On the Mechanism of Inhibition of Influenza Virus Replication by Amantadine Hydrochloride

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SUMMARY

The results of analyses of fowl plague virus-specific RNA and protein synthesis in infected chick embryo fibroblasts incubated in amantadine hydrochloride are reported. They indicate that provided amantadine is present from the time of virus addition no expression of the virus genome occurs and that the synthesis of even the first detectable transcripts catalysed by the polymerase of the infecting virus particles is prevented. In agreement with previous reports it is concluded that amantadine prevents an unknown event which occurs immediately following virus infection.

INTRODUCTION

The mechanism of inhibition of influenza virus replication by amantadine hydrochloride is unknown. The generally agreed conclusions from the results of several studies, for example those of Davies et al. (1964), of Hoffmann et al. (1965) of Kato & Eggers (1969) and of Dourmashkin & Tyrrell (1974), are that binding of virus particles to cells is not affected by the drug and that the inhibition of an undefined initial step in virus replication is involved. Since these results were obtained more sensitive procedures have been developed for detecting virus-specific molecules synthesized in the early stages of virus replication and the effects of amantadine hydrochloride on these stages have, therefore, been re-examined.

METHODS

Cells and viruses. Primary chick embryo fibroblast cells (CEF) were prepared as described by Porterfield (1960). The Rostock strain of fowl plague virus (FPV), Sendai virus, and influenza B/Hong Kong/8/73 were from the stocks of the World Influenza Centre in this Institute. The viruses were grown in the allantoic cavity of 10-day-old embryonated hens' eggs and infected allantoic fluid, clarified by centrifugation at 1000 g for 10 min, was used as inoculum in tissue culture experiments to give multiplicities of infection of about 100 p.f.u./cell. Unless stated otherwise, infection was for 30 min at 20 °C. Radioactive virus labelled with 3H-uridine during replication in CEF (100 μc/culture) was purified by sucrose density gradient centrifugation as described by Hay (1974).

Analyses of the uptake of amantadine hydrochloride by CEF. CEF in monolayer culture (5 × 10^6 cells/culture) were incubated in medium containing 3H-amantadine (5 μc/culture, 200 μg/ml) for different periods between 2 and 60 min following which they were washed with saline TCA and ethanol as described previously (Skehel et al. 1967).
Analyses of polypeptide synthesis. These were as described previously (Skehel, 1972) using $^{35}$S-methionine (5 μc/culture) as the labelled precursor and 10 min labelling periods. The labelled polypeptides in the CEF extracts, solubilized in 8 M-urea, 1 % SDS, 0·2 % β-mercaptoethanol, were separated by electrophoresis in polyacrylamide gels. The buffer system used was that described by Laemmli (1970), the gels contained 15 % acrylamide, and electrophoresis was at 5 V/cm for the times specified in the figure legends.

Analyses of RNA synthesis

RNA extraction. In these experiments $^3$H-uridine (20 μc/culture) was used as labelled precursor. After labelling for the times indicated in the figure legends, cells were washed three times with cold saline and dissociated in 0·01 M-sodium acetate, pH 5·0, 0·5 % SDS. The solution was extracted twice with equal volumes of water-saturated phenol and RNA was precipitated from the aqueous phase at $-20\,^\circ\text{C}$ following the addition of ethanol. The precipitated RNA was dissolved in 0·01 M-sodium acetate, pH 5·0, containing 0·02 M-sodium pyrophosphate and re-precipitated by adding cetyl trimethyl ammonium bromide (CTAB) to 0·2 % (Ralph & Bellamy, 1964). The precipitate was washed with 0·01 M-sodium acetate, pH 5·0, 0·05 M-NaCl, 0·2 % CTAB and then twice with 70 % ethanol, 0·1 M-NaCl.

RNA-RNA hybridization was as described by Ito & Joklik (1972). RNA mixtures in 0·01 M-tris-HCl, pH 7·5, 0·01 M-NaCl, 0·001 M-EDTA were denatured by adding 9 vol. of dimethyl sulphoxide and incubating at 45 °C for 30 min. The salt solutions were then adjusted to 0·01 M, 0·03 M and 0·0015 M respectively, the dimethyl sulphoxide concentration reduced to 63 % and the incubation continued at 37 °C for 20 h. RNA was precipitated from this solution and washed in 70 % ethanol, 0·02 M-NaCl.

Fractionation of RNA-RNA hybrids by LiCl precipitation. Mixtures of RNA-RNA hybrids in 0·01 M-tris-acetate, pH 7·8, 0·001 M-EDTA were adjusted to 2 M-LiCl. Soluble and insoluble molecules were separated by centrifugation at 2000 g for 15 min.

Polyacrylamide gel electrophoresis. Before analysis, RNA samples in 0·01 M-sodium acetate, pH 4·5, 0·1 M-NaCl, 0·0005 M-ZnSO$_4$ were incubated at 37 °C for 4 h with S$_1$ nuclease (1000 units/ml, 200 units/sample) and then re-precipitated in ethanol. Dry RNA samples were dissolved in 0·01 M-tris-acetate, pH 7·8, 0·005 M-EDTA, 7 M-urea. The RNA components were separated by electrophoresis in one of two systems, as specified in the figure legends: (i) in gels containing 7·5 % acrylamide, 6 M-urea, 0·04 M-tris-acetate, pH 7·8, for 40 h at 3 V/cm; (ii) in gels containing 4·0 % acrylamide 0·04 M-tris-acetate, pH 7·8, for 16 h at 5 V/cm. Following electrophoresis the gels were processed for fluorography as described by Bonner & Laskey (1974) using pre-exposed film (Laskey & Mills, 1975).

Electron microscopy. CEF monolayers were fixed in situ using the simultaneous osmium tetroxide and glutaraldehyde method of Hirsch & Fedorko (1968); they were subsequently embedded in Spurr resin, sectioned and stained with uranyl acetate and lead citrate. FPV infected cells, with or without added amantadine hydrochloride at 200 μg/ml, were fixed 15 min, 45 min and 4 h after addition of the virus; others were examined after 4 h in the presence of amantadine followed by a 2 h period in fresh amantadine-free medium. These cultures were compared with cells at similar time points after exposure to amantadine alone, and with untreated control monolayers.

Materials. $^5$H-uridine (49 Ci/mmol) and L-$^{35}$S-methionine (340 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Polyacrylamide gel components were from Eastman Kodak Co., Rochester, New York. Amantadine hydrochloride was a gift of Dr A. Galbraith, Pharmaceutical Development Unit, Geigy Pharmaceuticals, Macclesfield,
Amantadine and influenza replication

Table 1. The effect of amantadine hydrochloride at different concentrations on the release of haemagglutinin from FPV infected cells*

<table>
<thead>
<tr>
<th>Amantadine hydrochloride concentration (µg/ml)</th>
<th>Haemagglutinin titre at different times after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td>800</td>
<td>2</td>
</tr>
</tbody>
</table>

* CEF cells in monolayer cultures (5 x 10⁶ cells/culture) were incubated in Gey's balanced salt solution containing amantadine hydrochloride at the concentrations indicated for 1 h at 37 °C before infection with FPV. Absorption of inoculum (100 p.f.u./cell) containing the appropriate amounts of amantadine was at 20 °C for 30 min, after which the cultures were re-incubated at 37 °C in Gey's balanced salt solution containing amantadine. Samples of the culture fluid were withdrawn at the indicated times for haemagglutination assays.

Cheshire. Samples of ³H-amantadine hydrochloride were gifts of Dr D. C. Wiley and Dr E. Kantrowitz, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass., and Dr R. Grunert and Dr C. C. Whitney, E. I. duPont de Nemours and Co., Newark, Delaware.

RESULTS

Virus production

Preliminary analyses showed that at a concentration of 50 µg/ml, or greater, amantadine hydrochloride inhibited FPV production in chick embryo fibroblasts as judged by the release of haemagglutinin from infected cells (Table 1) and that at concentrations between 50 and 400 µg/ml the production of radioactively labelled virus was gradually prevented (Fig. 1). Results such as these were only obtained when the drug was added at the time of infection or before; addition to the same concentration later than 30 min after infection at 37 °C had essentially no effect on the final yield of virus.

Protein synthesis

The results of previously reported studies have shown that analysis of the synthesis of virus-specific proteins is a useful procedure for detecting influenza virus gene expression in infected cells (Follett et al. 1974; Lomniczi et al. 1977). Using similar techniques of polyacrylamide gel electrophoresis and autoradiography the following results were obtained with amantadine hydrochloride treated cells. (a) At concentrations above 200 µg/ml amantadine hydrochloride completely inhibited virus-specific protein synthesis in FPV-infected chick embryo fibroblasts and at 50 µg/ml 4 h after infection approx. 50 % inhibition was observed (Fig. 2). A concentration of 200 µg/ml was chosen for subsequent experiments. At this level no inhibition of host protein synthesis was detected (Fig. 2) and similar observations were made after 8 h of incubation. For the effects on virus-specific protein synthesis to be obtained the drug had to be added to the incubation medium either before or together with the inoculum. As shown in Fig. 3, addition at later times, even as short as 10 min after infection, caused essentially no inhibition. (b) The inhibition of
Fig. 1. The effect of amantadine hydrochloride at several concentrations on the production of radioactive virus by FPV-infected cells labelled with $^3$H-uridine. Cells were infected and incubated in amantadine hydrochloride as described in Table 1. At 1 h after infection 100 $\mu$Ci of $^3$H-uridine was added to each culture and 10 h later virus was purified from the culture medium by sucrose density gradient centrifugation (Hay, 1974). The gradient fractions containing virus were pooled and samples taken for determination of radioactivity. In the gradient used for the control culture medium these fractions contained 56 000 cts/min.

virus gene expression was readily reversed after several hours of infection in the presence of the drug (Fig. 4). Following removal of the amantadine hydrochloride from the medium the synthesis of virus proteins appeared to proceed normally after a delay of approx. 1 h. A similar delay was observed in cells treated with amantadine hydrochloride before infection but incubated in the absence of the drug after infection, and may reflect the time of clearance of the drug from the cells. In this regard, experiments with $^3$H-amantadine hydrochloride have indicated that the compound rapidly enters cells and that its efflux is restricted during extensive washing with saline or medium (Table 2).

In addition to demonstrating the reversibility of the inhibition, the results in Fig. 4 also imply that binding of the infecting virus particles was not inhibited since the inoculum was removed during the normal infection and washing procedure, in this case, 4 h before removal of the drug.

In conclusion then, virus-specific protein synthesis is completely but reversibly prevented in cells incubated in amantadine hydrochloride from the time of infection. Addition of the drug at later times has no effect on subsequent replication and clearly the translation of neither host nor virus messenger RNAs appears to be directly affected. Finally in this section, the results shown in Fig. 5 indicate that inhibition by amantadine hydrochloride is not restricted to type A influenza viruses since virus-specific protein synthesis was prevented under similar conditions, in Sendai and influenza B/Hong Kong/8/73-infected cells.
Fig. 2. The synthesis of polypeptides in FPV-infected cells incubated in different concentrations of amantadine hydrochloride. Cells were pre-treated, infected and incubated in medium containing different concentrations of amantadine as described in Table 1. At 4 h after infection [35S]-methionine (5 μCi) was added to each culture and after 10 min the cells were washed with salt solution (0.15 M-NaCl) and dissociated in a solution containing urea, β-mercaptoethanol and SDS (8 M, 2 and 1 % respectively) at 100 °C as described previously (Skehel, 1972). The labelled polypeptides in the cell extracts were separated by electrophoresis for 14 h (5 V/cm) on polyacrylamide gels containing 15 % acrylamide and the buffers described by Laemmli (1970). The gel was processed for autoradiography as described previously (Skehel, 1972). The direction of migration was towards the anode, from top to bottom, in this and all other figures presented. The virus-specific polypeptides are designated as described in Inglis et al. (1976) and Lomniczi et al. (1977). The figure shows the labelled polypeptide components of: A, uninfected cells; B, uninfected cells treated with 200 g/ml amantadine; C, uninfected cells at 400 μg/ml; and D, E, F, G, H and I, FPV-infected cells treated with 0, 25, 50, 100, 200 and 400 μg/ml of amantadine respectively.
Fig. 3. The synthesis of polypeptides in FPV-infected cells incubated in amantadine hydrochloride either before or at different times after infection. Cells were incubated at the indicated times in medium containing 200 µg/ml amantadine hydrochloride for the duration of the experiment including the infection period which was for 10 min at 37 °C. The experimental details and processing of the samples were as described for Fig. 2. The lanes from left to right contained the extracts from cells incubated with amantadine at: A, 20 min before infection; B, the time of infection; C, 10 min after infection; and D, E, F, G, H, I and J, 20, 30, 40, 60, 80, 100 and 120 min after infection, respectively.

RNA synthesis

The above experiments on FPV-specific protein synthesis were accompanied by analyses of the effects of amantadine hydrochloride on virus messenger RNA synthesis. These molecules can be detected in extracts of radioactively labelled cells and conveniently analysed following hybridization with an excess of virion RNA (vRNA), to which they are complementary in sequence. The results shown in Fig. 6 indicate that, in cells treated
before infection with amantadine hydrochloride at concentrations greater than 200 μg/ml, the synthesis of RNA complementary to vRNA is completely prevented.

The results of detailed analyses of virus-specific RNA synthesis in FPV-infected cells have indicated that RNA complementary in sequence to vRNA (cRNA) is made up of two classes of transcript one of which is polyadenylated and functions as messenger RNA
Table 2. Removal of amantadine hydrochloride from cells during saline, trichloroacetic acid and ethanol washes*

<table>
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<tr>
<th>Wash number</th>
<th>Radioactivity</th>
<th>% of added radioactivity</th>
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<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7854</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1437</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>479</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>366</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>294</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10421</td>
<td>12.7</td>
</tr>
<tr>
<td>5 % TCA</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1445</td>
<td></td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>670</td>
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<tr>
<td>5</td>
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</tr>
<tr>
<td>Total</td>
<td>10548</td>
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<tr>
<td>Ethanol</td>
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<tr>
<td>4</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>455</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The washing procedure was as described previously (Skehel et al. 1967). Triplicate CEF cultures (5 x 10⁶ cells/culture) were used and the volume of each wash was 2.0 ml/culture. The time of incubation in ³H-amantadine was 5 min, and similar results were obtained between 2 and 60 min of incubation. No radioactivity remained in the insoluble cell fraction.

and the other of which does not bind to oligo-dT cellulose and probably serves as template in genome replication (A. J. Hay, B. Lomniczi, A. R. Bellamy and J. J. Skehel, submitted for publication). Hybrids formed between vRNA and molecules of the former class of cRNA precipitate in 2 M-LiCl; those formed using the latter class remain soluble since they are completely double-stranded molecules and these solubility properties are the basis of a simple procedure for distinguishing between the two. Consequently, since the results presented in Fig. 6 were obtained using mixed populations of cRNA, additional analyses were made to determine whether or not amantadine hydrochloride preferentially affected either class of transcript. The results presented in Fig. 7 and 8 clearly indicate that the synthesis of cRNA molecules of either type is equivalently inhibited. They also show that the inhibitions are reversible and, moreover, that amantadine appears to be without effect on transcription if added later than 30 min after the virus inoculum. In addition, since these results indicate that, to be effective, amantadine hydrochloride must be present at the time of infection, they also imply that the transcription processes per se which are operative at 1 h after infection are not affected by the drug. They do not necessarily indicate, however, that the transcription mediated by the polymerase of the infecting virus particles is similarly unaffected. This possibility was, therefore, examined directly both in vitro and in infected cells treated with cycloheximide to prevent protein synthesis and in which, as a consequence, all transcription is catalysed by the input virion polymerase. The results in Fig. 9 show that amantadine hydrochloride reversibly inhibits virus-specific messenger RNA synthesis in cycloheximide-treated cells. They also indicate conclusively that amantadine hydrochloride has no effect on RNA synthesis when added after infection in the presence of cycloheximide and, therefore, it does not directly inhibit transcription of the genetic material of infecting virus particles by the virion associated polymerase.
Fig. 5. The synthesis of polypeptides at different times after infection of cells in the presence or absence of amantadine (200 µg/ml) with B/Hong Kong/8/73, Sendai virus or FPV. The polypeptides synthesized in B/Hong Kong/8/73 infected cells at 2, 4, 6 and 8 h after infection in the absence of amantadine are shown in lanes A, B, C and D and in the presence of amantadine in lanes E, F, G and H. Extracts of Sendai virus infected cells at the same times are in lanes I, J, K and L and extracts of Sendai virus infected cells incubated in amantadine in lanes M, N, O and P. Lanes Q and R contain polypeptides extracted from FPV-infected cells at 4 h after infection in the absence or presence of amantadine, respectively. Other experimental details are as for Fig. 2.

Table 3. The effect of amantadine hydrochloride on the transcriptase activity of virus particles and infected cell extracts

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Amantadine concentration in polymerase assay (mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Virion enzyme</td>
<td></td>
</tr>
<tr>
<td>Virion enzyme + GpG</td>
<td></td>
</tr>
<tr>
<td>Infected cell cytoplasm enzyme</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1361</td>
</tr>
<tr>
<td></td>
<td>12985</td>
</tr>
<tr>
<td></td>
<td>6587</td>
</tr>
</tbody>
</table>

* The virion enzyme was assayed in 100 µl reaction mixtures containing 0·1 M-tris-HCl, pH 8·0; 0·001 M-ATP, -CTP and -GTP; 0·0005 M-3H-UTP, 2·5 µc; 0·008 M-MgCl₂; 0·15 M-NaCl; 0·1 % β-mercaptoethanol; 0·1 % NP40; 20 % glycerol; and 25 µg FPV. Where indicated 0·0005 M-GpG was used (McGeoch & Kitron, 1975). The enzyme from FPV-infected cell cytoplasm extracted 4 h after infection was assayed in 100 µl reaction mixtures containing 0·1 M-tris-HCl, pH 8·0; 0·0002 M-ATP, -CTP and -GTP; 0·00002 M-3H-UTP, 2·5 µc; 0·002 M-MgCl₂; 0·1 % β-mercaptoethanol; 200 µg Macaloid; and enzyme from 5 × 10⁶ cells. In all cases amantadine was mixed with the enzyme before adding to the reaction mixture. Incubation was at 31 °C and TCA-insoluble radioactivity was determined on 30 µl samples after 30 min.
Fig. 6. The synthesis of RNA complementary in sequence to virion RNA in FPV infected cells incubated in different concentrations of amantadine hydrochloride. Cells were pre-treated, infected and incubated in medium containing different concentrations of amantadine as described in Table 1. At 1.5 h after infection [3H]-uridine (20 μC/culture) was added to each culture for 1 h following which the medium was replaced with 2 ml of sodium acetate, 0.01 M, pH 5.0, containing 0.5% SDS. The disrupted cell suspension was then extracted twice with phenol and RNA precipitated from the aqueous phase in ethanol and hybridized with vRNA as described in Methods. The final ethanol precipitates were dissolved in a solution of 7 M-urea, 0.01 M-tris-acetate, pH 7.8, 0.005 M-EDTA and the labelled double-stranded RNA molecules were separated by electrophoresis for 40 h (3 V/cm) on polyacrylamide gels containing 7.5% acrylamide, 6 M-urea, 0.04 M-tris-acetate, pH 7.8. The gel was processed for fluorography as described by Bonner & Laskey (1974). The hybrid molecules are numbered in decreasing order of their molecular weights. The figure shows labelled hybrids from infected cells treated with A, 800 μg/ml; B, 400 μg/ml; C, 200 μg/ml; D, 100 μg/ml; E, 50 μg/ml amantadine hydrochloride. Lane F contains hybrids formed from untreated infected cell material.

Fig. 7. The synthesis of polyadenylated cRNA in FPV-infected cells incubated in amantadine hydrochloride either at the time of infection or after infection. Cells were incubated at the indicated times in medium containing 400 μg/ml amantadine hydrochloride. The infection

[Continued on facing page]
Amantadine and influenza replication

The results of analyses of the effects of amantadine hydrochloride on the in vitro activity of both the virion transcriptase and the virus-specific transcriptase isolated from the cytoplasm of infected cells are in complete agreement with this conclusion (Table 3).

DISCUSSION

Analyses of the earliest synthetic events in FPV-infected cells have, therefore, indicated that amantadine hydrochloride prevents the expression of the virus genome in cells incubated in the drug at the time of infection. The inhibition is reversible and it does not appear to involve binding of virus particles to the cells. This conclusion is compatible with the previous suggestions of others referred to in the Introduction, that 'uncoating' of the infecting virus particles is impaired. The process of 'uncoating' in influenza infections is, however, not well understood, primarily because distinct subviral components have not been observed following the initial membrane association. In a study of the effects of amantadine hydrochloride on influenza infected cells Dourmashkin & Tyrrell (1974), using the electron microscope, observed no differences between control and drug-treated cells. Similar observations have been made of chick embryo fibroblasts infected and incubated in amantadine hydrochloride under the conditions detailed here. The electron micrographs shown in Fig. 10 lead simply to the conclusion that at this level of resolution no differences can be discerned between cells infected in the presence of amantadine hydrochloride and untreated infected controls. Added virus particles attach to the treated cells normally, are observed within vacuoles and become indistinct in the cell cytoplasm at the same times after infection as in untreated cells. Clearly no distinct 'uncoating' intermediates can be seen to accumulate in the amantadine-treated cells.

From the results presented here it can, again, only be concluded that amantadine hydrochloride inhibits an unknown event which occurs immediately following virus infection. Once this initial key process is complete the drug appears to have no effect on virus replication. In this connection, although the transcriptase activity of infecting virus particles might be considered to be the early function involved, contrary to a recent report (Kalninya & Indulen, 1976) no effect on polymerase activity either in vivo or in vitro could be demonstrated. The reason for this difference is not clear.

Finally, the results in Fig. 5 concerning the effects of amantadine hydrochloride on Sendai virus and influenza B virus-infected cells imply that the observations of others (e.g. Davies et al. 1964) regarding the variation in sensitivity between strains of virus may extend beyond the influenza type A viruses and in this connection it is hoped that com-
Fig. 8. The synthesis of non-polyadenylated cRNA in FPV-infected cells incubated in amantadine hydrochloride either at the time of infection or after infection. The samples analysed contain the hybrids soluble in 2 M-lithium chloride in the experiment described in Fig. 7 obtained using RNA from cells labelled between 1·25 and 2 h after infection. The figure shows labelled hybrids formed with RNA from: A, control cells; B, cells maintained in medium containing amantadine throughout the experiment from the time of virus addition; C, cells incubated in amantadine during the 30 min infection period at 20 °C and thereafter incubated in medium lacking amantadine, and D, cells incubated in amantadine from 30 min after infection onwards. Electrophoresis conditions were as for Fig. 7.

Fig. 9. The effects of amantadine hydrochloride on the synthesis of cRNA in FPV-infected cells incubated in cycloheximide. The experimental procedure for this experiment was exactly as described in Fig. 7 except that precipitation in 2 M-lithium chloride was not done. All cells were incubated in cycloheximide (100 μg/ml) for 1 h before addition of virus and maintained throughout in medium containing cycloheximide. The figure shows labelled hybrids formed with RNA from: A, control cells not incubated in amantadine; B, cells maintained in medium containing amantadine throughout the experiment; C, cells incubated in medium containing amantadine from 30 min after infection onwards and D, cells incubated in medium containing amantadine during the 30 min infection period at 20 °C and thereafter incubated in medium lacking amantadine. All cells were labelled between 1·25 and 2 h after infection.
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Fig. 10. Virus–cell relationships found in sectioned CEF cells 15 min after infection in the presence of amantadine at 200 µg/ml. Profiles showing attachment (a), endocytosis (b), and localization of the virus particles in small or sometimes larger vacuoles (c and d), were essentially similar in the presence of amantadine to when it was absent. At the 45 min stage a majority of the vacuoles and their contained virus particles seemed to have disappeared.

Comparative analyses of amantadine hydrochloride sensitive and resistant strains of virus, such as those described recently by Tuckova et al. (1973) and Appleyard (1977), may give additional information on the mechanism of action of the compound.

We thank David Stevens, Bernard Precious, Erick Fernandes and Stephen Griffiths for excellent assistance.
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