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Effect of Rimantadine on Influenza Virus Replication[1] (40428)

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Rimantadine hydrochloride (α-methyl-1-adamantane methylamine hydrochloride) has been reported to possess antiviral activity against influenza A, rubella, rubeola, respiratory syncytial, and parainfluenza types 2 and 3 viruses (1). Previous studies have suggested that the mode of action and antiviral spectrum of rimantadine is similar to its structural analog amantadine hydrochloride (1-amino-adamantane hydrochloride) (2).

The exact mode of action of either rimantadine or amantadine against influenza infections is still unknown. *In vitro*, both compounds appear to prevent virus replication by blocking an early step in the viral replicative cycle. Based on studies with amantadine, this effect is presumed to result from inhibition of penetration of the virus into the host cell (3, 4), or by suppressing the uncoating of the virus particle (5). In view of findings that show rimantadine to be more active against influenza than amantadine in tissue culture (1, 6), in animal (7) and clinical studies (8), and the paucity of information on its mode of action, further investigation was made of its antiviral action *in vitro*.

Materials and methods. Virus and cell cultures. Influenza A/WSN (HON1) was obtained form Dr. Purnell Choppin, Rockefeller University, New York, N.Y., and inocula used in these experiments were grown in 10-day old embryonated eggs. Madin Darby canine kidney cells (MDCK) were grown in Eagles minimum essential medium (MEM) supplemented with 10% fetal calf serum in 150 × 25 mm plastic tissue culture dishes (Falcon Plastics, Oxnard, CA). Confluent monolayers of MDCK cells were inoculated with 2.0 ml of 1:100 dilution of A/WSN influenza virus (initial titer $10^{8.5}EID_{50}$/0.2 ml). Following absorption for one hour at 37°, the inoculum was poured off and replaced with 15 ml of Eagles MEM with 2% fetal calf serum and allowed to incubate for the amount of time indicated in each figure.

Hemagglutination titration. This procedure was performed by standard methods in plastic microtiter plates obtained from Cooke Engineering Company, Alexandria, VA, as described previously (9).

Structural integrity experiments. A/WSN influenza labelled with [^{3}H]leucine was purified by a modification of a procedure described previously (10). Eagles MEM was substituted for reinforced Eagles medium, and the virus was grown in MDCK cells. Radiolabelled virus was treated with either distilled water, rimantadine 100 μg/ml, or 1% SDS for 1 h at 37°, and then centrifuged at 106,000g in a 10–60% linear sucrose gradient for 1 h at 4°. The gradient was fractionated by bottom hole puncture, and fractions were collected and counted in a liquid scintillation counter.

DNA-dependent-RNA-polymerase assay. Nuclei were extracted from cell cultures as previously described (11). DNA-dependent-RNA polymerase II activity was assayed by modification of a method described previously (12). The reaction mixture contained in a final volume of 0.4 ml: 56mm Tris–HCl pH 7.9, 8 mm KCl, 6 mm NaCl, 125 mm $(NH_4)_2SO_4$, 2.5 mm $MnCl_2$, 2.5 mm dithiothreitol, 0.6 mm ATP, 0.6 mm CTP, 0.6 mm GTP, 0.03 mm (^{3}H + cold) UTP, final specific activity 1400 cpm/pmole, 72 μg calf thymus DNA, and 50 μg nuclear protein. Two reaction mixtures were set up for each time point, and one mixture from each point received 2.5 μg of α-amanitin. Reaction mixtures were incubated at 31° for 15 min, and triplicate aliquots of 100 μl were mixed with 0.1 ml of saturated sodium pyrophosphate and 1.0 ml of trichloroacetic acid (10% TCA). After precipitating on ice for 1 hr, the precipitates were collected on Whatman 3 MM filter disks (23 mm) and rinsed 5× with 10% TCA. The filters were dried at 60° for 30 min, and shaken in Aquasol (New England Nuclear)

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for one hour before being counted in a liquid scintillation counter. RNA polymerase II activity was calculated as total incorporation (cpm/mg protein) minus incorporation in the presence of 2.5 μg of α-amanitin.

RNA-dependent-RNA-polymerase assay. RNA-dependent RNA polymerase activity was isolated from the microsomal extract of infected MDCK cells by a method described previously (13). At 8 hr post-infection, cells were rinsed 2× with RSB (0.01 *M* Tris–HCl pH 7.4, 0.01 *M* KCL, 0.0015 *M* $MgCl_2$) and scraped from the plates. The cells were homogenized on ice with 10–20 strokes of a Ten-Broeke homogenizer and centrifuged at 2000*g* for 10 min to pellet the nuclei. The supernatant was centrifuged for 10 min at 10,000*g* to remove mitochondria, and the final supernatant was centrifuged for 2 hr at 100,000*g* to pellet the microsomes. The microsomal extract was resuspended in a small volume of RSB. The RNA-dependent RNA polymerase assay was a modification of previously described methods (14, 15). In a final volume of 0.4 ml, the following compounds were mixed: 50 mm Tris–HCl pH 7.9, 50 mm KCl, 10 mm NaCl, 0.2% Triton N-101, 5 mm $MgCl_2$, 1.6 mm ATP, 0.8mm UTP, 0.8 mm CTP, 0.03 mm GTP (^3H and cold), 2.5 mm dithiothreitol, 0.4 mm ApG, and 100 μg microsomal extract. The mixture was incubated at 30° for 15 min, and triplicate aliquots of 100 μl were mixed with 0.1 ml saturated sodium pyrophosphate and 1.0 ml 10% TCA to precipitate the acid-insoluble RNA. Washing of the acid-insoluble material and radioactivity determinations were as described for the DNA-dependent RNA polymerase assay. Values expressed in graphs and tables represent the means of three or more experiments.

Pulse and chase experiments. Experiments were performed by a method reported previously in this laboratory (16). All plates were rinsed 2× with Hanks Balanced Salt Solution before pulsing. Fifteen ml of pulse fluids contained: 1.5 ml rimantadine (125, 250, or 500 μg/ml) or distilled water, 1.5 ml of MEM containing 2% fetal calf serum, 11.85 ml of MEM without leucine, and 0.15 ml [^3H]leucine (75 μCi per plate). Infected cells were pulsed for 15 min at 8 hr after infection and, similarly, uninfected cells from the same lot were pulsed for 15 min after incubation for 8 hr. Cells were incubated in MEM containing 2% fetal calf serum. Following the pulse period, the cells were rinsed 2× with phosphate buffered saline (PBS) and incubated for 30 min with 15 ml of MEM containing 2% fetal calf serum. The media was then poured off and the cells were rinsed 2× with PBS and scraped from the plates with a rubber policeman. Cell harvests were sonicated on ice 3× for 20 sec at setting 43 (Biosonik). The sonicates were centrifuged for 10 min at 12,062*g* at 4° and the supernatants were saved to be analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Viral and cellular proteins were analyzed by a discontinuous polyacrylamide gel electrophoresis system using Tris–glycine buffer with SDS (17). Polyacrylamide gels, 8.75%, were prepared in siliconized glass tubing to a length of 10 cm. Samples were mixed with buffer (0.0625 *M* Tris–HCl pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 3.0% SDS) and heated for 2 min in a boiling water bath. They were then applied to the gels and electrophoresed at 2 mA/gel for 3.5 hr. Following electrophoresis, gels were frozen at −70° for 30 min and then sliced into 1 mm fractions. To facilitate solubilization, each gel fraction was incubated overnight at 37° in 0.5 ml of 0.5% SDS. Five ml of Aquasol was added and the slices were counted in a Beckman LS-250 liquid scintillation counter. Molecular weights of viral proteins were determined by the method of Weber and Osborn (18).

Protein determination. Sonicates were precipitated in 5% TCA, washed twice, dissolved in diluted NaOH and assayed for protein by the Lowry procedure (19).

Chemicals. Radioisotopes were obtained from Schwarz-Mann, Orangeburg, N.J. Rimantadine hydrochloride was obtained from E. I. du Pont de Nemours and Co., Inc., Newark, N.J. Calf thymus DNA and α-amanitin were obtained from the Sigma Chemical Co., St. Louis, MO.

Results. Effect of rimantadine on influenza replication. Figure 1 shows the effect of increasing concentrations of rimantadine on the replication of A/WSN influenza virus in MDCK cells. At a concentration of 50 μg/ml, virus replication was maximally inhibited during 22 hr of incubation without visible

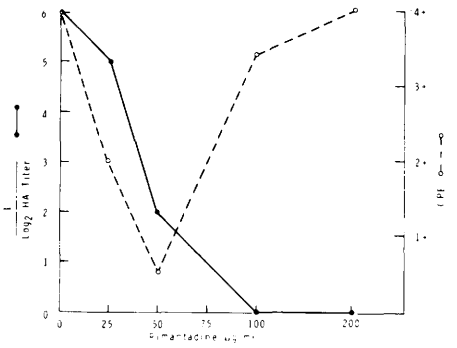

FIG. 1. Dose response curve of rimantadine on the infection of MDCK cells with A/WSN influenza virus. Drug was added immediately after the one-hour viral adsorption period. At 22 hr postinfection, samples were harvested and hemagglutination titrations were performed.

toxicity of the drug on the cell culture. At concentrations greater than 50 μg/ml, toxicity consisting of rounding, granulation and destruction of the cell sheet was observed in infected and uninfected preparations.

The time course of the effect of rimantadine on virus replication with two different multiplicities of infection (m.o.i.) are shown in Fig. 2. Rimantadine added immediately after virus adsorption strongly inhibited viral replication with the larger m.o.i. as measured by reduced formation of hemagglutinin in the supernatant fluid. When added at 2 hr or 8 hr postinfection, the drug had no effect on viral replication. With the lower m.o.i., viral replication was completely inhibited by the drug. It was also observed (data not shown) that rimantadine added during the one-hour viral adsorption period and thereafter rinsed out did not inhibit the replication of the virus.

Effect of rimantadine on structural integrity of the virus. Figure 3 shows the sedimentation profiles of purified influenza virus preparations centrifuged on 10–60% linear sucrose gradients following treatment with 1% SDS or rimantadine 100 μg/ml as described in Materials and Methods. Whereas the SDS disrupts the virus, thereby altering its position on the gradient relative to an untreated preparation, the rimantadine treatment did not change the sedimentation profile of the virus. Hemagglutinin titers were also determined for each fraction of sucrose gradient. The peak titer of hemagglutinin corresponded with the peak of radioactivity at fraction 4 to 5. This peak approximates the density of intact influenza virus approximately 1.018. Since hemagglutinin would sediment at this density only if associated with intact virus, it is reasonable to suppose that rimantadine does not have a significant effect on the structural integrity of the virus.

Effect of rimantadine on DNA-dependent RNA polymerase II activity. DNA-dependent RNA polymerase II activity was measured in nuclei of influenza-infected cells for periods up to 4 hr after infection and compared with the activities measured in uninfected cell nuclei taken at each time point (Fig. 4). RNA polymerase II activity in infected cells increased to approximately 120% above the activity in uninfected cells one to two hours after infection. This 120% increase in RNA polymerase II activity was significantly inhibited when rimantadine 50 μg/ml was present in infected cells immediately following the viral adsorption period. These data indicate that the drug acts very early in the viral replicative cycle. The drug had no effect on RNA polymerase II activity measured in nuclei of uninfected cells, nor did it inhibit RNA polymerase II activity when added directly to the assay mixture at concentrations as high as 200 μg/ml (data not shown).

Effect of rimantadine on RNA-dependent RNA polymerase activity in influenza-infected cells. Influenza virions contain an RNA-dependent RNA polymerase (20–23). RNA-de-

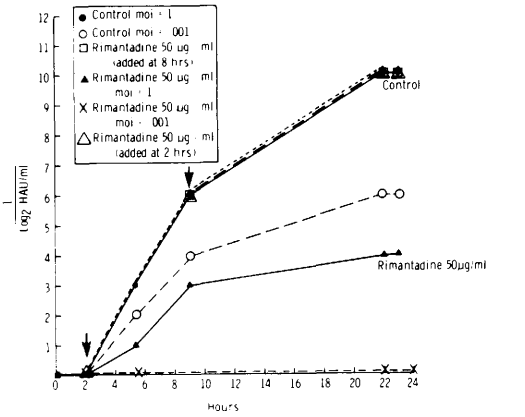

FIG. 2. Multicycle growth curve of A/WSN influenza in MDCK cells. Rimantadine was added immediately following infection except where arrows indicate addition of drug either 2 hr or 8 hr postinfection.

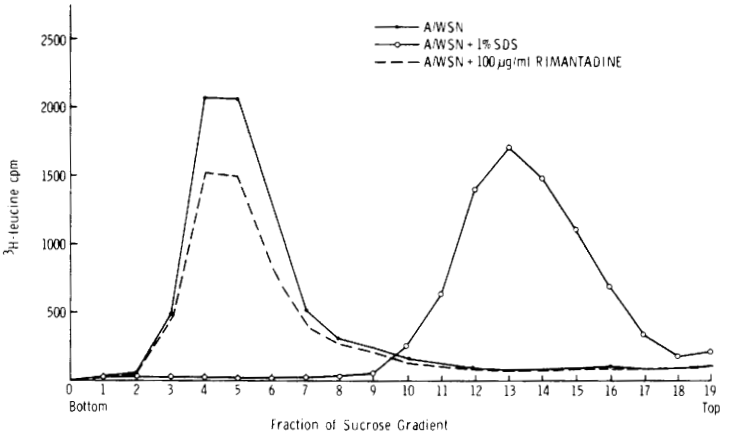

FIG. 3. Effect of rimantadine on the structural integrity of A/WSN influenza. Experimental procedures are described in Materials and Methods.

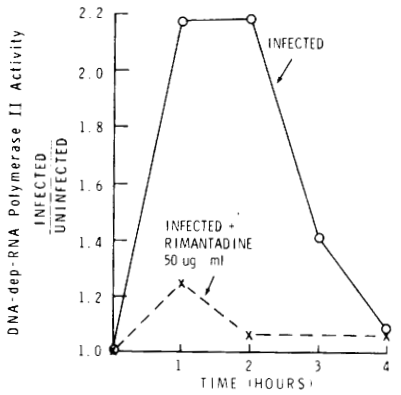

FIG. 4. Effect of rimantadine on the stimulation of DNA-dependent RNA polymerase II activity in influenza-infected cells. Drug was added immediately following adsorption of the virus to the host cells, nuclei were extracted at hourly intervals after infection, and RNA polymerase II activity was determined as described in Materials and Methods. Values represent the means of three sets of experiments.

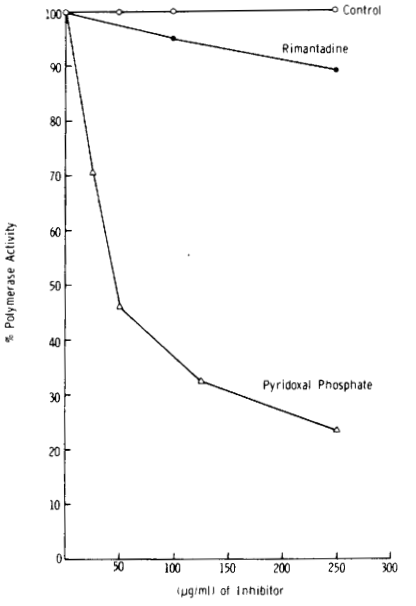

FIG. 5. Effect of rimantadine on the *in vitro* RNA-dependent RNA polymerase activity isolated from microsomal extracts of A/WSN infected MDCK cells. Assay conditions are described in Materials and Methods.

pendent RNA polymerase activity not present in uninfected cells has been isolated from microsomal extracts of various influenza infected cell systems (13, 14, 24, 25, 26). Figure 5 shows the effect of rimantadine on the *in vitro* RNA-dependent RNA polymerase activity isolated from microsomal extracts of A/WSN infected MDCK cells. Rimantadine at concentrations as high as 250 µg/ml did not appreciably inhibit this polymerase activity. Pyridoxal phosphate, although observed to inhibit polymerase activity, does not reduce the yield of influenza virus in a replicative cycle when added to the cells immediately following the viral adsorption period (data not shown).

Although rimantadine did not inhibit the RNA-dependent RNA polymerase activity when added directly into the *in vitro* assay mixture, it significantly inhibits the formation of this enzyme when incubated with influenza-infected cells for an 8-hr period begin-

ning immediately after viral adsorption, as shown in Table I.

Effect of rimantadine on protein synthesis. Figure 6 shows a gel electropherogram of influenza-infected and uninfected MDCK cells pulsed with [^{3}H]leucine and chased with cold medium as described in Materials and Methods. Infected cell preparations were co-electrophoresed with purified [^{14}C]labelled A/WSN influenza virus to identify the presence of viral proteins in infected cells (data not shown). At 8 hr postinfection, the viral NP and M proteins are easily distinguished from host cell protein background by the 15-min pulse.

When rimantadine 50 μg/ml was included during the 15-min pulse, influenza infected cell protein synthesis was suppressed as shown in Fig. 7a. This suppression appears to be non-specific, as uninfected cells pulsed with rimantadine also showed a similar, but less pronounced decrease in protein synthesis (Fig. 7b). The difference in degree of suppression between influenza infected and uninfected cells may be related to the difference in rates of protein synthesis between the two systems. The relationship between the observed inhibition of protein synthesis and the antiviral effect of the drug is discussed below.

TABLE I. EFFECT OF RIMANTADINE ON FORMATION OF RNA-DEPENDENT RNA POLYMERASE ACTIVITY IN A/WSN-INFECTED MDCK CELLS.[a]

Cell culture	Polymerase activity (picomoles/mg protein)	% Polymerase activity
Control—no rimantadine	73.7 ± 3.0	100
Rimantadine 25 μg/ml	48.8 ± 4.2	66.3
Rimantadine 50 μg/ml	42.2 ± 4.6	57.2

[a] Rimantadine added immediately following adsorption of the virus to the host cells; cells were harvested 8 hr post infection as described in Materials and Methods. Specific activity of [^{3}H]GTP was 11 Ci/mmole.

Discussion. Most reports agree that for amantadine or rimantadine to inhibit influenza virus replication *in vitro*, the drugs must be added early in the viral replicative cycle (3–5). One exception is a report that influenza virus infection of rhesus monkey kidney cells was inhibited by rimantadine added 24 hr after infection (2). It is noted, however, that influenza virus infection in monkey kidney cells requires several days for maximum growth. Our results showed that rimantadine

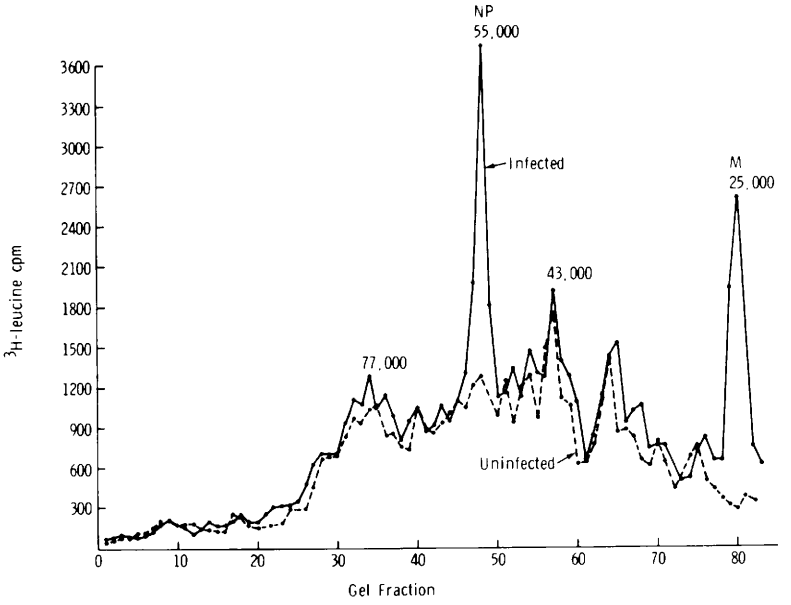

FIG. 6. Polyacrylamide gel electropherogram of infected MDCK cells 8 hr postinfection with A/WSN virus, and uninfected MDCK cells following 8-hr mock infection. Cells were pulsed for 15 min with ^{3}H-leucine and chased for 30 min with cold medium as described in Materials and Methods. Abbreviations indicate influenza nucleoprotein (NP) and membrane protein (M). Molecular weight determination as described in Materials and Methods.

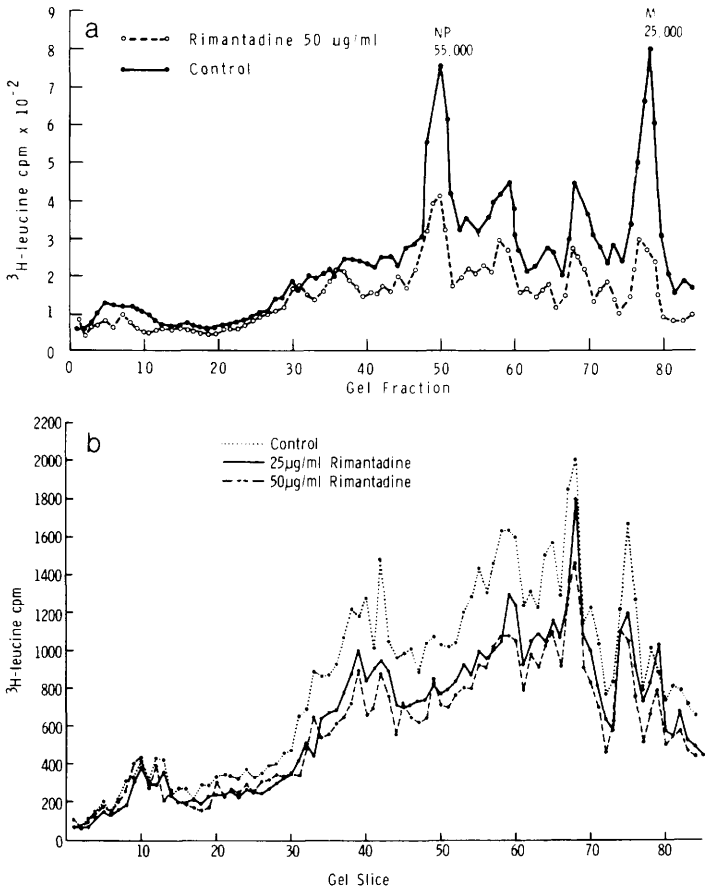

FIG. 7a and 7b. Effect of rimantadine on viral and cellular protein synthesis. Equal amounts of protein were analyzed in each gel. (a) Infected cell culture. Rimantadine was added during a 15-min pulse period as described in Materials and Method. Abbreviations indicate influenza nucleoprotein (NP) and membrane protein (M). (b) Uninfected cell culture, otherwise as in (a).

inhibited influenza virus replication when added immediately after viral adsorption, but had no effect on virus yield when added 2 hr later.

Rimantadine did not alter the structural integrity of influenza virus as measured by virus sedimentation profiles in linear sucrose gradients. Wallbank observed no direct inactivation of either Rous sarcoma virus or Esh sarcoma virus by rimantadine, although the drug inhibited virus replication in permissive cells (6).

Previous studies have shown that influenza virus stimulates cellular DNA-dependent RNA polymerase II activity early in the infectious cycle (12). Recently, it has been suggested that a product of RNA polymerase II is required for the primary transcription of the influenza genome (27). Our results show that A/WSN influenza virus infection of MDCK cells stimulates the cellular DNA-dependent RNA polymerase II activity 1–2 hr after infection, and that this stimulation can be blocked by rimantadine, 50 µg/ml, added immediately following the viral adsorption period. Preliminary experiments have shown that cycloheximide, an inhibitor of protein synthesis, did not block the stimulation of cellular RNA polymerase II activity in influenza-infected cells when added following the viral adsorption period. This finding suggests that the increased transcriptional activity in influenza-infected cells is not the result of an increase in synthesis of RNA polymerase II molecules. Since rimantadine does not effect RNA polymerase II

activity directly when assayed *in vitro*, it appears that the drug acts prior to transcriptional events of the influenza virus replicative cycle. Similarly, formation of RNA-dependent RNA polymerase in influenza infected cells was inhibited by addition of rimantadine after the viral adsorption period, but preformed RNA-dependent RNA polymerase was not inhibited by rimantadine when assayed *in vitro*.

We observed a moderate interference by rimantadine with protein synthesis of both influenza infected and uninfected MDCK cells. Oxford and Schild (28) showed that amantadine partially inhibited synthesis of all the polypeptides of A/NJ/76 *in vero* cell cultures when the drug was added immediately after viral adsorption. It appears that the inhibition of protein synthesis caused by rimantadine is not significant enough to account for the antiviral effect of the drug *in vitro*, since addition of the drug eight hours postinfection had no effect on virus yield (Fig. 2), at a time when suppression of protein synthesis was observed. Skehel *et al.* (29) did not observe any inhibition of viral or host cell protein synthesis with amantadine in a fowl plague virus-infected chick embryo fibroblast system when the drug was added late in infection.

Our results are in agreement with previous studies (1) that rimantadine, like amantadine, acts at an early stage in the influenza replicative cycle. Since drug added during the viral adsorption period and thereafter removed showed no inhibition of virus replication, it appears that rimantadine acts after viral adsorption and before primary transcription of the influenza genome. The events during this interval consist of penetration and uncoating of the virus, and the stimulation of cellular DNA-dependent RNA polymerase activity II activity. Although rimantadine inhibits the stimulation of RNA polymerase II activity in influenza-infected cells, it is unknown whether the drug is acting directly at this point or at an earlier step in the influenza replicative cycle. These questions are currently under investigation in our laboratory.

Summary. Rimantadine hydrochloride in concentrations up to 50 μg/ml inhibits the replication of A/WSN influenza virus in MDCK cells without causing visible cytopathic effects. Virus replication was inhibited when the drug was added immediately following infection, but not when added late in the replicative cycle. Rimantadine does not alter the structural integrity of influenza virus as measured by sedimentation profiles of purified virus in linear sucrose gradients. Addition of drug following the viral adsorption period blocked the stimulation of cellular DNA-dependent RNA polymerase II activity observed in untreated infected cells, but had no effect on RNA polymerase II activity isolated from uninfected cell nuclei. Rimantadine present 0–8 hr postinfection inhibited the formation of viral RNA-dependent RNA polymerase activity in infected cells, but when this enzyme was assayed *in vitro*, rimantadine at concentrations as high as 250 μg/ml did not significantly inhibit polymerase activity. Finally, rimantadine interferes with viral and cellular protein synthesis, but this inhibition appears not to be of sufficient magnitude to account for the antiviral effect of the drug. We conclude that the drug inhibits influenza replication *in vitro* by acting at a step following viral adsorption and preceding primary transcription of the influenza virus genome.

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