thereby elevating pH (Ohkuma and Poole, 1978). Perturbation of the pH within lysosomes and related vesicles has been shown (Helenius *et al.*, 1980; Marsh *et al.*, 1983) to inhibit the fusion of the prelysosomal (or endosomal) membrane with the viral envelope of the togavirus, Semliki Forest virus. Such fusion inhibition thereby interferes with virion uncoating and liberation of viral genome into the cytosol.

The role of lysosomes and related acidic compartments in the replication of coronaviruses is not well understood. Coronavirus infection frequently involves the intracellular accumulation of virus-filled, electron-dense structures which have been interpreted to be lysosomes (David-Ferreira and Manaker, 1965; Ducatelle and Hoorens, 1984). Such structures are observed within 2-3 hr postinoculation and have, therefore, been suggested to be involved in virus uptake (David-Ferreira and Manaker, 1965). Further evidence for a lysosomal role in coronavirus replication was provided by studies in which lysosomotropic agents were found to inhibit production of virus progeny and cytopathic effect in cells infected with mouse hepatitis virus (MHV; Mallucci, 1966; Krzysztyniak and Dupuy, 1984).

Besides affecting early events, lysosomotropic agents including ammonium chloride have been reported to inhibit other stages in virus replication, such as protein glycosylation (Kousoulas *et al.*, 1982) and expression of cytopathic activity (Holland and Person, 1977). In order to investigate more precisely the mode of action of ammonium chloride on the replication of MHV, we undertook a systematic examination of its effects on the normal se-

quence of events occurring during the replication of MHV in a lytic, syncytiogenic infection of mouse L-2 cells.

MATERIALS AND METHODS

Infection and culture conditions. L-2 cells (Rothfels *et al.*, 1959) maintained as described previously (Lucas *et al.*, 1977) were inoculated with the A59 strain of mouse hepatitis virus (MHV), adsorbed for 30 min at 4°, washed with minimum essential medium (MEM), and then incubated at 37° in MEM supplemented with 5% fetal calf serum (FCS). Culture medium was made 20 mM with respect to ammonium chloride by the addition of a 200 mM stock solution.

Assays of intracellular and extracellular MHV. Cultures of L-2 cells, inoculated with MHV (see above) at a multiplicity of infection (m.o.i.) of 20 in 35-mm tissue culture plates were used. To determine levels of extracellular MHV, aliquots were removed from culture media for subsequent plaque assay (Lucas *et al.*, 1977). To assay intracellular MHV levels, monolayers were thrice washed with MEM and harvested on ice in 2 ml MEM with 5% FCS by scraping with a Teflon policeman. Harvests were forced twice through a 21-gauge needle fitted to a 3-ml plastic syringe and subjected to a single cycle of freezing (-70°) and rapid thawing. The thawed samples were finally forced through a 30-gauge needle and directly assayed for infectious virus by plaque assay (Lucas *et al.*, 1977).

Assay of virus internalization. Cultures of L-2 cells in 35-mm plates were adsorbed for 30 min with MHV at an m.o.i. of 5, washed to remove excess inoculum, then warmed to 37° for varying intervals in the presence or absence of ammonium chloride. Cultures were subsequently treated with proteinase K (0.5 mg/ml) in phosphate-buffered saline (PBS) for 45 min at 4° in order to remove external virus (Helenius *et al.*, 1980). After terminating the protease treatment with 1 mM PMSF, 3% bovine serum albumin (BSA) in PBS, cells were transferred to a centrifuge tube, spun into pellets (1 min at

650 g), and washed twice with 0.2% BSA in PBS. The final cell pellets were assayed for internalized virus by infectious center assay on L-2 monolayers.

Assay for infectivity of internalized virus inoculum. Cultures of L-2 cells in 35-mm plates were adsorbed for 30 min with MHV at an m.o.i. of 5, washed to remove excess inoculum, and subsequently incubated for 20 min at 37° in medium either containing or lacking ammonium chloride. Cells were then treated with proteinase K (as above) to remove external virus, washed, and then returned to incubation at 37° in the presence or absence of ammonium chloride. At various intervals, cells were harvested by centrifugation, washed twice, and assayed for intracellular MHV as described above.

Infectious center assays. Infected cell monolayers, cultured in the presence or absence of ammonium chloride, were washed, trypsinized, and assayed for infectious centers as previously described (Lucas *et al.*, 1977) except that, in some cases, ammonium chloride (20 mM) was included in the assay medium.

Dot-blot hybridization assay of viral RNA synthesis. Culture dishes (35 mm) of L-2 cells inoculated with MHV at an m.o.i. of 20 were harvested at various times post-inoculation (PI) and subjected to dot-blot hybridization analysis as previously described (Cheley and Anderson, 1984) using a ³²P-labeled cDNA prepared against MHV nucleocapsid-protein mRNA (Cheley *et al.*, 1981). It should be noted that this cDNA detects sequences present in all (+)-sense, intracellular MHV RNAs (Cheley *et al.*, 1981).

Gel electrophoresis analysis of viral RNA. Cultures of L-2 cells, inoculated with MHV at an m.o.i. of 20 were incubated at 37° in MEM supplemented with 5% FCS. At 0, 1, 2, or 3 hr PI culture medium was changed to fresh MEM supplemented with 5% FCS and containing 20 mM ammonium chloride. Cultures were supplemented with actinomycin D (2 µg/ml) at 4 hr PI and subsequently radiolabeled at 5 hr PI with [5-³H]uridine (10 µCi/ml). Cultures were harvested at 6 hr PI as described (Cheley and Anderson,

1984) except that the RNA pellets were taken up in 1 ml 50% dimethyl sulfoxide (DMSO), 7 M urea, 10 mM Tris, pH 5. Aliquots of 10 µl were applied to the wells of a 1-mm-thick 1% agarose gel prepared in 50% DMSO, 7 M urea, 10 mM Tris, pH 5, and electrophoresed in a vertical gel apparatus for 5-6 hr at 15 mA. Following electrophoresis, the gel was treated with EN³HANCE (New England Nuclear), dried, and autoradiographed.

Analysis of viral protein synthesis. Cultures of L-2 cells in 35-mm plates, inoculated with MHV at an m.o.i. of 20, were incubated at 37° in 2 ml MEM with 5% FCS with or without ammonium chloride. At various times PI culture medium was replaced with 0.5 ml of labeling medium ([³⁵S]methionine (100 µCi/ml) in methionine-free MEM, with or without ammonium chloride). After further incubation for 30 min at 37°, cultures were harvested and samples subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography as previously described (Cheley and Anderson, 1981). For *in vitro* translations, postmitochondrial ("S-30") extracts were prepared from infected cells at 5 hr PI, according to the procedure of Fischer and Moldave (1981). The resultant S-30 preparations, following desalting by Sephadex G-25 gel filtration, were used directly (i.e., without micrococcal nuclease digestion) for *in vitro* translation of endogenous mRNA. Final incubation mixtures contained, in volumes of 60 µl: 20 µl S-30, 6.3 mM HEPES, pH 7.4, 107 mM KCl, 3.4 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 20 mM creatine phosphate, 24 µg creatine phosphokinase, 0.1 mM each of 19 non-isotopic amino acids (excluding methionine), 30 µCi [³⁵S]methionine, and either 0, 10, or 20 mM ammonium chloride. Mixtures were incubated 60 min at 30° and subjected to SDS-PAGE followed by fluorographic analysis.

RESULTS

Effect of ammonium chloride on MHV replication. The presence of ammonium chloride in the culture medium at a con-

centration of 20 mM delayed the production of progeny MHV by roughly 4 hr in MHV-inoculated L-2 cells (Fig. 1). As shown in Fig. 1A, intracellular MHV progeny is first detectable under normal culture conditions at approx 4–5 hr PI. In contrast, intracellular MHV progeny does not appear until approx 8 hr PI in the presence of ammonium chloride. From the times of initial appearance of progeny virus, however, the replication profiles, either in the presence or absence of ammonium chloride, are very similar. Moreover, the final titers of MHV produced in either case differ only by a fraction of a log.

A similar delaying effect of ammonium chloride was observed on the production of extracellular virus. Under normal conditions, MHV-infected L-2 cells first produce detectable extracellular virus at approx 5 hr PI (Fig. 1B). In the presence of ammonium chloride, however, extracellular virus is not seen until approx 9–10 hr PI. Again, as in the case of intracellular

virus, the subsequent replication curves and final virus titers are very similar, whether monitored from control or ammonium chloride-treated cultures.

The above results suggest that the action of ammonium chloride is to attenuate a step in MHV replication, which once overcome, permits normal virus production. If this step is an early one, the normal chronology for the majority of the events associated with MHV replication will be displaced by a 4- to 5-hr time interval similar to that observed with virus replication. In agreement with this prediction, the manifestation of cell-cell fusion, a hallmark of MHV infection of L-2 cells (Mizzen *et al.*, 1983) was found to be delayed by ammonium chloride (Table 1) to an extent similar (approx 5 hr) to that observed with the production of virus progeny. The final levels of cell fusion in both ammonium chloride-treated and control cultures, were, nevertheless, similar, i.e., engulfing the total cell monolayer. There would, therefore, appear to be no specifically inhibitory effect of ammonium chloride on MHV-mediated cell fusion *per se*.

In order to confirm that the effect of ammonium chloride on MHV replication was one of attenuation rather than suppression, the number of productively infected L-2 cells was monitored by an infectious center assay. Cultures of MHV-inoculated L-2 cells were incubated for 3 hr at 37° in the presence or absence of ammonium chloride, then trypsinized and plated on uninfected L-2 cell monolayers in order to quantitatively screen for numbers of productively infected cells. As shown in Table 2, ammonium chloride-treated cultures showed similar numbers of infected cells as untreated cultures. Moreover, little difference was observed even when ammonium chloride was included in the medium used for the infectious center assay (Table 2). The size of the plaque produced by each infectious center, however, was considerably smaller in the case of the cells which were cultured in the presence of ammonium chloride. This result is entirely consistent with the

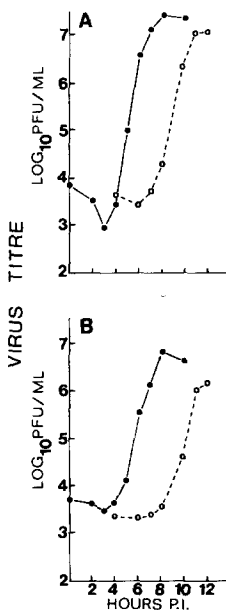


FIG. 1. Production of intracellular (A) and extracellular (B) virus from MHV-infected L-2 cells maintained at 37° in the absence (●—●) or presence (○---○) of 20 mM ammonium chloride.

TABLE 1
MHV-INDUCED CELL FUSION IN THE ABSENCE AND PRESENCE OF AMMONIUM CHLORIDE

Time PI (hr)	Approx area percentage of monolayer fused ^a	
	Control	NH ₄ Cl
0	0	0
2	0	0
3	0	0
4	10	0
5	60	0
6	90	0
7	100	5
8	—	10
9	—	50
10	—	70
11	—	90
12	—	100

^a Dish cultures (35 mm) of MHV-inoculated (m.o.i. = 20) L-2 cells were incubated in the absence (control) or presence (NH₄Cl) of 20 mM ammonium chloride. Dashes indicate detachment of the fused monolayer from the plastic substrate.

idea that ammonium chloride attenuates or delays MHV replication, without imposing an absolute block.

Effects of ammonium chloride on early stages of MHV infection. In agreement with the studies by Krzystyniak and Dupuy (1984), our preliminary experiments had shown that MHV adsorption to L cells was unaffected by the presence of ammonium chloride. Accordingly, all studies reported in the present paper were performed with the addition of ammonium chloride immediately following the adsorption period. The entry of MHV into the host cell appears to be one involving viropexis (David-Ferreira and Manaker, 1965) in which virus inoculum is internalized within a cytoplasmic vesicle or endosome. In order to investigate whether ammonium chloride affected the process of virus internalization, MHV-adsorbed cells were warmed to 37° for various intervals in the presence or absence of ammonium chloride, then stripped of external virus with proteinase K, and as-

sayed for internalized virus by infectious center assay. The results shown in Fig. 2A demonstrate that virus internalization occurred with equal efficiency, whether or not ammonium chloride was present. In either case, uptake of MHV into the cell occurred rapidly upon exposure to 37° and was essentially complete after 2 hr.

Events subsequent to virus uptake, which result in the liberation of viral genome into the cytosol have not been characterized in coronavirus replication. One possibility, analogous to that observed with many enveloped viruses, is that the viral envelope undergoes fusion with the membrane of the surrounding vesicle (e.g., Marsh *et al.*, 1983). Since this latter fusion event is known to be inhibited by lysosomotropic amines in the case of infection with certain viruses, such as the enveloped togaviruses (Helenius *et al.*, 1982), we monitored the effect of ammonium chloride on the eclipse phase of MHV infection. Virus uncoating, as well as any other degradative processes occurring during the eclipse phase, can be monitored by following the decline in infectivity of internalized MHV inoculum. In order to allow a reasonable degree of virus internalization to occur MHV-adsorbed cells were warmed

TABLE 2
AMMONIUM CHLORIDE DOES NOT MARKEDLY REDUCE THE NUMBER OF CELLS INFECTED WITH MHV

Initial medium (0-3 hr PI)	Number of cells infected ^a assay medium	
	Control	NH ₄ Cl
Control	6.8 × 10 ⁸	6.1 × 10 ⁸
NH ₄ Cl	5.2 × 10 ⁸	4.9 × 10 ⁸

^a Dish cultures (35 mm) of L-2 cells were inoculated with MHV at an m.o.i. = 0.01, adsorbed 30 min, and incubated 3 hr in the absence (control) or presence (NH₄Cl) of 20 mM ammonium chloride. Cells were recovered intact by trypsinization and aliquots were plated out for infectious center assay on monolayers of L-2 cells; assays were performed for 24 hr in the absence (control) or presence (NH₄Cl) of 20 mM ammonium chloride in medium containing methylcellulose.

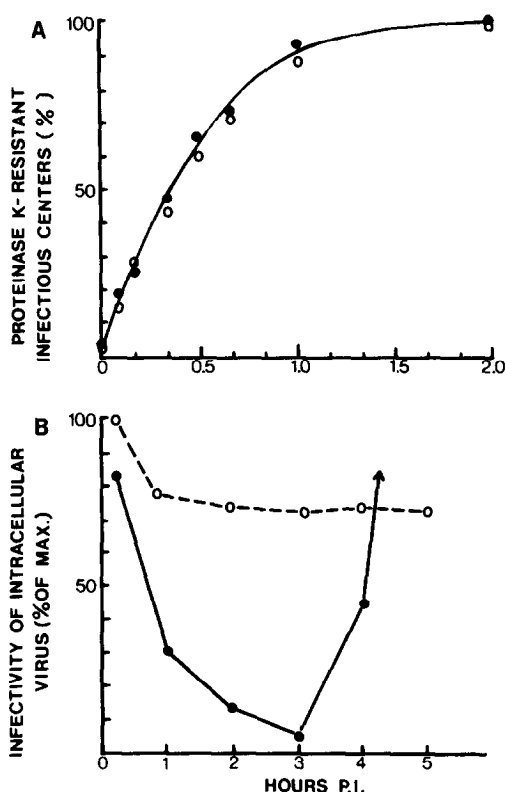


FIG. 2. Effect of ammonium chloride on virus internalization (A) and infectivity of internalized virus (B). (A) Cultures of MHV-adsorbed L-2 cells were warmed to 37° for various times in the absence (●—●) or presence (○---○) of ammonium chloride. After treatment with proteinase K to remove external virus, cells were assayed for internalized virus by infectious center assay. (B) Cultures of MHV-adsorbed L-2 cells were warmed to 37° for 20 min in the absence (●—●) or presence (○---○) of ammonium chloride in order to permit virus internalization. After removal of external virus by proteinase K treatment, the cells were returned to incubation at 37° in the absence (●—●) or presence (○---○) of ammonium chloride. Cells were harvested and lysed for assay of infectivity of intracellular virus. Results are expressed as a percentage of the maximal titer obtained (20 min, ammonium chloride).

to 37° for 20 min in medium with and without ammonium chloride. Under these conditions, approximately 50% of cell-bound virus is internalized (Fig. 2A). Cells were then treated with proteinase K to remove external virus, incubated at 37° in the presence or absence of ammonium

chloride, then subsequently lysed and assayed for infectivity of internalized inoculum. While this procedure does not permit analysis of events occurring during the first 20 min, it is evident from the results shown in Fig. 2B, that the eclipse phase in MHV infection is strikingly affected by the presence of ammonium chloride. At the first time point examined (20 min) virus eclipse was seen to occur more rapidly in the absence of ammonium chloride, as judged by an approx 17% decrease in relative infectivity of intracellular inoculum. The effect of ammonium chloride on virus eclipse became more pronounced with time. Thus, while in normal MHV infection, virus eclipse was complete by 3 hr PI, the process occurred with clearly less efficiency in the presence of ammonium chloride (Fig. 2B), so that even as late as 5 hr PI considerable infectivity was associated with the internalized virus inoculum. We interpret these data on the eclipse stage of MHV infection as suggesting an inhibitory effect of ammonium chloride on one or more processes involved in the uncoating of MHV. It is not possible to say at present as to which proportion of the eclipsed virus represents legitimate endosomal uncoating as opposed to a possible "dead-end" pathway of lysosomal degradation. Indeed, in light of the infectious center data (Table 2), it is apparent that, even in the presence of ammonium chloride, MHV must eventually uncoat to a similar degree as that attained in untreated cells. Given the evidence that ammonium chloride inhibits the activities of both lysosomes (Ohkuma and Poole, 1978) and endosomes (Maxfield, 1982) the results of Fig. 2B are best explained on the basis of combined inhibitory actions on lysosomal/endosomal processing of internalized MHV.

Effects of ammonium chloride on MHV-RNA synthesis. If the eclipse phase is the primary site of action of ammonium chloride during MHV replication, then attenuation or delay of infection at this early stage would be expected to give rise to similar delays in subsequent virus-encoded functions. Since the first easily assayable

function following eclipse is viral RNA synthesis, we examined levels of MHV RNA produced in infected cells maintained in the presence or absence of ammonium chloride. Radiolabeled RNA obtained at 6 hr PI from MHV-infected L-2 cells, maintained in the presence or absence of ammonium chloride, was subjected to agarose gel electrophoresis and autoradiographic analysis. The normal pattern of MHV-encoded RNAs (Cheley *et al.*, 1981) numbered according to the convention of Siddell *et al.* (1983) was obtained (Fig. 3, lane C). When ammonium chloride was present immediately following adsorption, the level of radiolabeled MHV RNA was reduced (Fig. 3, lane 0) to 8% of the control level, as determined by comparative densitometric scanning of lanes 0 and C. When the addition of ammonium chloride was delayed by 1, 2, or 3 hr following adsorption, thus permitting progressively

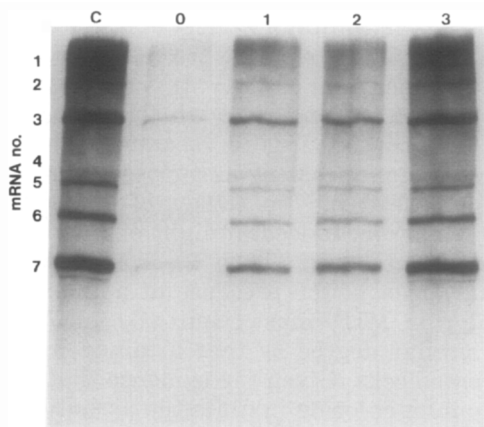


FIG. 3. Effect of ammonium chloride on the production of virus-specific RNAs. Cultures of L-2 cells in 100-mm dishes were inoculated with MHV at an m.o.i. of 20 and incubated in the absence (C) or in the presence of ammonium chloride, added either immediately after adsorption (0), 1 hr PI (1), 2 hr PI (2), or 3 hr PI (3). Viral RNA was radiolabeled by culturing the infected cells with [3 H]uridine in the presence of actinomycin D. Cultures were harvested at 6 hr PI, and the RNA was extracted and analyzed by agarose gel electrophoresis. An autoradiograph of the dried gel is shown. Numbering of the MHV-specific RNA bands follows the convention of Siddell *et al.* (1983).

more virus uncoating, the levels of radiolabeled MHV RNA were seen to increase (Fig. 3, lanes 1, 2, and 3) to 34, 52, and 85%, respectively, of that obtained in the normal MHV infection (lane C). Furthermore, the normal electrophoretic pattern of MHV RNA was obtained in the presence of ammonium chloride.

In order to determine whether the effect of ammonium chloride on viral RNA synthesis was one of inhibition or attenuation, a quantitative time-course analysis of viral RNA levels was performed on MHV-infected cells maintained in the presence or absence of ammonium chloride. As shown in Figs. 4A and B, MHV-infected L-2 cells, maintained in normal medium, first showed detectable levels of viral RNA at 3 hr PI; this was delayed until approx 6 hr PI when ammonium chloride was present. Nevertheless, the ammonium chloride-treated culture eventually (at 12 hr PI) produced levels of viral RNA very similar to the maximum levels produced in the untreated culture. Although the kinetics of RNA synthesis were noticeably slower in the presence of ammonium chloride, this is likely due to a certain degree of desynchronization in the initiation of RNA transcription, as a consequence of the protracted eclipse (uncoating) stage (Fig. 2B). Thus, it would appear that ammonium chloride does not inhibit viral RNA synthesis in a quantitative manner, but rather delays its onset.

Effects of ammonium chloride on MHV protein synthesis. In a time-course manner analogous to that used for viral RNA quantitation, viral protein synthesis was monitored in infected L-2 cells maintained in the presence or absence of ammonium chloride. As shown in Figs. 4C and D, the intracellular synthesis of viral polypeptides is delayed by some 4-6 hr in the presence of ammonium chloride. Nevertheless, all three structural polypeptide classes (E_2 , N, and E_1) are synthesized in normal proportions (Fig. 4C) and in eventual levels (Fig. 4D) similar to those seen in untreated cells.

In order to test whether ammonium chloride had a specific, inhibitory effect

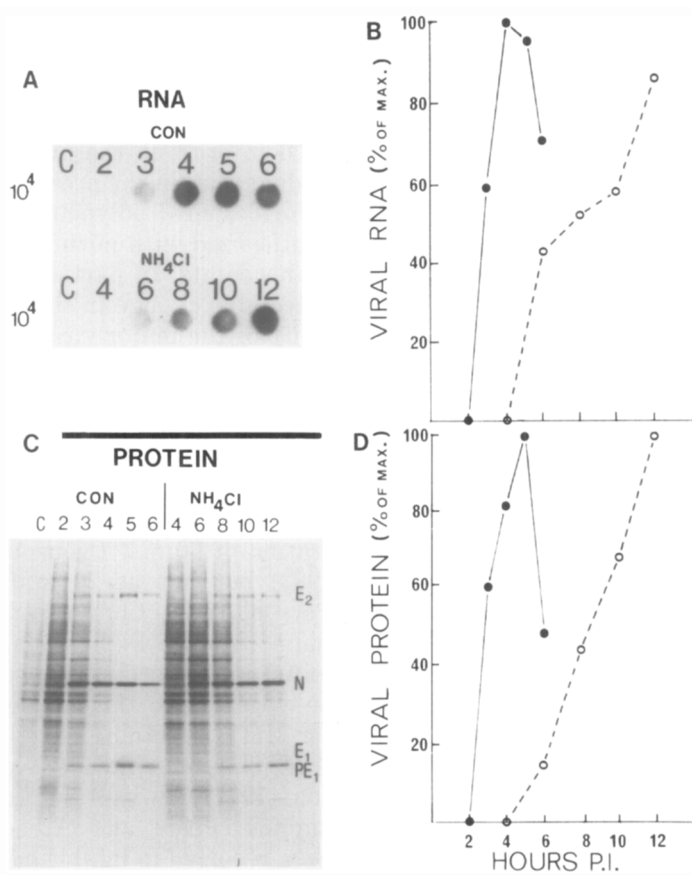


FIG. 4. Time-course study of MHV-RNA and -polypeptide synthesis in the absence or presence of 20 mM ammonium chloride. Viral RNA was detected by autoradiographic dot-blot analysis (A) of cell extracts from cultures maintained in the absence (Con) or presence (NH₄Cl) of ammonium chloride. Viral polypeptides were detected by autoradiographic SDS-PAGE (C) of cell extracts from [³⁵S]methionine-labeled cultures. Relative quantitation of viral RNA (B) and viral polypeptides (D) was performed by densitometric scanning of the autoradiograms shown in (A) and (C), respectively. Symbols (B, D) represent data from cultures maintained in the absence (●—●) or presence (○---○) of ammonium chloride.

on translation of viral proteins from MHV-messenger RNA, cell-free translation was performed using an S-30 prepared from MHV-infected L-2 cells harvested at 5 hr PI. The S-30 was "self-translated" (i.e., using its endogenous mRNA) in the presence of 0, 10, or 20 mM ammonium chloride. The major viral translation products were the nucleocapsid (N) protein and the envelope membrane (E₁ and its precursor PE₁) proteins. The addition of ammonium chloride to the S-30 did not have an inhibitory effect on the synthesis of any of these viral proteins (data not shown).

DISCUSSION

Certain compounds such as amines have been noted to enter cells and become sequestered within specific vesicular compartments, such as lysosomes (Wibo and Poole, 1974), endosomes, or receptosomes (Helenius and Marsh, 1982; Dickson *et al.*, 1981), and phagosomes (Young *et al.*, 1981). Within these normally acidic organelles, amines effectively raise the local pH (Ohkuma and Poole, 1981; Helenius and Marsh, 1982; Young *et al.*, 1981), thereby affecting a number of pH-dependent activities. One such activity is the pH-de-

pendent fusion step between the vesicular membrane and the lipid envelope of a variety of viruses, such as alphaviruses (Helenius *et al.*, 1980), rhabdoviruses (Fan and Sefton, 1978), and orthomyxoviruses (Matlin *et al.*, 1981).

Early steps in coronavirus replication have not been thoroughly characterized. Studies with both avian (Chasey and Alexander, 1976) and murine (David-Ferreira and Manaker, 1965; Krzystyniak and Dupuy, 1984) coronaviruses have demonstrated that uptake of viral inoculum occurs predominantly by viropexis. As a result, virions are observed in cytoplasmic vesicles within 1 to 2 hr postinoculation (David-Ferreira and Manaker, 1965). There has to date, however, been no documentation, by electron microscopic or other means, of the uncoating process of intracellular coronaviruses. A role for lysosomal involvement in coronavirus replication was first suggested by Mallucci (1966) who noted that the lysosomotropic agent, chloroquine, reduced MHV yields from inoculated mouse peritoneal macrophages. In a recent study, Krzystyniak and Dupuy (1984) described an inhibitory effect of the lysosomotropic agent, ammonium chloride, on the replication of MHV. These authors noted that when the addition of ammonium chloride to MHV-infected cells was delayed until 3 hr PI, the inhibitory effect was largely abrogated, suggesting that an early event in the replication cycle was affected.

The results of the present study offer the novel finding that the inhibitory action of ammonium chloride on MHV replication can be explained largely on the basis of an inhibitory effect on the eclipse phase, particularly uncoating. All posteclipse parameters examined (progeny MHV production, cell fusion, viral protein synthesis, and viral RNA synthesis) were found to be chronologically displaced by about 4-7 hr when the infection was performed in the presence of ammonium chloride. These results demonstrate that ammonium chloride intervenes primarily at a stage prior to, or including, synthesis of viral RNA (positive-sense species) during

the MHV replication cycle. This stage is likely that of uncoating as suggested by the protracted eclipse (Fig. 2) in the presence of ammonium chloride.

Of interest in the present study is our finding that, in the presence of ammonium chloride, viral polypeptides appear to be synthesized with normal apparent molecular weights. In particular, there was no detectable change in the SDS-PAGE mobility of the major viral glycoprotein (E_2) which could be ascribed to altered glycosylation. Apparent alterations in glycosylation of viral glycoproteins have been observed in herpesvirus infections of human embryonic lung cells, when maintained in ammonium chloride at a concentration of 50 mM (Kousoulas *et al.*, 1982, 1983) but not at 20 mM (Holland and Person, 1977). Concentrations of ammonium chloride higher than 20 mM were found in our preliminary experiments to be cytotoxic to the mouse L-2 cells used for MHV infection.

Ammonium chloride (Holland and Person, 1977) as well as certain tertiary amines (Poste and Allison, 1973) have been reported to inhibit membrane fusion, presumably by direct action on membrane components or their immediate ionic environment. In the present study, MHV-induced L-2 cell fusion is delayed in the presence of ammonium chloride. This delay, however, appears to be a consequence of inhibitory activity at an early stage, particularly uncoating, rather than a direct inhibitory action on membrane fusion.

The molecular events involved in pH-dependent virus uncoating are best characterized in the case of influenza virus. Here, pH-dependent fusion with the endosomal membrane is accompanied by a conformational change in the hemagglutinin glycoprotein, resulting in exposure of a fusion-active, hydrophobic peptide (Skehel *et al.*, 1982). In the case of the alphavirus, Sindbis, evidence has also been presented (Edwards *et al.*, 1983) suggesting that one of the envelope glycoproteins (the E_2) undergoes conformational rearrangement upon exposure to acidic conditions. It remains uncertain, however, as

to whether the alphaviral E₂ or another glycoprotein (e.g., the E₁; Kondor-Koch *et al.*, 1983) possesses the activity required for fusion with the endosomal membrane. The most likely candidate for analogous function in the coronavirus, MHV, is the E₂ glycoprotein, which has been shown to be responsible for manifestation of cell-cell fusion activity (Collins *et al.*, 1982).

ACKNOWLEDGMENTS

We are grateful to the Medical Research Council of Canada for research support (Grant MA-5941 to R.A.) and for a MRC Studentship (to L.M.).

REFERENCES

- CHASEY, D., and ALEXANDER, D. J. (1976). Morphogenesis of avian infectious bronchitis virus in primary chick kidney cells. *Arch. Virol.* **52**, 101-111.
- CHELEY, S., and ANDERSON, R. (1981). Cellular synthesis and modification of murine hepatitis virus polypeptides. *J. Gen. Virol.* **54**, 301-311.
- CHELEY, S., and ANDERSON, R. (1984). A reproducible, microanalytical method for the detection of specific RNA sequences by dot-blot hybridization. *Anal. Biochem.* **137**, 15-19.
- CHELEY, S., ANDERSON, R., CUPPLES, M. J., LEE CHAN, E. C. M., and MORRIS, V. L. (1981). Intracellular murine hepatitis virus-specific RNAs contain common sequences. *Virology* **112**, 596-604.
- CHOPPIN, P. W., and COMPANS, R. W. (1975). Reproduction of paramyxoviruses. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 4, pp. 95-178. Plenum, New York.
- COLLINS, A. R., KNOBLER, R. L., POWELL, H., and BUCHMEIER, M. J. (1982). Monoclonal antibodies to murine hepatitis 4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology* **119**, 358-371.
- DALES, S. (1973). Early events in cell-animal virus interactions. *Bacteriol. Rev.* **37**, 103-135.
- DAVID-FERREIRA, J. F., and MANAKER, R. A. (1965). An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells. *J. Cell Biol.* **24**, 57-78.
- DICKSON, R. B., WILLINGHAM, M. C., and PASTAN, I. (1981). α_2 -Macroglobulin adsorbed to colloidal gold: A new probe in the study of receptor-mediated endocytosis. *J. Cell Biol.* **89**, 29-34.
- DUCATELLE, R., and HOORENS, J. (1984). Significance of lysosomes in the morphogenesis of coronaviruses. *Arch. Virol.* **79**, 1-12.
- EDWARDS, J., MANN, E., and BROWN, D. T. (1983). Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. *J. Virol.* **45**, 1090-1097.
- FAN, D. P., and SEFTON, B. M. (1978). The entry into host cells of Sindbis virus, vesicular stomatitis virus and Sendai virus. *Cell* **15**, 985-992.
- FISCHER, I., and MOLDAVE, K. (1981). Preparation and characterization of a cell-free system from Chinese hamster ovary cells that translates natural messenger ribonucleic acid and analysis of intermediary reactions. *Anal. Biochem.* **113**, 13-26.
- GOLDSTEIN, J. L., ANDERSON, R. G. W., and BROWN, M. S. (1979). Coated pits, coated vesicles and receptor-mediated endocytosis. *Nature (London)* **279**, 679-685.
- HELENIUS, A., KARTENBECK, J., SIMONS, K., and FRIES, E. (1980). On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* **84**, 404-420.
- HELENIUS, A., and MARSH, M. (1982). Endocytosis of enveloped animal viruses. In "Membrane Recycling," Ciba Foundation Symposium 92, Pitman, London, pp. 59-76.
- HELENIUS, A., MARSH, M., and WHITE, J. (1982). Inhibition of Semliki Forest virus penetration by lysosomotropic weak bases. *J. Gen. Virol.* **58**, 47-61.
- HOLLAND, T. C., and PERSON, S. (1977). Ammonium chloride inhibits cell fusion induced by syn mutants of herpes simplex virus type 1. *J. Virol.* **23**, 213-215.
- KATO, N., and EGGERS, H. J. (1969). Inhibition of uncoating of Fowl-plague virus by 1-adamantanamine hydrochloride. *Virology* **37**, 632-641.
- KONDOR-KOCH, C., RIEDEL, H., SODERBERG, K., and GAROFF, H. (1982). Expression of the structural proteins of Semliki Forest virus from cloned cDNA microinjected into the nucleus of baby hamster kidney cells. *Proc. Natl. Acad. Sci. USA* **79**, 4525-4529.
- KOUSOULAS, K. G., BZIK, D. J., DELUCA, N., and PERSON, S. (1983). The effect of ammonium chloride and tunicamycin on the glycoprotein content and infectivity of herpes simplex virus type 1. *Virology* **125**, 468-474.
- KOUSOULAS, K. G., PERSON, S., and HOLLAND, T. C. (1982). Herpes simplex virus type 1 cell fusion occurs in the presence of ammonium chloride-inhibited glycoproteins. *Virology* **123**, 257-263.
- KRZYSTYNIAK, K., and DUPUY, J. M. (1984). Entry of mouse hepatitis virus 3 into cells. *J. Gen. Virol.* **65**, 227-231.
- LUCAS, A., FLINTOFF, W., ANDERSON, R., PERCY, D., COULTER, M., and DALES, S. (1977). In vivo and in vitro models of demyelinating diseases: Tropism of the JHM strain of murine hepatitis virus for cells of glial origin. *Cell* **12**, 553-560.
- MAEDA, T., KAWASAKI, K., and OHNISHI, S. (1981).

- Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. *Proc. Natl. Acad. Sci. USA* **78**, 4133-4137.
- MALLUCCI, L. (1966). Effect of chloroquine on lysosomes and on growth of mouse hepatitis virus (MHV-3). *Virology* **28**, 355-362.
- MARSH, M., and HELENIUS, A. (1980). Adsorptive endocytosis of Semliki Forest virus. *J. Mol. Biol.* **142**, 439-454.
- MARSH, M., BOLZAU, E., and HELENIUS, A. (1983). Penetration of Semliki Forest virus from acidic prelysosomal vacuoles. *Cell* **32**, 931-940.
- MATLIN, K. S., REGGIO, H., HELENIUS, A., and SIMONS, K. (1981). Infectious entry pathway of influenza virus in a canine kidney cell line. *J. Cell Biol.* **91**, 601-613.
- MAXFIELD, F. R. (1982). Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* **95**, 676-681.
- MIZZEN, L., CHELEY, S., RAO, M., WOLF, R., and ANDERSON, R. (1983). Fusion resistance and decreased infectability as major host cell determinants of coronavirus persistence. *Virology* **128**, 407-417.
- OHKUMA, S., and POOLE, B. (1978). Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA* **75**, 3327-3331.
- OHKUMA, S., and POOLE, B. (1981). Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J. Cell Biol.* **90**, 656-664.
- PAZMINO, N. H., YUHAS, J. M., and TENNANT, R. W. (1974). Inhibition of murine RNA tumor virus replication and oncogenesis by chloroquine. *Int. J. Cancer* **14**, 379-385.
- POSTE, G., and ALLISON, A. C. (1973). Membrane fusion. *Biochim. Biophys. Acta* **300**, 421-465.
- ROTHFELS, K. H., AXELRAD, A. A., SIMINOVITCH, L., McCULLOCH, E. A., and PARKER, R. C. (1959). The origin of altered cell lines from mouse, monkey and man as indicated by chromosome and transplantation studies. *Canad. Cancer Conf.* **3**, 189-214.
- SCHLEGEL, R., DICKSON, R. B., WILLINGHAM, M. C., and PASTAN, I. H. (1982). Amantadine and dansylcadaverine inhibit vesicular stomatitis virus and receptor-mediated endocytosis of α_2 -macroglobulin. *Proc. Natl. Acad. Sci. USA* **79**, 2291-2295.
- SHIMIZU, Y., YAMAMOTO, S., HOMMA, M., and ISHIDA, N. (1972). Effect of chloroquine on the growth of animal viruses. *Arch. Ges. Virusforsch.* **36**, 93-104.
- SIDDELL, S. G., ANDERSON, R., CAVANAUGH, D., FUJIIWARA K., KLENK, H. D., MACNAUGHTON, M. R., PENSART, M., STOHLMAN, S. A., STURMAN, L., and VAN DER ZELJST, B. A. M. (1983). Coronaviridae. *Intervirology* **20**, 181-189.
- SIMPSON, R. W., HAUSER, R. E., and DALES, S. (1969). Viropexis of vesicular stomatitis virus by L cells. *Virology* **37**, 285-290.
- SKEHEL, J. J., BAYLEY, P. M., BROWN, E. B., MARTIN, S. R., WATERFIELD, M. D., WHITE, J. M., WILSON, I. A., and WILEY, D. C. (1982). Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci. USA* **79**, 968-972.
- WHITE, J., MATLIN, K., and HELENIUS, A. (1981). Cell fusion by Semliki Forest virus, influenza and vesicular stomatitis viruses. *J. Cell Biol.* **89**, 674-679.
- WIBO, M., and POOLE, B. (1974). Protein degradation in cultured cells II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B₁. *J. Cell Biol.* **63**, 430-440.
- WILLINGHAM, M. C., and PASTAN, I. (1980). The receptosome: An intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. *Cell* **21**, 67-77.
- YOUNG, M. R., D'ARCY HART, P., and GEISOW, M. J. (1981). Action of weak bases on phagosomes of cultured macrophages. Suppression by ammonium ions of an early increase in phagosomal pH. *Exp. Cell Res.* **135**, 442-445.