J. Exp. Med., 1956, v104, 881.

17. Weil, M. H., Clin. Obstet. Gynec., 1961, v4, 971.

18. Zweifach, B. W., Functional Behavior of the Microcirculation, Chas. C Thomas, Springfield. Ill., 1961.

19. Abell, R. G., Page, I. H., J. Exp. Med., 1942, v75, 305.

20. Berde, B., Weidman, H., Cerletti, A., Helv. physiol. pharmacol. acta, 1961, v19, 285.

21. Cort, J. H., Hammer, J., Ulrych, M., Pisa, Z., Dousa, T., Rudinger, J., Lancet, 1964, 840.

Received February 11, 1965. P.S.E.B.M., 1965, v119.

Antiviral Activity of Amantadine Hydrochloride in Tissue Culture and *in ovo*. (30191)

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The antiviral activity of 1-adamantanamine hydrochloride† (amantadine HCl) in tissue culture, *in ovo* and *in vivo* has been briefly reviewed by Davies *et al*(1) and Maassab and Cochran(2). Demonstration of corresponding antiviral activity in human studies has been presented by Jackson *et al*(3) and Wendel(4). In this paper is presented a detailed account of the antiviral activity of amantadine HCl in tissue culture and *in ovo*.

Materials and methods. Compound. Solutions of amantadine were made either from the free base (neutralized with HCl) or from the hydrochloride salt.

Media. All media contained 100 units of penicillin G and 100 μg of streptomycin sulfate. The basic maintenance medium consisted of 90% Earle's saline(5) modified to contain 0.9% NaCl, 0.45% glucose, 0.015% phenol red and 0.5% enzymatic lactalbumin hydrolysate (Nutritional Biochemicals) and 10% Difco tryptose phosphate broth with 0.1% yeast extract and 290 mg% glutamine added. For studies on plaque formation 0.9% agar and for hemadsorption studies 0.3% agar was added to the liquid maintenance medium. Growth medium consisted of 2% normal chicken serum, 8% horse serum, 10% tryptose phosphate broth and 80% modified Earle's saline plus an additional 0.31% $NaHCO_3$.

Cells. Chick embryo cells were prepared from decapitated whole chick embryos according to the method of Dulbecco(6) and 25×10^6 cells in 10 ml of growth medium were placed in petri dishes. After a 48-hour incubation period the cell layers were washed with phosphate-buffered saline (PBS), inoculated with test virus and returned to the incubator for a one-hour virus adsorption period. Unadsorbed virus was then removed and the cell layers covered with 10 ml of maintenance medium and reincubated. All tissue cultures were incubated at 37°C in a humidified atmosphere (RH 90-95%) containing 10% CO_2. Other cell layers were treated in a similar manner. Plaque formation was established after an appropriate incubation time, usually 3 to 5 days, by addition of either 0.007% neutral red or 0.15% of 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT). Hemadsorption tests were developed by washing the infected monolayers with PBS after removal of the soft agar overlay and adding 3 ml of a 0.5% suspension of chicken red blood cells (RBC) in PBS. Excess RBC were washed off after 20 minutes and the monolayers checked for areas of adsorbed RBC. Tests for total virus were carried out with liquid maintenance medium. At the end of the desired incubation period the infected cells were scraped into the medium and subjected to 3 cycles of freezing at —70°C and thawing at 37°C. The cell debris was removed by centrifuga-

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TABLE I. Viruses Inhibited by 25 μg/ml or Less of Amantadine* in Tissue Culture.

Viruses	Host cell system†	Test method†
Influenza		
A/PR8	CE	HaI, HA
A/Swine/S15	CE	HaI, HA
	MK	CPE
A/WS	CE	HaI, HA
A/WSN	CE	HaI, HA, PI, PAF
	CK, MK, HK	CPE
A1/FM-1/47	CE	HaI, HA
A2/Japan/305/57	CE	HaI, HA
	HK	CPE
A2/Jp^c	CE	PI
A2/AA/2/60	CE	HaI, HA
C/1233	MK	CPE
Parainfluenza 1/Sendai	MK	CPE
	CE	HA
Pseudorabies, Aujeszky	HuA	CPE
	CE	PAF

* Amantadine base.

† Abbreviations: MK, monkey kidney; CK, canine kidney; CE, chick embryo; HK, hamster kidney; HuA, human amnion; HaI, hemadsorption inhibition; HA, hemagglutination titer; CPE, cytopathic effects; PI, plaque inhibition; PAF, plaque assay of fluid.

tion and the supernatant fluid examined for virus by appropriate methods.

Rabbit and hamster kidney cell monolayers were prepared from kidneys of young adult animals. HeLa, monkey kidney, canine kidney and human amnion cells were obtained from commercial sources.

In ovo studies were carried out in 10-day embryonated White Leghorn eggs obtained from a commercial hatchery and standard methods of disinfecting, drilling and inoculation were used.

Experimental. Toxicity. Toxicity of amantadine to cell culture was determined by 3 methods. The first was by means of a dye exclusion test using 0.15% Eosin Y and determining the number of viable cells by direct count. The second was a determination of the effect of amantadine on the multiplication rate of cells over a 3-day period and the third was a check for cytotoxicity by direct microscopic examination of treated, stained cell layers after incubation periods of one to 14 days. The highest well-tolerated concentration of amantadine base (that which caused no observable difference from control cells) by all 3 criteria was 25 μg/ml, although viable cells capable of producing virus remained at concentrations up to 100 μg/ml. Toxicity of amantadine *in ovo* was determined by injecting solutions of amantadine into either the allantoic cavity or the yolk sac of embryonated eggs with daily checks for mortality. Five hundred μg per embryo caused no mortality and normal chicks were hatched. One mg of amantadine per embryo resulted in slight mortality and 2 mg per embryo in 60% mortality.

Antiviral spectrum. The viruses sensitive to amantadine at 25 μg/ml in tissue culture systems are listed in Table I. Those sensitive at well-tolerated concentrations *in ovo* are listed in Table II. Viruses tested and found to be insensitive at these concentrations in tissue culture or *in ovo* are shown in Table III. It is evident that inhibition by amantadine occurred predominantly in the A, A1 and A2 strains of influenza virus and that sensitivity was not dependent on the host system used to propagate the virus. Although the correlation of actual sensitivity in different systems was good, the degree of susceptibility *in ovo* (Table II), varied among the sensitive strains. This was also the case

TABLE II. *In ovo* Activity of Amantadine.

Influenza strain*	Compound,† μg/embryo	Hemagglutination at 48 hr: % Eggs positive‡	Hemagglutination at 48 hr: Avg titer
A/WS	500	33	2048
	—	65	4096
A/WSN	200	15	256
	—	62	256
A/PR8	500	23	4096
	—	76	16384
A/Swine/S15	200	10	4
	—	77	4096
A1/FM-1/47	500	39	1024
	—	59	8192
A2/Japan/305/57	200	57	320
	—	87	2176
C/1233	500	95	256
	—	96	1024

* Inoculated with approximately one EID_{50} (egg infectious dose) of virus *via* the allantoic cavity.

† Amantadine base inoculated *via* the yolk sac 30 min prior to infection. Comparable results are obtained with treatment *via* the allantoic cavity.

‡ Spot hemagglutination test carried out on groups of 13 to 60 eggs per treatment.

TABLE III. Organisms Found Insensitive to Amantadine* in Tissue Culture or *in ovo*.

Organism	Host system	Test method
Adeno 2 & 4	HeLa	CPE
Coxsackie A-9 & B-2	MK, HeLa	CPE, PI
ECHO 4 & 22	MK	CPE
Herpes simplex, HF	MK	PI
Influenza B/Lee & GL	CE† Chick embryo†	PI HA
Canine hepatitis, Led. 255	CK	CPE
Mouse hepatitis, MHV-3	CE	PI
Myxoma	CE	PI
NDV, Bonney & Roakin	CE Chick embryo	PI HA
Parain- 1/C35 fluenza 2/Greer 3/C243	MK	CPE
Polio, Type 2, Lansing	HeLa	PI
Reo, Type 1, Lang	MK	CPE
Respiratory syncitial, Long	HeLa	CPE
Rhinovirus, HFP	MK	CPE
Semliki Forest	CE	PI
Vaccinia, Lederle‡	CE	PI
Vesicular stomatitis	CE	PI
Yellow Fever, 17-D	CE	PI
Feline pneumonitis	Chick embryo	MDD + % survivors§
Laryngotracheitis	" "	Pock count on CA membrane
Lymphogranuloma venereum	" "	MDD + % survivors
Rickettsia, akari & prowazekii	" "	MDD + % survivors
Rous sarcoma	" "	Count on CA membrane

* Amantadine base at 25 μg/ml in tissue culture and 500 μg/embryo *in ovo* administered 15 min and 30 min prior to infection.
† CE—chick embryo cell monolayer.
Chick embryo—*in ovo* test.
‡ Variable results.
§ Mean day of death.

in tissue culture and the dose-response relationship of several representative strains of virus to amantadine are shown in Fig. 1. Under these conditions there are major differences in sensitivity among the strains tested, although all were sensitive at 25 μg/ml of amantadine. These differences appear to be more a reflection of the test system used rather than an indication of the absolute sensitivities of the various strains.

With the test conditions illustrated in Fig. 1, 25 μg/ml of amantadine were required for complete inhibition of influenza A/WSN. By comparison, in a plaque inhibition system with the inoculum of influenza A/WSN diluted to provide 20 plaques per monolayer only 0.8 to 1.6 μg/ml of amantadine HCl, added 15 minutes prior to infection, were required to completely suppress plaque formation.

Delayed treatment. Delay of treatment with amantadine until after infection results in loss of antiviral activity. Preliminary trials suggested that once virus penetrated into the host cell, amantadine was no longer effective (1,6) indicating a strictly prophylactic activity of the compound at the cellular level. The effect of delayed addition of amantadine in a plaque formation test, in which multiple cycles of virus proliferation occur, was studied with influenza A/WSN. Results of such a test in which 40 μg/ml of amantadine were added to infected chick embryo cell monolayers at intervals after infection are shown in Table IV. Treatment at day 0 was a few minutes after infection and resulted both in a reduction in plaque size and plaque number. No reduction in plaque number oc-

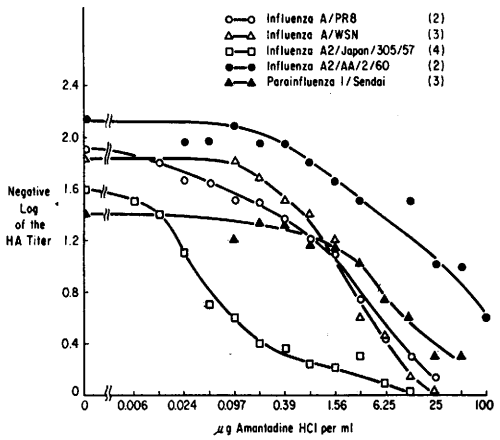

FIG. 1. Dose-response of sensitive viruses to treatment with amantadine. Figures in parentheses represent number of tests averaged for each curve. Amantadine HCl added at indicated concentrations 15 min prior to infection. Inoculum consisted of a 10^{-1} dilution of allantoic fluid stock virus. Incubation time 20-22 hr. Virus content determined by HA titration of cells and medium.

TABLE IV. Effect of Delayed Treatment with Amantadine* on Size† and Number of Plaques of Influenza A/WSN.

Treatment start post-infection day	Control‡		Treated‡		Control§	
	No. plaques	Size, mm	No. plaques	Size, mm	No. plaques	Size, mm
0‖	16	15	6	9.5	—	—
1	16	15.2	12	10.3	0	—
2	15	16.3	14	11.9	14	2.8
3	13	15	11	13	11	9.5
4	11	16	14	15.4	12	12.2

* 40 μg/ml amantadine HCl.
† Avg of 10 largest plaques.
‡ Plaque number and size determined on day 5. Controls received medium free of compound and treated with compound at indicated times.
§ Plaque number and size determined at indicated time.
‖ Treatment immediately after infection.

curred with delayed treatment but plaque size was reduced to a value intermediate between those of full incubation controls and those present on the control plates at the time of compound addition. Also striking was the observation that 40 μg/ml of compound added shortly after infection only reduced plaque formation, whereas complete suppression of plaque formation occurs with pretreatment with 0.8 to 1.6 μg/ml of amantadine.

Discussion. Amantadine exerts significant antiviral activity against a number of viruses in tissue culture and *in ovo*. Corresponding antiviral activity has also been reported for mouse infections(7) and for both challenge (3) and natural(4) infection studies in man.

No explanation is available for the specificity of action of amantadine, nor for the varying sensitivities of susceptible strains. The absolute susceptibility or resistance of viruses to amantadine is not host-dependent since correlation between sensitivity in tissue culture and *in vivo* is excellent. Relative sensitivity of susceptible strains is dependent on the test system employed and a virus found highly sensitive in one type of test may not be so in another system. Although some tissue toxicity is observed at higher concentrations of amantadine, the sensitive strains are inhibited at concentrations far below those that cause cellular damage according to the most sensitive criteria of cell toxicity used. All of the results obtained thus far are compatible with the concept(1) that the antiviral activity of amantadine is exerted at a very early stage in the infectious process. Although an infected cell cannot be protected by addition of amantadine, antiviral activity can be observed with delayed treatment in multicellular systems with multiple cycles of virus replication.

Summary. Reproducible antiviral activity in tissue culture and *in ovo* has been demonstrated with well-tolerated concentrations of amantadine HCl. All strains tested of the A, A1 and A2 groups of influenza were susceptible although antiviral activity was not limited to these since influenza C, parainfluenza 1/Sendai and pseudorabies were also inhibited. Greatest activity occurs with compound present at time of infection, although a reduction in the size of plaques produced by a sensitive virus was demonstrable with delayed treatment.

1. Davies, W. L., Grunert, R. R., Haff, R. F., McGahen, J. W., Neumayer, E. M., Paulshock, M., Watts, J. C., Wood, T. R., Hermann, E. C., Hoffmann, C. E., Science, 1964, v144, 862.

2. Maassab, H. F., Cochran, K. W., ibid., 1964, v145, 1443.

3. Jackson, G. G., Muldoon, R. L., Akers, L. W., Antimicrobial Agents & Chemotherapy, 1963, p703.

4. Wendel, H., Fed. Proc., 1964, v23, 387.

5. Earle, W. R., Schilling, E. L., Stark, T. H., Straus, N. P., Brown, M. F., Shelton, E., J. Nat. Cancer Inst., 1943, v4, 165.

6. Hoffmann, C. E., Haff, R. F., Neumayer, E. M., Fed. Proc., 1964, v23, 387.

7. Grunert, R. R., McGahen, J. W., Davies, W. L., Virology, 1965, in press.

Received February 12, 1965. P.S.E.B.M., 1965, v119.