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Further Observations on the Inhibitory Action of Ammonium Chloride on Influenza Virus.* (32067)

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Ammonium salts at low concentration inhibit the growth and cytopathic effect of type A influenza viruses(3,10). Other myxoviruses are affected slightly or not at all and the only other species for which similar results have been reported is a member of EMC group(8). Inhibition of viral penetration has been suggested as a common mode of antiviral action between NH_4Cl, adamantanamine, and a piperazine derivative(1,6,7,9). In earlier experiments with a different cell-virus system (3) we had been unable to demonstrate a significant effect of ammonium phosphate on penetration of influenza virus. Also inhibition of a type B virus in chorioallantoic tissue was observed although it has been reported by others that neither adamantanamine nor NH_4Cl are effective against this type of influenza(6). Since an accurate knowledge of the mode of action of effective inhibitors is of fundamental importance it seemed desirable to investigate the reasons for these discrepancies.

Materials and methods. The media and methods used for preparation of roller tube cultures of chorioallantoic tissue have been described(2,4).

Virus. The PR_8 type A strain and the Lee type B strain of influenza virus were propagated in the allantoic sac of chick embryos. The allantoic fluids had hemagglutinin (HA) titers of 1,000 to 5,000 HA units per ml. Virus/cell multiplicities were estimated as follows. For the PR_8 strain one HA unit is approximately 10^6EID_{50}. The roller tubes each contained 40 mg (wet weight) of tissue in 4 ml of medium and a final dilution of virus giving 40 HA units per ml would represent an input multiplicity of 10^6EID_{50}/mg of tissue. Assuming that there are 10^5 cells/mg and that all cells come in contact with virus the resulting EID_{50}/cell would be approximately 10.

Antiserum. Sera from rabbits immunized with type A influenza virus had hemagglutination inhibition (HI) antibody titers between one and five thousand as measured by a standard method. Since these sera contained antibodies to chicken tissue adsorption with 10% erythrocyte suspensions was necessary before they could be used in experiments with chorioallantoic tissue. When antiserum having a titer of 1:5000 *vs* 8 HA units was used at a final dilution of 1:200 this gave 25 HI units per ml or an antibody excess of approximately 10-fold over virus input.

Viral replication can be limited to a single cycle by placing preinfected cells in a medium containing antiviral antibody. The yield of cell-associated virus after a period of replication is then proportional to the amount of virus that has penetrated cells. When antiserum is removed after an hour's contact with the cells, as has been done in other laboratories, multiple growth cycles occur unless all cells are infected initially with a large excess of virus. Treatment of infected cells with antiserum was performed in two different

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TABLE I. Effect of Pretreatment with Antiserum and NH_4Cl on Yield of Extracellular and Cell-Associated Hemagglutinins in Chorioallantoic Tissue Culture.

EID_{50}/mg tissue	Antiserum		Cells (C) or supernat. (S)	24-hr HA titers with (A) nil	(C) A.S.	(B) NH_4Cl†	(D) A.S. + NH_4Cl	B ÷ D / A ÷ C
	Conc, %	Time*						
10^6	1.0	3–4	S	32	4	32	0	—
"	0.5	"	S	64	32	64	32	1
"	0.5	"	S	128	64	64	32	1
10^4	2.0	"	C	512	8	256	<4	—
"	"	"	S	64	0	64	0	—
"	1.0	"	C	512	128	256	16	4
"	"	"	S	64	0	32	0	—
"	0.5	"	C	512	512	512	256	2
"	"	"	S	128	64	64	8	4
10^6	0.5	1–24	C	1024	128	512	4	16
"	"	"	C	256	8	512	4	4
"	"	4–24	C	1024	128	1024	32	4
"	"	"	C	256	16	512	32	1

* Antibody present at indicated interval in hours.
† NH_4Cl 200 μg/ml added at zero hour.

ways: (A) Cells incubated in the presence of virus with or without NH_4Cl for varying periods were resuspended in a medium containing antiserum and incubated with the antibody for a period of 24 hours. Controls treated in the same way were incubated without antibody.

(B) Cells preinfected at 5C were then resuspended with or without NH_4Cl. After a period of 3 hours at 36C antiserum was added. The cells were centrifuged after another hour, resuspended in medium without NH_4Cl or antiserum, and incubated in roller tubes for 24 hours.

Measurements of cell associated hemagglutinins were done on extracts obtained as previously described(2,4).

Results. Antiserum and NH_4Cl in chorioallantoic tissue. Experiments were done with inhibitory concentrations of NH_4Cl which must be higher than in Krebs 2 ascites tumor cells (4) or cultures of mammalian kidney. The results of representative experiments are presented in Table I. When method B, as described in the previous section, was used neutralization of virus remaining on cell surfaces at 3 hours was reflected in reduced HA yields as compared with the controls (Columns A and C Table I). Columns B and D represent the neutralization of virus adsorbed in the presence of NH_4Cl. It is of interest that an increase in antibody, although already in excess, resulted in considerably more neutralization of "adsorbed" virus. The reduction in titer due to the action of antibody on adsorbed virus is obtained by dividing HA titers in Column A by those in Column C. Similarity division of the values in Column B by those in D gives the fold reduction in titer by antiserum after treatment with NH_4Cl. The increase of neutralizable surface virus due to NH_4Cl is then estimated by the comparison ratio shown in the right had column of Table I. In some of the experiments the virus neutralizable in the presence of NH_4Cl was estimated to be 2 to 4 times that in its absence but sometimes no effect of NH_4Cl on penetration could be demonstrated. As shown previously NH_4Cl at the concentration used in these experiments when present during the 24 hour period consistently reduces the HA yield by 50- to 100-fold but has no effect if added one hour after the virus(3,4).

In general antiserum caused a greater reduction of HA titer in the supernatant than of the cell associated HA. Some residual antibody may have remained in the tubes after centrifuging the cells and resuspending them in new medium. This might reduce somewhat the HA yield in the supernatants without affecting cell associated hemagglutinins.

In single cycle growth experiments antiserum was added at various times from 10 minutes to 4 hours and left in for the entire

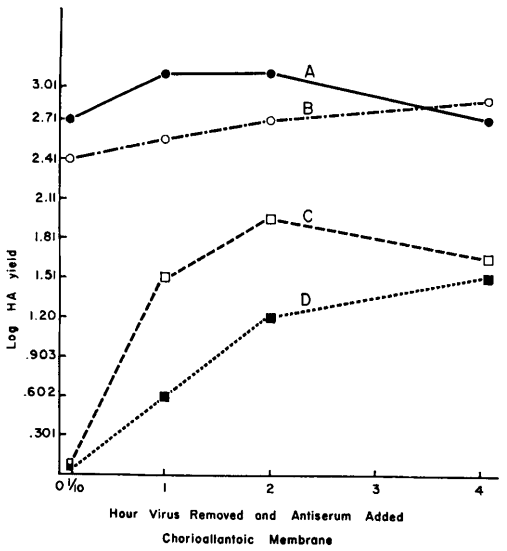

FIG. 1. Effect of exposure to NH_4Cl for various periods on neutralization of adsorbed virus. Curves correspond to columns in Table I. A and C, infected tissue incubated without NH_4Cl for time indicated, centrifuged to remove virus, antiserum added (C). B and D, infected tissue incubated with NH_4Cl, centrifuged to remove virus and NH_4Cl, antiserum added (D). Points represent titers of cell associated hemagglutinins after 24 hours (See method A).

period of 24 hours (Method A). The yields of cell associated hemagglutinins were then measured. The results are presented in the last lines of Table I and in Fig. 1. In only one experiment was a large reduction of penetration by NH_4Cl observed. A duplicate experiment gave results which were much less significant (Table I). A major portion of the virus is adsorbed within 10 minutes even in the presence of NH_4Cl. Neutralizable virus decreases rapidly after 10 minutes but penetration does not appear to be complete until about 2 hours. In the presence of antiserum viral growth was limited to a single cycle (Curves C and D) and yields of cell associated HA were therefore lower than in the absence of antiserum (Curves A and B) where multiple cycles of growth could occur. Comparison of Curves C and D (antiserum present) indicates that more neutralizable virus remained on cell surfaces at 1 and 2 hours in the presence of NH_4Cl (D) but penetration had occurred to almost the same extent by 4 hours. Addition of antiserum at that time resulted in very little neutralization either with or without NH_4Cl. The ratio $\frac{B \div D}{A \div C}$ was maximum at one hour and tended to approach unity at 4 hours.

Results with type B influenza virus. A comparison of types A and B viruses with respect to NH_4Cl sensitivity and reversibility of inhibition is presented in Table II. High and low input multiplicites were investigated. At the higher multiplicity the HA titers of the supernatants, when NH_4Cl at 200 μg/ml was present, did not increase, and at the lower multiplicity no HA appeared. With NH_4Cl at 100 μg/ml there was a delayed increase in HA titer between 24 and 72 hours of incubation. The results indicate that types A

TABLE II. Sensitivity of Influenza Virus Types A and B to NH_4Cl. Reversal of inhibition supernatant hemagglutination titers.

Dilution*	NH_4Cl, μg/ml	Fluid change	Virus: Type A (PR_8) 24 hr	Type A (PR_8) 48-72 hr	Type B (Lee) 24 hr	Type B (Lee) 48-72 hr
10^{-2}	0	none	512	512	128	256
	100	"	32	128	4	16
	200	"	32	16†	4	4†
	200	24 hr‡	32	256	4	64
	0	24 "	1024	64	128	16
10^{-4}	0	none	256	512	32	64
	50	"	64	512	—	16
	100	"	0	32	0	8
	200	"	0	0	0	0
	200	24 hr‡	0	512	0	32
	0	24 "	256	32	32	16

* Final dilution of infected allantoic fluid in tissue culture medium.
† These titers are approximately the same as HA titers in the fluid at zero hours.
‡ Changed to medium without NH_4Cl.

and B are almost identical in sensitivity to inhibition by NH_4Cl in this cell-virus system. Newcastle disease virus was not inhibited by NH_4Cl at 500 μg/ml in this system.

Reversal of the NH_4Cl effect by centrifuging the tissue and resuspending in medium without NH_4Cl indicates that the inhibition was not due to cell damage. Hemagglutinins were either absent or low in titer at 24 hours with NH_4Cl but showed a marked increase after its removal. Conversely most of the hemagglutinin was formed in the controls during the first 24 hours and when the fluids were changed additional hemagglutinins appeared in reduced amounts.

Discussion. These experiments and others to be reported elsewhere(5) raise questions about the significance of minor and irregular effects of NH_4Cl on viral penetration as the mode of inhibition of influenza virus replication. It is possible that NH_4^+ or NH_3 causes a physicochemical change at the cell surface which influences uptake of viral particles but it is unlikely that such a non-specific effect would be limited to strains of myxovirus. The surface action might vary with the species of cell, possibly being greater in monkey kidney tissue culture(6). An analogous inhibition of membrane transport by NH_4Cl in excluding diphtheria toxin from entry into cells in tissue culture with consequent prevention of cytopathic effect has been reported(11). Since toxin in the presence of NH_4Cl remains neutralizable for long periods these experiments are quite convincing, and differ from our results where virus remains neutralizable for only a short time.

It has been reported that adamantanamine is ineffective against type B strains of influenza virus and resistance of influenza B to NH_4Cl in monkey kidney tissue cultures has been taken as evidence of a common mode of action between NH_4Cl and adamantanamine(6). Our observations with the Lee strain of influenza type B in chorioallantoic tissue do not confirm that it is resistant to NH_4Cl.

As established in previous publications NH_4Cl does not inactivate extracellular influenza virus nor prevent adsorption. If a significant effect on viral penetration is in doubt then the possibility of action at an intracellular site must be considered. If, as previously supposed, an early event during the eclipse phase is involved(3) this might relate to RNA polymerase or the messenger action of viral RNA(12). It is of interest that inhibition by $(NH_4)_2SO_4$ at 15% saturation has been used as one of the criteria to distinguish the RNA polymerase of Mengo virus from the corresponding polymerase of the host cell (13). Indeed Columbia SK virus, which like Mengo virus is a member of the encephalomyocarditis group, is inhibited by ammonium salts(8) but only at concentrations up to 50 times those effective against influenza virus. Unfortunately the methods developed for study of RNA and polymerase of picorna viruses are not entirely applicable to myxoviruses. Development of new methods would facilitate an investigation of the possible intracellular action of NH_4Cl.

Summary. The effect of NH_4Cl on viral penetration was measured in chorioallantoic tissue *in vitro* by neutralizing absorbed virus with antiserum and determining the subsequent 24 hour yield of hemagglutinins. In some experiments conditions for single cycle replication were obtained by leaving the antibody in the supernatant during the entire period of growth and then measuring cell-associated hemagglutinins. Although some reduction of the amount of penetrated virus was demonstrable one hour after adding virus and NH_4Cl this later became insignificant. The prevention of viral penetration by NH_4Cl was considered too inefficient and irregular to account for the over-all inhibitory effect of NH_4Cl on viral replication. Inhibition by NH_4Cl of a type B (Lee) strain was comparable with that for Type A and reversibility of inhibition was demonstrated.

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Response of the Coyote to Endotoxin. (32068)

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The coyote *(Canis latrans)* is a shaggy, dog-like animal, somewhat smaller than a Collie dog, with erect pointed ears and bushy, drooping tail. Its taxonomy, habitat, and general characteristics have been described in detail(1,2). Pathophysiological studies contrasting its responses with those of the dog *(Canis familiaris)* appear to be lacking in the literature. Recent research has described the various responses of dogs to endotoxin(3,4,5) as well as other species; including cats, bears, monkeys, rabbits and chickens(4,6-11). Dogs respond to a lethal injection of endotoxin with portal hypertension leading to a marked drop in venous return and systemic arterial pressure(4), followed by generalized splanchnic congestion, hemorrhage, edema, acidosis, and hemoconcentration. Notable species differences have been observed in dogs and monkeys and are primarily concerned with responses of the hepatosplanchnic bed(6,9). It was thought to be of interest to study the response of the coyote to endotoxin in terms of its resistance, hemodynamic alterations, and histopathology.

Materials and methods. Experiments were carried out on 9 adult coyotes obtained from the Oklahoma City Zoo. Animals were of either sex, weighing between 8.2 and 12.1 kg. They were intravenously anesthetized with 30 mg/kg sodium pentobarbitol. *E. coli* endotoxin obtained from Difco (Detroit, Mich.) was administered to all animals after obtaining control measurements. Four animals were used for survival studies. An LD_{80} dose, recently established in dogs in this laboratory, was 0.4 mg/kg; however, the first coyote survived a 2 mg/kg injection. The remaining 3 animals received 4 mg/kg; one animal died within 24 hours and the remaining, within 3 days. This latter dose was administered to 5 other coyotes and a variety of parameters were measured. Systemic arterial pressure was obtained by advancing a polyethelene catheter from the femoral artery into the aorta, connecting it to a Statham pressure transducer, and recording pressures on a Sanborn direct writing recorder. Portal vein pressure was measured by advancing a catheter from a splenic vein to the main portal vessel following a laparotomy. Hematocrit, pH, and heart rate were periodically recorded after endotoxin. Liver function was evaluated by a colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminase (SGOT)(12). Sigma reagents were used in a procedure specifically outlined by the Sigma Technical Bulletin (Sigma Chemical Co., St. Louis, Mo.). Blood urea nitrogen (BUN) determinations were carried out by the automatic autoanalyzer diacetyl method(13). Gross and histological observations were made on animals in the latter group in which blood pressures were followed for 4 hours.

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