INHIBITION OF INFLUENZA VIRUSES *IN VITRO* AND *IN VIVO* BY 1-ADAMANTANAMINE HYDROCHLORIDE

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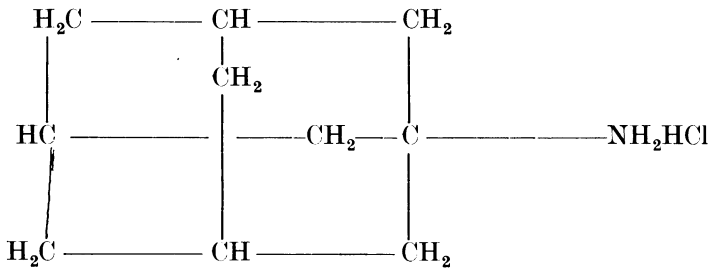
Received for publication September 3, 1964

1-ADAMANTANAMINE HYDROCHLORIDE (1-AH) is a synthetic organic compound of molecular weight 187, having the following structural formula :



Preliminary studies (Davies *et al.*, 1964) have indicated that 1-AH has a selective antiviral activity against a number of laboratory strains of influenza virus. The compound appeared to act by blocking virus penetration and evidence of viral inhibition was obtained in both tissue culture and animal experiments. Since 1-AH is highly soluble, relatively non-toxic and biologically stable it merited further extensive investigation.

The object of the present studies was firstly, to compare a number of different human and animal strains of influenza A virus in their degree of sensitivity to 1-AH in tissue cultures and in the experimental infection of mice. Secondly to investigate the spectrum of activity of the compound in tests with other myxoviruses and representative strains of other virus families. Thirdly, to study quantitative aspects of inhibition *in vitro* with a highly sensitive virus strain (A2/Scot/49/57) and a strain of moderate sensitivity (NWS virus).

MATERIALS AND METHODS

1-Adamantanamine hydrochloride.—A sterile 10 per cent stock solution was made up in deionised water and stored at 4° until required. A single stock was used throughout the series of experiments.

Tissue cultures.—Primary or secondary monolayer culture of rhesus monkey kidney (MK) cells, chick embryo fibroblasts (CEF) and HEpII cells in tubes or 4 oz. medical bottles were prepared as described by Hobson and Schild (1960). Human diploid fibroblast cells (WI 38 strain) were grown as a continuous cell line by the method of Hayflick and Moorhead (1961). On reaching confluence the growth medium in tissue cultures was replaced by a maintenance medium (1·5 ml./tube ; 10 ml./4 oz. medical bottle). For experiments involving myxoviruses this was medium " 199 " containing 0·88 g./l. $NaHCO_3$; for other

viruses the maintenance medium was Hanks' saline containing 1·1 g./l. $NaHCO_3$ (97·5 per cent), inactivated calf serum (2 per cent) and lactalbumin hydrolysate (0·5 per cent). Penicillin, streptomycin and polymyxin (100 units/ml.) were incorporated in all media.

Virus strains.—The following strains of influenza A virus of human origin were used; neutrotrophic variant NWS (Stuart-Harris, 1939) was virus of the 4th allantoic passage after adaptation in the mouse brain (103 passages). A/PR8 was a strain obtained from Dr. Himmelweit, St. Mary's Hospital, London, and had a long history of laboratory passage in the ferret, mouse lung and allantois. A1/FM1, A2/Sing./1/57 and B/Lee were strains with numerous allantoic passages in this laboratory. A2/Tokyo/1/62 was 5th allantoic passage virus originally obtained from the Central Public Health Laboratory, Colindale, London. A2/Sing./4/57 was a mouse-adapted strain supplied by Dr. H. G. Pereira of the National Institute for Medical Research, Mill Hill, London. A2/Scot/49/57 was obtained from Dr. D. A. J. Tyrrell of the Common Cold Research Unit, Salisbury, and had a history of 6 allantoic passages in embryonated eggs. This was the same strain as used in human volunteer experiments with 1-AH (Tyrrell, Bynoe and Hoorn, 1965). 442/63 was an Asian influenza strain isolated in this laboratory from a case of respiratory disease in 1963. The isolation and subsequent two passages of this agent were carried out in MK tissue cultures.

The following influenza A strains of animal origin were used: A/Swine (Shope strain) was a laboratory strain with a history of numerous allantoic passages since its original isolation in 1931. A/Duck/Prague/56, A/Duck/Eng/62, A/Equi/Prague/56 and A/Equi/Miami/63 were strains supplied by Dr. H. G. Pereira and had a history of laboratory passage in the allantois. Except for 442/63, working pools of the influenza viruses were prepared in 10 day fertile eggs incubated for 48 hr. at 35° after inoculation and the infected allantoic fluids removed after chilling at 4°. 442/63 was an infected MK tissue culture fluid.

Other viruses: Parainfluenza 1-Sendai-(T'ang strain) was a strain obtained from Dr. Jensen, Communicable Disease Center, Atlanta, Georgia, U.S.A., and had been passed in mice and the allantois of embryonated eggs. Parainfluenza 2 (C.A. virus) and parainfluenza 3 (HAI virus-Moss strain, Sutton, Clarke and Tyrrell, 1959) were infected tissue culture fluids of strains with numerous MK passages. HGP and B632 (Tyrrell and Bynoe, 1961) were rhinovirus (M) strains obtained from Dr. D. A. J. Tyrrell and had been passed in MK cultures. Rhinovirus 16/60 was an H strain of virus isolated in this laboratory (Hobson and Schild, 1960) and passed 3 times in human embryo kidney tissue cultures. Adenovirus 7 was a laboratory strain with numerous passages in HeLa tissue cultures. Polio I virus was Sabin's L.Sc.2ab oral vaccine strain. Coxsackie B1 was a recent (1963) laboratory isolate passed in MK tissue cultures. Respiratory syncytial (RS) virus was the "Long" strain and had numerous laboratory passages in HeLa and HEpII cultures.

Cell toxicity of 1-AH.—Before experiments with viruses the cytotoxicity of 1-AH was estimated by incorporating amounts varying from 12·5 μg./ml. to 200 μg./ml. in the maintenance medium of tissue cultures and examining the cells daily for toxic effects.

Virus infectivity titrations.—Serial 10-fold dilutions of virus were prepared in phosphate buffered saline (PBS. Dulbecco and Vogt, 1954) and each dilution inoculated into 4 or 5 tube tissue cultures. Tubes were incubated at 36·5° for 2 hr. when the inoculum was removed and replaced by fresh maintenance medium. Microscopic examination of cultures for c.p.e. was carried out at intervals, however, since certain myxoviruses produced poor c.p.e., the presence of haemadsorption (Vogel and Shelokov, 1957) in inoculated cultures was taken as evidence of infection with virus. End-point infectivity titres were calculated by the method of Reed and Muench (1938). Rhinoviruses were assayed by counting focal lesions (microplaques) in cultures maintained at 33° (Parsons and Tyrrell, 1961).

Measurement of virus sensitivity to 1-AH.—Virus strains were titrated in parallel in tube tissue cultures with and without 1-AH. In most experiments a standard concentration (25 μg./ml.) was used in sensitivity assays. 1-AH was added to the cultures 4 hr. before inoculation with virus and was present for the whole of the incubation period. Final examination of the cultures for c.p.e. or haemadsorption was routinely 7–9 days after inoculation. The difference in end-point infectivity titre of a virus in cultures with and without 1-AH was taken as a measure of sensitivity.

Growth curve experiments.—Monolayer cultures of MK cells in 4 oz. medical bottles (containing approx. $2·5 \times 10^6$ cells/bottle) were maintained in medium "199" containing known concentrations of 1-AH. Cultures were inoculated with $10^5 TCID_{50}$ of virus (multiplicity of infection approx. 0·04) and incubated for 3 hr. at 36·5° during virus adsorption: the inoculum was then replaced by 10 ml. volumes of "199" after washing the monolayers

with two serial aliquots of PBS. At 12 or 24 hr. intervals 1 ml. volumes of tissue culture fluids were removed from each bottle for assay of free haemagglutinin (W.H.O. Expert Committee on Influenza, 1953) with chick red blood cells. Growth curves of the viruses were plotted using the geometric means of HA titres detected in groups of 2–3 cultures treated in parallel.

It was considered possible that the presence of 1-AH fluids from infected cultures might interfere with the measurement of HA titres. Consequently tests were carried out in which HA titres of NWS and A2/Scot/49/57 in fluids from infected MK cultures were assayed before and after the addition of 100 μg./ml. of 1-AH. No significant difference in the titres was observed.

Mouse experiments.—The strain of Swiss mice used was derived in 1954 from the Compton strain. Animals were randomly bred in a closed colony and used for experiments at 4–6 weeks old. Inoculations of virus intranasally and intracerebrally were carried out as described by Lindenmann, Lane and Hobson (1963) and groups of 6 mice were inoculated with each dilution of virus. LD_{50} virus titres were calculated (Reed and Muench, 1938) from the numbers of animals dying within 12 days of inoculation. In the case of intranasal inoculations surviving mice were killed on the 12th–14th day and the degree of gross pulmonary consolidation noted. ID_{50} titres were calculated from the total deaths plus visibly infected survivors. In treated mice 1-AH was given by intraperitoneal inoculation. A single dose was 70 mg./kg. in 0·3 ml. PBS. Two doses were given, at 24 and 2 hr. before inoculation with virus, and 3 doses at 24 hr. intervals after inoculation. Control animals received PBS instead of 1-AH or 1-AH but no virus.

RESULTS

Toxicity of 1-AH in tissue cultures

The results of tests on the toxicity of 1-AH in MK cultures are shown in Table I. Concentrations of 100 μg./ml. or greater were rapidly toxic, producing

TABLE I.—*Toxic Effects of 1-Adamantanamine Hydrochloride in MK Tissue Cultures*

Concentration of 1-adamantanamine hydrochloride	Days of incubation				
	1	3	4	7	9
0	—	—	—	—	—
12·5 μg./ml.	—	—	—	—	—
25 ,, ,,	—	—	—	—	—
50 ,, ,,	—	—	+	+	++
100 ,, ,,	++	+++	..	..	..
200 ,, ,,	+++	..	..	..	..

— no toxic effects.
\+ 25 per cent or less of cells affected.
++ 25–50 per cent of cells affected.
+++ 50 per cent or more cells affected.

rounding up of cells and granularity in the cytoplasm. However, with 25 μg./ml. there was no evidence of toxic effects after 9 days incubation also the metabolism of the cells as indicated by development of acid conditions in the medium remained unchanged. Toxic effects appeared to approximately the same extent in CEF and WI 38 cultures. As a result of these tests the concentration of 1-AH used in tissue culture studies with viruses in no case exceeded 25 μg./ml.

Sensitivity of virus strains to 1-AH

End-point infectivity titres of test strains of virus in the presence and absence of 1-AH (25 or 8 μg./ml.) are shown in Tables II and III. Considerable differences

TABLE II.—*Sensitivity of Myxoviruses to* 1-*Adamantanamine in Tissue Culture*

	Virus strain	Tissue culture system	Incubation period (days)	Dosage of 1-AH μg./ml.	Virus titre* without 1-AH	Virus titre with 1-AH	Reduction in titre
Human influenza A strains	A/PR8	MK	7	25	$10^{3.5}$	$10^{3.0}$	$10^{0.5}$
	NWS	,,	6	25	$10^{6.2}$	$10^{4.9}$	$10^{1.3}$
	A1/FM1	,,	7	25	$10^{4.6}$	$10^{3.2}$	$10^{1.3}$
	,,	CEF	7	25	$10^{3.2}$	$10^{1.4}$	$10^{1.8}$
	A2/Sing/1/57	MK	9	25	$10^{4.2}$	$10^{2.8}$	$10^{2.4}$
	,,	CEF	7	25	$10^{5.2}$	$10^{3.2}$	$10^{2.0}$
	A2/Sing/4/57	MK	7	25	$10^{4.7}$	$10^{2.2}$	$10^{2.5}$
	A2/Tokyo/1/62	,,	7	25	$10^{5.4}$	$10^{1.4}$	$10^{4.0}$
	A2/Scotland/49/57	,,	10	25	$10^{6.2}$	$<10^{\circ}$	$10^{6.2}$
	442/63	,,	7	8	$10^{6.3}$	$10^{2.8}$	$10^{3.5}$
	,,	,,	7	25	$10^{6.0}$	$10^{2.0}$	$10^{4.0}$
Animal influenza A strains	A/Swine	MK	7	25	$10^{7.2}$	$10^{6.7}$	$10^{0.5}$
	A/Duck/Prague/56	,,	6	25	$10^{5.0}$	$10^{2.7}$	$10^{2.3}$
	A/Duck/Eng/62	,,	9	25	$10^{5.0}$	$10^{3.7}$	$10^{1.3}$
	A/Equi/Prague/56	,,	9	25	$10^{4.0}$	$10^{3.0}$	$10^{1.0}$
	A/Equi/Miami/63	,,	9	25	$10^{5.2}$	$10^{2.2}$	$10^{3.0}$
Other myxoviruses	B/Lee	,,	7	25	$10^{4.5}$	$10^{4.3}$	—
	Parainfluenza 1	CEF	7	25	$10^{3.7}$	$10^{3.5}$	—
	,, 2	MK	7	25	$10^{4.8}$	$10^{4.2}$	$10^{0.6}$
	,, 3	,,	7	25	$10^{2.5}$	$10^{2.8}$	—

* $TCID_{50}$/ml.

TABLE III.—*Effect of* 1-*Adamantanamine Hydrochloride on Viruses Other Than Myxoviruses*

Virus strain	Tissue culture system	Incubation period (days)	Dosage of 1-AH μg./ml.	Virus titre without 1-AH	Virus titre with 1-AH	Reduction in titre
Rhinovirus H.G.P.	MK	2	25	$1{\cdot}1\times10^{4}$p.f.u./ml.	$1{\cdot}5\times10^{4}$p.f.u./ml.	—
,, B632	,,	2	25	$4{\cdot}0\times10^{4}$p.f.u./ml.	$4{\cdot}5\times10^{4}$p.f.u./ml.	—
,, 16/60	WI 38	7	25	$10^{3.5}TCID_{50}$/ml.	$10^{3.5}TCID_{50}$/ml.	—
Polio I	MK	9	25	$10^{7.5}$,,	$10^{7.3}$,,	—
Coxsackie BI	,,	7	25	$10^{7.9}$,,	$10^{8.2}$,,	—
RS virus	HEp II	9	25	$10^{4.8}$,,	$10^{4.6}$,,	—
Adenovirus 7	,,	9	25	$10^{6.5}$,,	$10^{6.8}$,,	—

in the degree of inhibition by 1-AH were found among the human strains of influenza A. In general the pre-Asian strains (NWS, A/PR8 and A1/FM1) were relatively insensitive whilst the Asian strains of virus were more strongly inhibited. The most sensitive strain was A2/Scot/49/57 which was completely inhibited by 25 μg./ml. of 1-AH in cultures inoculated with large doses (approx. $10^{6}TCID_{50}$) of virus. The recent A2 isolate, 442/63, was highly sensitive to 8 μg./ml. as well as 25 μg./ml. of 1-AH. The strains of influenza A virus from animal sources resembled the human influenza A viruses in their sensitivity and similarly a marked variation in the degree of inhibition from $10^{0.5}TCID_{50}$ (A/Swine) to $10^{3}TCID_{50}$ (A/Equi/Miami/63) was observed. Amongst the other myxovirus strains only parainfluenza 2 (CA virus) was slightly inhibited ($10^{0.6}TCID_{50}$). No evidence of inhibition was found with the representative strains of other families of viruses used in the experiments.

In tests with A2/Sing/1/57 and A1/FM1 a similar degree of inhibition was detected in both MK and CEF tissue cultures indicating that the mechanism of inhibition was not specific for MK cells.

Direct inactivation of virus

Tests were carried out to determine whether the inhibition observed in tissue cultures could be due to direct inactivation of free virus. Infected allantoic fluids containing two relatively sensitive strains A2/Sing/1/57 and A2/Tokyo/1/62 were incubated with 100 μg./ml. of 1-AH for 3 hr. at 36·5° before titration in MK tissue cultures. Approximately the same small loss of infectivity ($<10^{0.5}TCID_{50}$) was detected as in control virus suspensions with no 1-AH. It thus appeared that the mechanism of action of 1-AH did not depend on direct inactivation of the virus particle.

Quantitative studies with A2/Scot/49/57 and NWS virus

A2/Scot/49/57 was selected for use in further studies because of its high degree of sensitivity to 1-AH. The effect of 1-AH on HA production in infected tissue cultures was determined with this virus in experiments in which bottle monolayers of MK cells were infected with approximately 10^5TCID_{50} of virus. The results were compared with those obtained with NWS, a moderately sensitive virus, in similar experiments. Figs. 1 and 2 show the effect of 1-AH on the growth curves

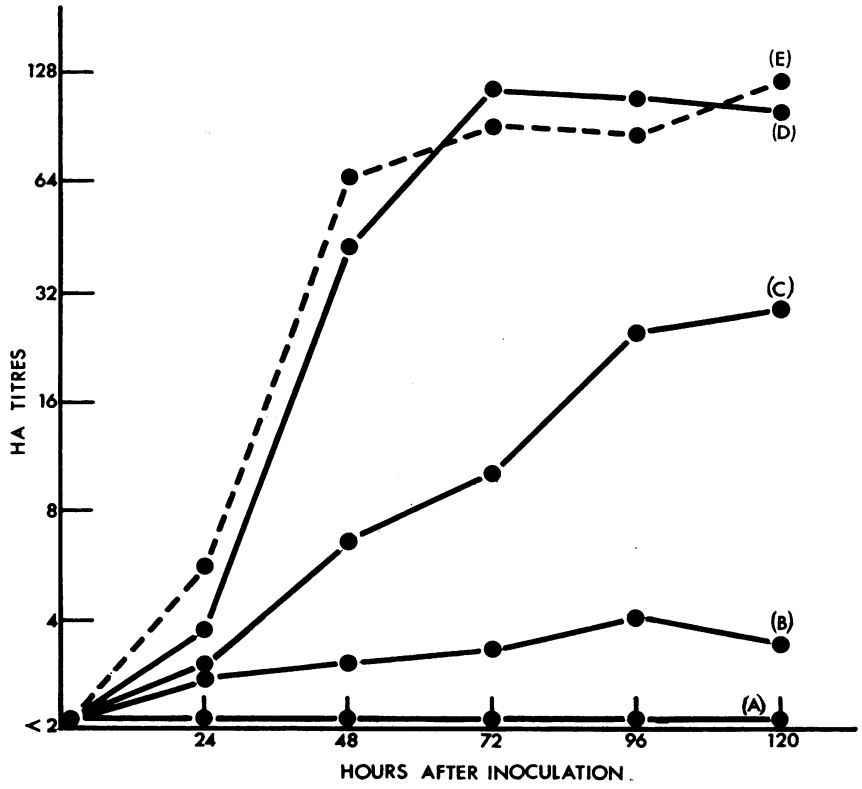


FIG. 1.—Effect of various concentrations of 1-AH on haemagglutinin production by A2/Scot/49/57 virus in MK cultures. Curve (A) 8 μg./ml., (B) 2·5 μg./ml., (C) 0·25 μg/ml., (D) 0·025 μg./ml., (E) control, no. 1-AH.

of the virus when added 4 hr. before infection with virus and present during the whole of the experiment. With both agents reduction in the yields of free haemagglutinin was observed in cultures containing 1-AH. With A2/Scot/49/57 no haemagglutinins were detected in cultures maintained in the presence of 8 μg./ml. of 1-AH. Concentrations of 2·5 μg. and 0·25 μg./ml. resulted in approximately 30 and 10-fold reductions respectively in the HA titre of the cultures when compared with control cultures at 96 hr. after infection. However, no obvious reduction in HA formation was observed with 0·025 μg./ml. With NWS virus a marked lag period (48 hr.) in the appearance of free haemagglutinin was observed with 25 μg./ml. of 1-AH and the peak HA titre, at 96 hr. after infection, was

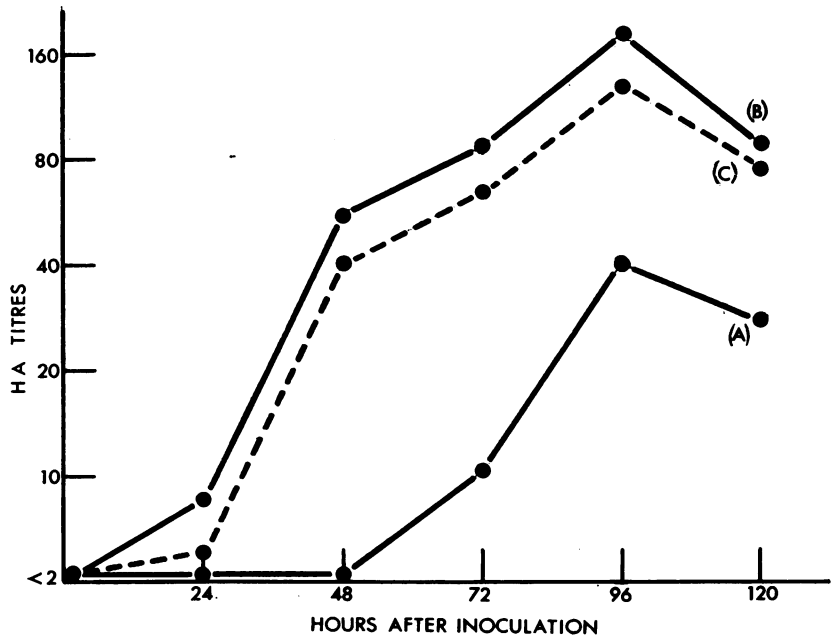


FIG. 2.—Effect of various concentrations of 1-AH on hæmagglutinin production by NWS virus. Curve (A) 25 μg./ml. (B) 2·5 μg/ml. (C) control, no 1-AH.

approximately one-third that of control cultures. 2·5 μg./ml. of 1-AH produced no obvious effect on the growth of NWS. It is of interest that a similar time lag in production of haemagglutinin was not observed with A2/Scot/49/57. Even with concentrations of 1-AH which produced marked depression of the final yield, it was possible to detect free HA as early as 24 hr. after infection.

Attempts were made to determine the stage in the growth cycle of A2/Scot/49/57 at which 1-AH is capable of producing optimal inhibitory effect on HA yield in MK cultures. In these experiments 1-AH was present in excess (25 μg./ml.) during only a specified period of the growth cycle. As in the previous experiment bottle monolayers were infected with $10^5 TCID_{50}$ of virus and 1-AH was present during the following periods : (a) for 1 hr. before infection with virus, (b) for 20 min. after inoculation, (c) from 20 min. until 3 hr. after inoculation, (d) from 3 hr. onwards.

In order that all experiments were comparable a period of 20 min. was allowed for virus adsorption after which the monolayers were washed with PBS and maintenance medium (containing 1-AH were appropriate) was added. The results are shown in Fig. 3. The greatest effect on the HA yield was observed when 1-AH was present for the first 20 min. after inoculation (curve (b)). Relatively little effect on HA yield occurred when 1-AH was added after this period (curves (c) and (d)). However, treatment of the monolayers for 1 hr. before addition of

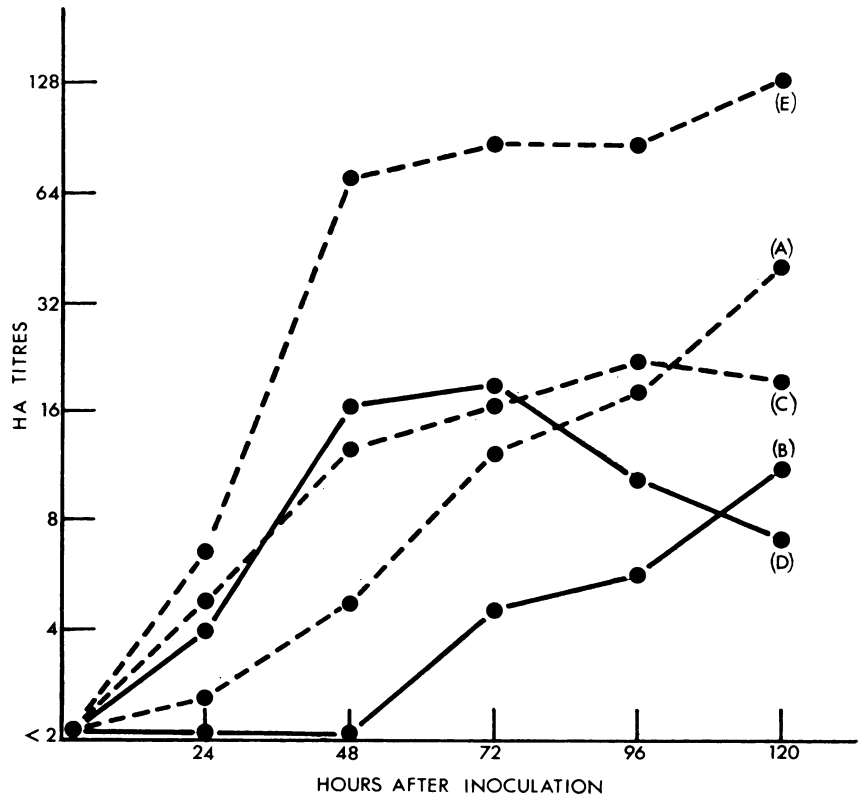


FIG. 3.—Effect of adding 1-AH (25 μg./ml.) at different times before and after infection of MK cultures with A2/Scot/49/57. (A) 1-AH present for 1 hr. prior to infection with virus, (B) 1-AH present for 20 min. after infection, (C) 20 min. to 3 hr. after infection (D) from 3 hr. after infection until end of experiment (E) control, no 1-AH.

virus (curve (a)) produced a marked effect on the rate of production and final yield of HA. In this series of tests the cell monolayers were washed with five serial 10 ml. aliquots of PBS before addition of virus. The results could not therefore be explained on the basis of small amounts of residual 1-AH in the maintenance medium.

A possible explanation of the effect of the pre-treatment of tissue culture cells is that 1-AH may exert a modifying effect on specific virus receptor sites at the cell surface resulting in blocking of adsorption or penetration of virus. Accordingly further tests were carried out to determine if 1-AH was capable of modifying

virus receptor sites on the surface of red blood cells. Chick and human group " O " red blood cell suspensions (0·5 per cent v/v) were treated by incubation with 100 μg./ml. of 1-AH for 3 hr. at 36·5°. Haemagglutinin titrations of allantoic fluids containing NWS and A2/Scot/49/57 viruses were performed in parallel with treated and untreated cells. Since no change in the HA titres of the viruses was detected using the treated batches of red cells it thus appeared that the receptor sites involved in the influenza virus haemagglutination reaction were not modified by 1-AH.

Mouse protection tests

Studies were carried out on the protective effects of 1-AH in mice inoculated with NWS, A/PR8 and A2/Sing/4/57 virus. Except for one experiment with A2/Sing/4/57 in which a single dose of 1-AH was given just before inoculation with virus, treated animals normally received 5 intraperitoneal injections of 1-AH at 24 hr. intervals. The results are shown in Table IV.

No protective effect was observed in two experiments in which A/PR8 virus was titrated intranasally in mice. In one experiment a slightly higher death rate ($10^{0.5}LD_{50}$/0·1 ml.) in the treated group of animals was observed. However, examination of the lungs at death of the animals in this group revealed gross consolidation due to influenza virus. With NWS virus inoculated intracerebrally a slight reduction in LD_{50} titre ($10^{0.9}LD_{50}$/0·03 ml.) was observed in the treated group in a single experiment.

A higher degree of protection was observed in mice inoculated intranasally with A2/Sing/4/57. This virus was found to have low LD_{50} titres and in one experiment no deaths occurred in treated or untreated animals. However, the infectivity titres of the virus (ID_{50}) as indicated by gross pulmonary lesions extended considerably beyond the LD_{50} end-point titre of the virus. In the two experiments in which deaths due to A2/Sing/4/57 occurred, the LD_{50} titre was lower in the treated animals. Furthermore, in all 3 experiments with A2/Sing/4/57 reductions in ID_{50} titres of the order of one hundredfold were detected. In the single experiment in which one dose of 1-AH was given immediately before infection with A2/Sing/4/57 the reduction in LD_{50} and ID_{50} titres was of the same order as in mice receiving 5 injections. This finding suggested that the protective effect of 1-AH may be manifested during the early stages of the infection.

No permanent ill effects were observed in a total of 30 control mice which received 5 injections of 1-AH and were not infected with virus.

DISCUSSION

Using end-point infectivity techniques, confirmation has been obtained that 1-AH at non-toxic concentrations is capable of inhibiting the growth of several different human and animal strains of influenza A virus. Furthermore, comparisons of the degree of inhibition revealed considerable differences between these strains in their sensitivity to the drug. The degree of inhibition extended over a wide range, from slight reduction in titre in the presence of 25 μg./ml. of 1-AH as with A/PR8 to complete inhibition by 8 μg./ml. of 1-AH with A2/Scot/49/57. In addition to depressing end-point infectivity titres 1-AH reduced the quantity of virus haemagglutinin produced in tissue cultures infected with one or other of

TABLE IV.—*Mouse Protection Experiments with 1-Adamantanamine Hydrochloride given by the Intraperitoneal Route*

Virus strain	Route of inoculation	LD_{50} Virus titres			ID_{50} Virus titres		
		1-AH treated mice	Untreated mice	protection (LD_{50})	1-AH treated mice	Untreated mice	Protection (LD_{50})
NWS	intracerebral	$10^{5.5}$/0·03 ml.	$10^{6.4}$/0·03 ml.	$10^{0.9}$/0·03 ml.	—	—	—
A/PR8	intranasal 1	$10^{5.0}$/0·1 ml.	$10^{5.2}$/0·1 ml.	insignificant	$10^{6.2}$/0·1 ml.	$10^{5.9}$/0·1 ml.	—
,,	,, 2	$10^{4.7}$/0·1 ml.	$10^{4.2}$/0·1 ml.	—	$10^{5.5}$/0·1 ml.	$10^{5.8}$/0·1 ml.	insignificant
A2/Sing./4/57	intranasal 1	no deaths	$10^{1.5}$/0·1 ml.	$10^{1.5}$/0·1 ml.	$10^{1.6}$/0·1 ml.	$10^{3.8}$/0·1 ml.	$10^{2.2}$/0·1 ml.
,,	,, 2	no deaths	no deaths	—	$10^{0.3}$/0·1 ml.	$10^{2.1}$/0·1 ml.	$10^{1.8}$/0·1 ml.
,,	,, 3*	$10^{0.3}$/0·1 ml.	$10^{1.7}$/0·1 ml.	$10^{1.4}$/0·1 ml.	$10^{1.9}$/0·1 ml.	$10^{4.4}$/0·1 ml.	$10^{2.5}$/0·1 ml.

* In one experiment with A2/Sing./4/57 a single dose of 70 mg./kg. of 1-AH was given, otherwise treatment was with 5 doses at 24 hr. intervals.

two strains of influenza virus. In general it appeared that virus strains which had often been passed in the laboratory were relatively insensitive, whilst those with few laboratory passages were highly sensitive. It is suggested by these observations that the outcome of tests with potential chemotherapeutic agents may be greatly influenced by the choice of test strains of virus and that examples of recent virus isolates should be included amongst the strains selected for testing such compounds. It is of interest that a recent A2 strain, 442/63, from a case of clinical influenza, which was recovered in MK tissue cultures, and thereafter serially passed twice in that system and in no other, was highly sensitive to 1-AH. The reason for this enhanced sensitivity of a recently-isolated strain may perhaps be sought in the lesser degree of virus adaptation to the host cells or perhaps in some other factor.

It was found that 1-AH produced no protective effect in mice inoculated intranasally with A/PR8 virus. However, this virus was only very slightly inhibited ($\log_{10}$ $TCID_{50}$ decreased by 0·5) in tissue culture experiments. With NWS virus inoculated intracerebrally in mice a slight reduction in LD_{50} titre was observed in animals treated with 1-AH and further, with A2/Sing./4/57 inoculated intranasally a consistent reduction in LD_{50} titre by 1·4–1·5 $\log_{10}$ was observed. It is of interest that these results correlated well with the sensitivity of the same virus strains in tissue culture systems (Table II). However, it should be stated that in the animal experiments the effect of different routes of inoculation of virus and drug were not further investigated so that optimal conditions for protection may not have been obtained. In attempts to simulate *in vivo* conditions Tyrrell *et al.* (1965) investigated the effect of 1-AH on A2/Pak./1/57 virus growing in organ cultures of human respiratory epithelium. Multiplication of the virus was slowed down only in the initial stages after infection although the same virus strain was inhibited by 1-AH in MK tissue cultures. Moreover, Tyrrell *et al.* (1965) failed to demonstrate a protective effect of 1-AH in human volunteers inoculated with A2/Scot/49/57. In the present studies this virus was found to be highly sensitive to 1-AH in MK cultures. However, in the human volunteer studies it was necessary to inoculate large doses of virus (approx. 10^6–$10^7 ID_{50}$) in order to obtain clinical evidence of infection.

Apart from parainfluenza 2 virus (CA strain) which was slightly inhibited in MK tissue cultures, representative strains of other families of viruses (including enteroviruses, rhinoviruses and an adenovirus) appeared to be unaffected by 1-AH The drug thus appeared to be highly selective for myxoviruses, and in particular for influenza A strains. Previous studies with anti-viral compounds have frequently demonstrated similar highly selective activity against a particular group of viruses. Eggers and Tamm (1961) showed that 2-(alpha-hydroxy benzyl) benzimidazole inhibited certain members of the enterovirus group but not others.

The present studies indicated that the mode of action of 1-AH did not depend upon direct inactivation of extracellular virus. However, it was shown in experiments with A2/Scot/49/57 growing in MK cultures that a marked reduction in virus yield occurred when 1-AH was present for a period of 20 min. immediately after infection of the tissue cultures. If 1-AH was not added until 20 min. or 3 hr. after infection, the reduction in virus yield was less. Furthermore, prior incubation of MK cultures with 25 μg./ml. of 1-AH reduced the yield of virus when the cultures were subsequently infected with A2/Scot/49/57 even though precau-

tions were taken to ensure that all traces of free 1-AH were removed before addition of virus. This finding suggested that 1-AH might have a direct effect on virus receptor sites at the cell surface. In previous studies with cell receptors for influenza viruses the red blood cell has frequently been employed as an experimental model. However, in the present studies there was no evidence that the presence of high concentrations of 1-AH interfered with haemagglutination titrations of A2/Scot/49/57 with chick or human red blood cells incubated with 1-AH (100 μg./ml.) for one hour before addition of virus. This finding may be taken to mean that the drug did not prevent attachment of virus (haemagglutinin) to the red cell surface. In conclusion, it appears that 1-AH acts by inhibiting an early stage in the virus replication cycle, presumably during adsorption or penetration, and that this effect may possibly be mediated directly through virus receptor sites at the cell surface. These findings are in general agreement with the observation of Davies *et al.* (1964) that 1-AH may act by inhibiting virus penetration into the cell.

SUMMARY

1-Adamantanamine hydrochloride has been found to produce highly selective inhibition of a number of different influenza A viruses in tissue culture and in the experimental infection of mice. Considerable variation in the degree of sensitivity to the drug was observed among different influenza A strains. It appeared that 1-Adamantanamine acted at an early stage in the virus growth cycle. Pretreatment of MK cells with the drug reduced the virus yield when the cells were subsequently infected with a sensitive virus strain, however no interference with the agglutination of human or chick erythrocytes by sensitive virus strains was observed in the presence of 1-Adamantanamine.

The authors are grateful to Dr. H. A. Wendel of E. I. du Pont de Nemours and Co., Delaware, U.S.A. for a gift of 1-Adamantanamine hydrochloride and to those who kindly supplied strains of virus. Receipt of supplies of 199 medium from Messrs. Pfizer is also gratefully acknowledged.

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