A (PR-8) infection than either of the compounds given alone.

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. Fletcher, R. D., Hirschfield, J. E., Forbes, M., Proc. Soc. Exp. Biol. and Med., 1966, v121, 68.

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Effect of 2-Diethylaminoethyl 4-Methylpiperazine-1-Carboxylate on Influenza Viruses in Tissue Culture. (30700)

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Significant protection of mice infected with influenza virus has been demonstrated with 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate(1). This report describes the effect of this compound on various types of influenza virus in tissue culture. In several experiments the compound was compared with 1-adamantanamine(2).

Materials and methods. The influenza viruses were grown in Rhesus monkey kidney monolayers in tubes containing Earle's lactalbumin hydrolysate. The 50% tissue culture infecting dose ($TCID_{50}$) was determined by the hemadsorption test(3). The strains of influenza virus used and their $TCID_{50}$ were as follows: Type A, PR-8 ($10^{7.7}$/ml); type A1, Ann Arbor 1/57 ($10^{6.2}$/ml); type A2, Japan 305/57 ($10^{5.7}$/ml); type B, Lee ($10^{4.9}$/ml); type B, Great Lakes ($10^{5.2}$/ml).

2-Diethylaminoethyl 4-methylpiperazine-1 carboxylate and 1-adamantanamine hydrochloride* were dissolved in Earle's lactalbumin hydrolysate and added to the tissue cultures in 0.1 ml volumes. The tissue culture systems except where indicated contained a final volume of 2 ml (pH 7.2).

The presence of virus or viral growth was determined by the hemagglutination titer and the infectivity titer. At the end of the incubation period, the tissue cultures were subjected to 2 cycles of freezing in the acetone-dry ice bath and thawing at room temperature to release intracellular virus. The hemagglutination titer of the supernatant fluid was then determined by the Salk procedure modified to use the Microtiter system(4) (Cooke Engineering Co., Alexandria, Va.). The concentration of infective virus was determined by titration in mice. Male white mice (Taconic Farms) weighing 18 to 22 g each were infected, while under slight ether anesthesia, by the intranasal instillation of 0.05 ml volumes of appropriate dilutions of the supernatant fluid of the frozen and thawed tissue cultures.

Antiserum against influenza A PR-8 strain virus was obtained from mice 21 days after infection with PR-8 virus. A 1:5 dilution of the serum in lactalbumin hydrolysate was used as stock.

The effect of the compounds on the adsorption of the virus to the cells was examined as follows: The virus inoculum was added to monkey kidney monolayers with and without the compounds and the hemagglutination titer of the supernatant fluid determined at intervals thereafter. The test was carried out at 5°C because at this temperature adsorption of the virus to the cells occurs, but not the subsequent steps of the infection process(5).

The effect of the piperazinecarboxylate on adsorption of the virus to the cells was also studied by exposing the virus inoculum in tissue cultures at 37°C to the compound for various periods. At the end of each exposure

* The compounds were synthesized by Dr. R. B. Angier, Dr. K. C. Murdock and Mr. W. V. Curran, Organic Chemical Research Section, Lederle Laboratories.

TABLE I. Effect of Time of Adding 2-Diethylaminoethyl 4-Methylpiperazine-1-carboxylate on Growth of Influenza A (PR-8) in Tissue Culture.

Time* of addition of compound, 50 μg/ml (hr)	Hemagglutination titer† (Log) at 96 hr	
	40‡	4‡
− ½	< .6	< .6
0	< .6	< .6
+ 1	.9	< .6
+ 3	1.5	< .6
+ 6	2.4	.5
+24	1.5	2.1
Virus control	2.1	1.8

* In relation to time of inoculation with virus.
† Mean value of results of duplicate tests.
‡ Multiplicity of infection.

period the tissue sheets were washed to remove the compound and unadsorbed virus; fresh medium was added and the cultures were reincubated. The amount of viral growth in the cultures treated with the compound, compared to that in the control cultures indicated whether or not the compound had inactivated the virus or had interfered with the adsorption of the virus to the cells.

The action of the compound on penetration of the cells by the virus was studied with the aid of specific antiserum. The virus inoculum in tissue cultures was exposed to the compound for various periods of time. Fifteen minutes prior to the end of each exposure period specific antiserum was added to the cultures and to control cultures without compound to neutralize the virus that had not become intracellular. At the end of each exposure period the tissue sheets were washed to remove the compound, antiserum and unadsorbed virus; fresh medium was added and the cultures were incubated. The amount of viral growth in the cultures treated with the antiserum and the compound, compared to that in the cultures treated with the antiserum alone, indicated whether or not the compound had interfered with the penetration of the cells by the virus.

The effect of compound on viral neuraminidase was determined by the use of sialolactose (General Biochemicals, Inc., Chagrin Falls, Ohio) as substrate. The supernatant fluid of PR-8 virus infected monkey kidney cells which had been ruptured by freezing and thawing served as source of enzyme. Sialic acid released by enzyme action was determined by the Aminoff procedure(6).

Results. Graded concentrations of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate produced a dose related inhibition of PR-8 virus growth as shown by the hemagglutination titers (Fig. 1) and the infectivity titers (Fig. 2) of the cultures.

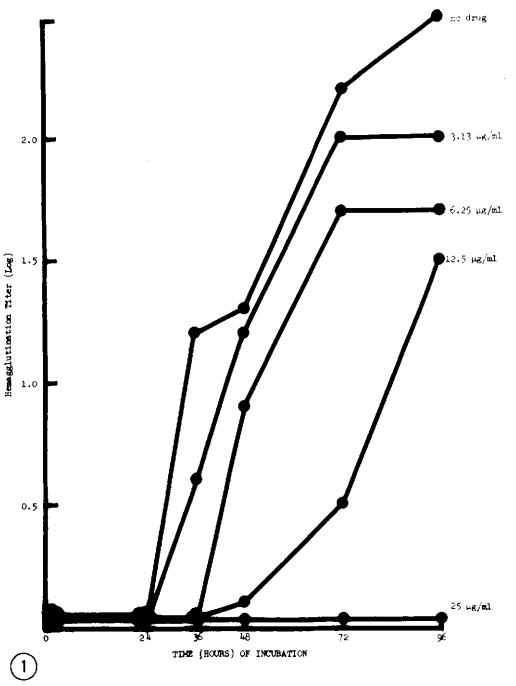
Best antiviral effect was obtained when the piperazinecarboxylate was added at the time of the virus inoculum. When the multiplicity of infection was high (40), the compound added to the cultures 3 hours after the inoculum had no appreciable inhibitory effect. When the multiplicity of infection was lower (4), the compound inhibited growth even when added as late as 6 hours after inoculum (Table I).

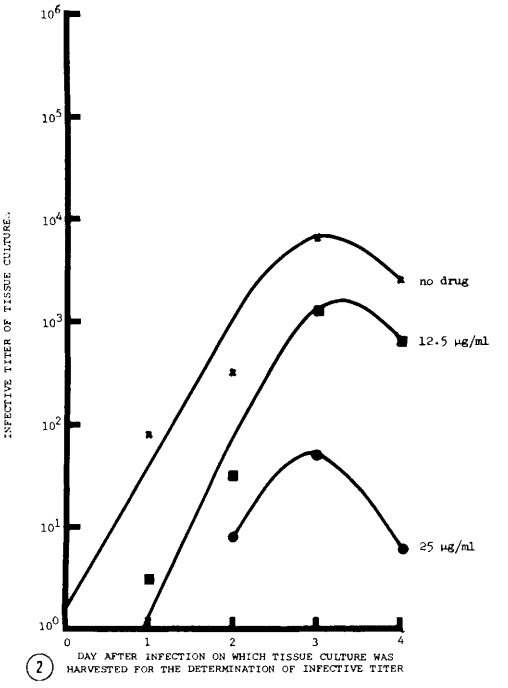
The piperazinecarboxylate was compared with 1-adamantanamine against various influenza viruses (Table II). It can be seen that the compounds had similar activities except that the piperazinecarboxylate inhibited the Great Lakes strain of influenza B, while 1-adamantanamine was ineffective against this strain. Both compounds were inactive

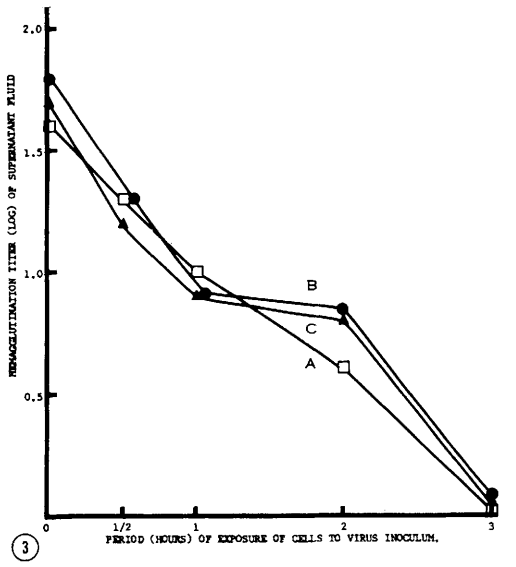
TABLE II. Minimum Concentration of 2-Diethylaminoethyl 4-Methylpiperazine-1-carboxylate or 1-Adamantanamine Required to Inhibit Growth of Various Influenza Viruses in Rhesus Monkey Kidney Tissue Culture.

Compound	Minimum inhibitory concentration,* (μg/ml)				
	Influenza A PR-8	Influenza A2 Japan 305/57	Influenza A1 Michigan Ann Arbor	Influenza B Great Lakes	Influenza B Lee
Piperazine-carboxylate	25	3.1	12.5	12.5	i
1-Adamantanamine	25	3.1	3.1	i	i

* Smallest concentration of drug which produced significant inhibition of viral growth determined by the hemagglutination titer at 96 hr.
i = inactive at 50μg/ml.







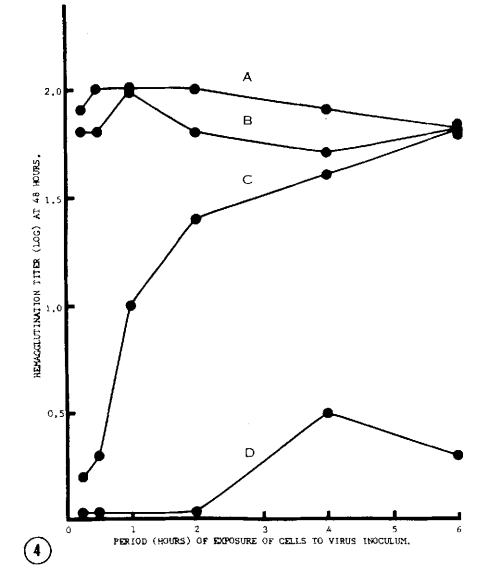


FIG. 1. Effect of graded concentrations of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate and exposure time on influenza A (PR-8) virus in Rhesus monkey kidney tissue culture. The piperazinecarboxylate was added 15 min prior to the infection with 0.1 ml of a 10^{-3} dilution of stock PR-8 virus. Cultures were incubated at 37°C for 24, 36, 48, 72 and 96 hours at which time their hemagglutination titer was determined.

FIG. 2. Effect of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate on influenza A (PR-8) virus in Rhesus monkey kidney tissue culture. Series of tissue culture tubes containing 25 μg/ml, or 12.5 μg/ml of the piperazinecarboxylate, or no drug were inoculated with 0.1 ml of a 10^{-3} dilution of stock PR-8 virus and incubated at 37°C. Each day thereafter 2 tubes from each group were combined. The combined tissue culture samples were frozen and thawed twice to release intracellular virus and the infectivity of each preparation was titrated in

against the Lee strain of influenza B. In these experiments no toxic effects were observed on the Rhesus monkey kidney cells with concentrations of the piperazinecarboxylate up to 80 μg/ml and with 1-adamantanamine to 160 μg/ml.

In studies of the mode of action, the effect of the piperazinecarboxylate on the adsorption of the virus to the cells was examined. Results (Fig. 3) show that the piperazinecarboxylate like 1-adamantanamine, did not affect the rate of adsorption.

Since influenza virus neuraminidase may be involved in the infection process(7), the effect of the piperazinecarboxylate on this enzyme was examined. The compound at 500 μg/ml did not affect neuraminidase activity.

In an experiment designed to determine the effect of the piperazinecarboxylate on the penetration of the cells by the virus, specific antiserum was used to neutralize extracellular virus. When the piperazinecarboxylate was added to the cultures at the time of inoculum and removed at intervals up to 6 hours later, it had no effect on subsequent viral growth (Fig. 4, curve B). This curve shows that the piperazinecarboxylate did not inactivate the virus on contact, and did not interfere with the adsorption of the virus to the cells. When specific antiserum was added at the time of inoculum, or 15 minutes thereafter, it inhibited growth. When it was added 45 minutes after the inoculum, or later, it did not inhibit growth (Fig. 4, curve C). In contrast, in the presence of the piperazinecarboxylate antiserum added as late as 5¾ hours after the inoculum still inhibited growth (Fig. 4, curve D). Thus, in the presence of the compound the virus remained susceptible to the neutralizing effect of antiserum. The piperazinecarboxylate did not inhibit adsorption. It must, therefore, be concluded that it blocked the penetration process.

Discussion. The piperazinecarboxylate inhibited influenza A viruses both in tissue cultures and in mice(1). The compound was also effective against the Great Lakes strain of influenza B in tissue cultures (Table II), but it did not protect mice infected with this virus(1). The reason for this apparent discrepancy is not known.

The compound was most effective when added at the time of infection. However, at a low multiplicity of infection the compound was effective even when added after the virus inoculum (Table I). In this case the cells are not all infected at the same time and the piperazinecarboxylate probably prevented further spread of the virus to the uninfected cells.

The piperazinecarboxylate has a mode of action similar to that reported for 1-adamantanamine(2). Both compounds appear to block the penetration of the cells by the virus. The compounds differ greatly in chemical structure, but both are basic amines. Ammonium ions and amines have been reported to inhibit viruses in tissue culture(8,9,10).

mice (10 mice per dilution). In this test each mouse surviving the entire 14-day test period was assigned a survival time of 14 days. The infective titer is the reciprocal of the dilution producing an average survival time of 8 days.

FIG. 3. Effect of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate and 1-adamantanamine on the rate of adsorption of influenza A (PR-8) virus to monkey kidney tissue culture. To drained Rhesus monkey kidney tissue cultures were added 0.9 ml of PR-8 stock virus suspension and 0.1 ml of a solution of either the piperazine carboxylate (1 mg/ml), or 1-adamantanamine (1 mg/ml), or Earle's lactalbumin hydrolysate. The tubes were rotated in a roller drum (3 rev/min) at 5°C. Hemagglutination titers of the supernatant fluid were determined at intervals indicated. Curve A: No drug; curve B: 1-adamantanamine; curve C: piperazinecarboxylate.

FIG. 4. Effect of exposure of the viral inoculum to 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate and specific antiserum on the subsequent growth of influenza A (PR-8) virus on Rhesus monkey kidney tissue cultures. The cultures were inoculated with 0.1 ml of a 10^{-3} dilution of stock PR-8 virus. The piperazinecarboxylate (final concentration of 50 μg/ml) was added 15 minutes before the virus. Antiserum was added 15 minutes before the end of the indicated exposure period. The final volume of the tissue-culture containing tubes was 2 ml (pH 7.2). At the end of each exposure period the tissue sheets were washed twice to remove the piperazinecarboxylate and the antiserum, fresh medium was added and incubation continued. The cultures were incubated at 37° for 48 hours at which time hemagglutination titers were determined. Curve A: control; curve B: piperazinecarboxylate; curve C: antiserum; and curve D: piperazinecarboxylate and antiserum.

Recently data were reported suggesting that these substances inhibit virus growth by blocking penetration of the cells by the virus (11). In contrast to the ammonium ions and other amines, which are active only against viruses in tissue culture, the piperazinecarboxylate and 1-adamantanamine are active against influenza A viruses in mice. This activity may be related to their ability to reach the site of infection in the body.

The antiviral action of both the piperazinecarboxylate or 1-adamantanamine can be reversed, at least during the early stages of infection, by simply washing the cells free of the compounds. This result suggests that the interaction between the compound and the virus and the cells is probably ionic in nature.

Summary. Graded concentrations of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate produced graded inhibition of influenza virus growth in Rhesus monkey kidney cultures as measured by hemagglutination and infectivity titration. The piperazinecarboxylate was effective against strains of influenza A, A1, A2, and the Great Lakes strain of influenza B, but it was not effective against the Lee strain in influenza B. The compound was most effective when added to the culture at the time of virus inoculum. When the multiplicity of infection was relatively low, the piperazinecarboxylate inhibited viral growth even when added after infection. The compound did not inactivate the virus on contact and it did not interfere with the adsorption of the virus to the cells. It appears to act by interfering with the penetration of the host cells by the virus. The compound has a mode of action and an antiviral spectrum similar to that reported for 1-adamantanamine.

1. Lindh, H. F., Forbes, M., Proc. Soc. Exp. Biol. and Med., 1966, v121, 65.

2. 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.

3. Shelokov, A., Vogel, J. E., Chi, L., Proc. Soc. Exp. Biol. and Med., 1958, v97, 802.

4. Sever, J. L., J. Immunol., 1962, v88, 320.

5. Choppin, P. W., Virology, 1963, v21, 342.

6. Aminoff, D., Biochem. J., 1961, v81, 384.

7. Johnson, C. A., Pekas, D. J., Winzler, R. J., Science, 1964, v143, 1051.

8. Jensen, E. M., Liu, O. C., Proc. Soc. Exp. Biol. and Med., 1961, v107, 834.

9. Eaton, M. D., Low, I. E., Scala, A. R., Uretsky, S., Virology, 1962, v18, 102.

10. Jensen, E. M., Liu, O. C., Proc. Soc. Exp. Biol. and Med., 1963, v112, 456.

11. Fletcher, R. D., Hirschfield, J., Forbes, M., Nature, 1965, v207, 664.

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Studies of Murine Leukemia Viruses I. Detection of Moloney and Rauscher Leukemia Viruses by Indirect Immunofluorescence.* (30701)

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The investigation of murine leukemia viruses has been hampered by dependence on time-consuming, cumbersome mouse inoculation methods for their detection and assay. Although the immunofluorescence method has been applied to detection of Friend leukemia virus (FLV) and Rauscher leukemia virus (RLV), results have been inconsistent. Some investigators have reported nuclear and cytoplasmic fluorescence in Friend leukemic cells (1) while others have described fluorescence confined to the cytoplasm in these cells and in FLV-infected mouse embryo cells(2,3). Both nuclear and cytoplasmic fluorescence have been observed in a wide variety of cells from RLV-infected animals but nuclear fluorescence only in megakaryocytes(4), where high concentrations of virus particles are

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