drase caused by progesterone. It seems that the antiprogestational ability of estrogen is almost the same in estradiol, ethinyl estradiol, hexestrol and estrone-3-methyl ether, but approximately 1/10 of the others in estriol-3-methyl ether, if given systemically. From the data, a linear log dose response relation of 2 antagonistic hormones was obtained in amounts ranging from 0.002 mg to 0.2 mg of estradiol, ethinyl estradiol, hexestrol and estrone-3-methyl ether and in doses of 0.02-2.0 mg in estriol-3-methyl ether, the regression about the line being highly significant. Thereby, quantitative differences in the potency of these estrogens to prevent progestational development may be provided.

Previously, Miyake and Pincus(2) showed that other estrogens (estradiol, estriol, estrone and stilbestrol) suppressed the enzymic response to progesterone, the extent of which correlated extremely well with the degree of pseudo-pregnant proliferation. The antiprogestational activities of these estrogens were all approximately the same. In their experiments, almost the same procedure as that reported here was employed. The response regression line of estradiol on progestational development is in satisfactory agreement with our data.

Considering that estrone and estriol were shown, according to them, to be the same in antagonistic potency, it would be expected that inhibitory abilities of estrone- and estriol-3-methyl ether would be the same because of certain similarities in chemical properties. However, this is not the case in our experiments. The results indicate a marked difference in the abilities of estrone- and estriol-3-methyl ether to affect the action of progesterone. Estriol-3-methyl ether seems to have approximately 1/10 the potency of estrone-3-methyl ether if the comparison is made from our regression line.

The authors are indebted to Professor T. Suzuki for many helpful suggestions and to Dr. K. Hirai for assistance throughout this study. We also wish to thank Dr. G. Pincus, Worcester Foundation for Experimental Biology, and Dr. W. O. Nelson, Population Council, Inc., Rockefeller Institute, for kindly supporting this work.

1. Lutwak-Mann, C., Adams, C. E., *J. Endocrinol.*, 1957, v15, 43.

2. Miyake, T., Pincus, G., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 478.

3. Clauberg, C., *Zentr. Gynäkol.*, 1930, v54, 2757.

4 Philpot, F. J., Philpot, J. St. L., *Biochem. J.*, 1936, v30, 2191.

5. Wilbur, K. M., Anderson, N. G., *J. Biol. Chem.*, 1948, v176, 147,

6. Lutwak-Mann, C., *J. Endocrinol.*, 1955, v13, 26.

7. Pincus, G., Miyake, T., Merrill, A. P., Longo, P., *Endocrinology*, 1957, v61, 528.

Received April 24, 1961. P.S.E.B.M., 1961, v107.

Inhibitory Effect of Ammonium Ions on Influenza Virus in Tissue Culture. (26653)

E. M. JENSEN, E. E. FORCE AND J. B. UNGER (Introduced by O. C. Liu)
Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pa.

While examining a series of pyrimidines for antiviral activity in a tissue culture system, it was observed that one compound exhibited a marked inhibition of the cytopathic effect (CPE) of influenza virus. Subsequent studies with other preparations of the same compound showed varying degrees of activity, and in some cases a complete lack of activity. This inhibitory effect was eventually shown to be due entirely to trace amounts of ammonium ion present in the active preparations as an impurity. Further studies have shown that comparable results can be obtained with a number of inorganic and organic ammonium salts. This report describes in detail the virus inhibition and discusses possible explanations for the observed effect. It is also shown that under normal growth conditions sufficient ammonia may be accumulated in the medium to pro-

TABLE I. Inhibition of Viral CPE by Several Ammonium Salts.

Compound	CPE at μg/ml of compound*								Minimum effective level	
	512	256	128	64	32	16	8	4	μg/ml	μmoles of ammonia
Ammonium chloride	Toxic	0	0	0	0	2+	3+	4+	16	.30
" sulfate	"	Toxic	0	0	2+	4+	4+	4+	32	.49
" citrate	"	0	2+	3+	4+	4+	4+	4+	128	1.08

* All tubes inoculated with 1000 $TCID_{50}$'s/ml influenza virus, PR-8.

tect the cells from influenza virus.

Materials and methods. The virus employed was Influenza A, strain PR-8, which was in its 126th passage in embryonated chicken eggs *via* the allantoic route. Virus dilutions were prepared in tissue culture maintenance medium described below. Infectivity titrations of virus were performed both in chick embryos and in tissue culture and were calculated by the method of Reed and Muench(1). Hemagglutination (HA) titrations were conducted by the method of Salk(2) and are expressed as the reciprocal of the dilutions.

The cell culture employed in this study was a stable line of epithelium-like cells originally isolated from a dog kidney, which has undergone 76 subpassages in our laboratory. The growth medium for these cells consisted of Eagle's basal medium(3) supplemented with 10% calf serum. Maintenance medium consisted of Eagle's basal medium supplemented with 2.5% calf serum.

Virus inhibition studies in tubes were conducted by inoculating 2 series of duplicate tissue culture tubes with 2-fold dilutions of the test compound ranging from 1024 μg/ml to 4 μg/ml. One series was retained as a toxicity control and the other series was inoculated with 1000 $TCID_{50}$'s of influenza virus, strain PR-8. Generally the compound was added one hour before addition of the virus. The results, observed 48 or 72 hours after infection are expressed in terms of CPE, graded on a scale of 0 for no cellular degeneration to 4+ for complete destruction of cells.

Ammonia assays were conducted by the method of Conway(4) and colorimetric determinations of the Nesslerized samples were made with a Bausch and Lomb Spectronic "20" colorimeter at 500 mμ.

Results. Table I shows the inhibitory effect of ammonium ions on the CPE of influenza virus. The results show that viral inhibition is roughly proportional to the actual amount of ammonia present in each salt. The lack of perfect correlation may be due to the limits of accuracy of the test system and to the difference in dissociation constants of the salts used. The possibility of the anions being inhibitory can be excluded by the fact that normal tissue culture medium contains approximately a thousand-fold greater concentration of chloride ion than is added in the form of ammonium chloride.

The degree of protection afforded by ammonium ion is shown in Fig. 1-3. These 3 photographs of unstained dog kidney monolayer cultures show clearly that 40 μg/ml of NH_4Cl is sufficient completely to protect the cells from an otherwise lethal inoculum of influenza virus. It is further shown that 40 μg ml of NH_4Cl has no apparent effect upon the cell layer. The protection was obtained only when the salt was added either before or simultaneously with the infection. A delay of one hour or more resulted in very little protection.

The protection observed as a function of CPE was also observed as a suppression of viral synthesis. Table II shows the inhibitory effect of 40 μg/ml of NH_4Cl as measured by CPE, hemagglutination and infectivity titers. The tissue cultures were infected one hour after addition of the NH_4Cl and

TABLE II. Inhibition of Viral Synthesis by 40 μg/ml of NH_4Cl.

NH_4Cl	Inoculum* $TCID_{50}$/ml	CPE	HA/ml	EID_{50}/ml
—	1000	3+	4096	$10^{5.0}$
40 μg/ml	"	0	<4	$10^{3.7}$

* Influenza virus, PR-8.

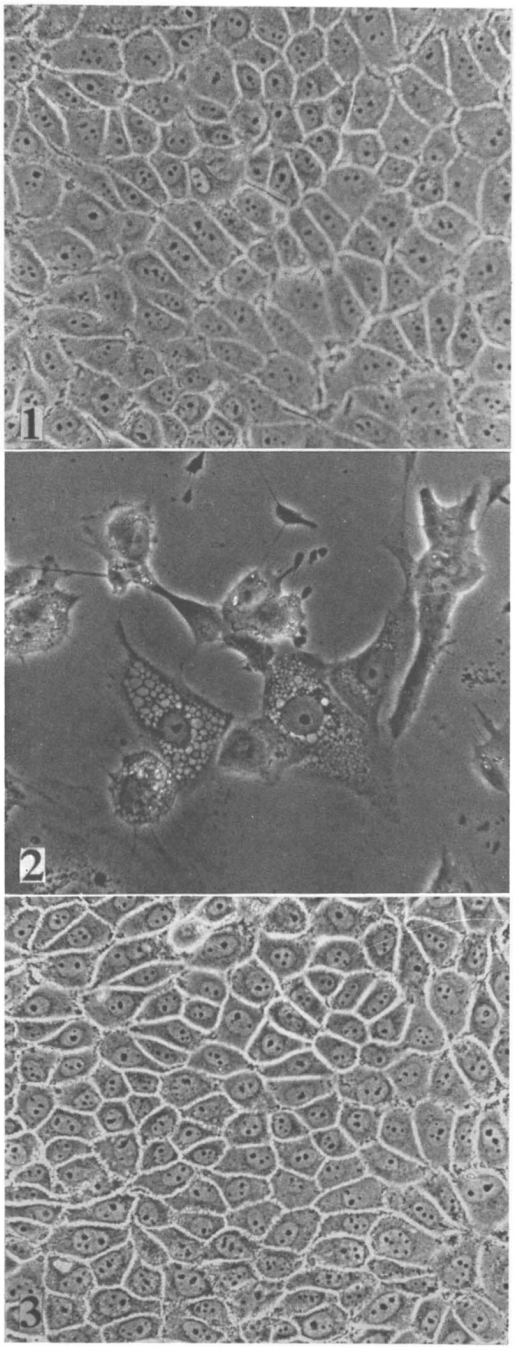

FIG. 1. Normal monolayer culture of dog kidney cells. (Phase contrast, magnif. 269 ×.)

FIG. 2. Dog kidney cell culture similar to Fig. 1 48 hr after infection with 1000 $TCID_{50}$'s of influenza virus, PR-8. Majority of the cells have been destroyed and dislodged from cover slip. Remaining cells show severe viral CPE. (Phase contrast, magnif. 336 ×.)

results were observed at 48 hours.

Preliminary attempts to establish the mechanism of action of the inhibition have been unsuccessful. Ammonium chloride is not virucidal at concentrations substantially higher than those required for inhibition. As much as 1000 μg/ml of NH_4Cl mixed with 10^5 EID_{50}'s of influenza virus in maintenance medium caused no reduction in chick embryo infectivity titers when incubated for 5 hours at 37°C. Furthermore, the effect cannot be attributed to a pH change. Addition of 1000 μg/ml of NH_4Cl to maintenance medium lowers the pH by less than 0.1 of a pH unit; a change substantially less than those encountered in normal tissue culture growth.

Table III shows the results of an experiment designed to determine the effect of ammonium ions on adsorption of influenza virus to host cells. Cultures 2 and 4 were treated with 40 μg/ml of NH_4Cl. One hour later all cultures were infected with 1000 $TCID_{50}$'s of influenza virus. Cultures 1 and 2 were incubated for 48 hours, whereas the remaining cultures were washed free of virus and NH_4Cl one hour after infection, refed and incubated for 48 hours. The results show that whether ammonium ion was present or not, the virus was adsorbed and possibly absorbed in a period of less than one hour. Protection of the cells from the virus was obtained only if the ammonium ion was left in contact with the cells for the full incubation period. This experiment shows that the virus inhibition was not due to an interference with virus adsorption and this conclusion was also supported by the observation that as much as 10,000 μg/ml of NH_4Cl did not interfere with influenza hemagglutination titrations.

During this work it was observed that actively growing cells released measurable amounts of ammonia into the medium and that after several days this reached levels which should be inhibitory to virus growth. As expected, it was shown that 3-day-old cul-

FIG. 3. Monolayer dog kidney cell cultures showing protective effect of ammonium ions. Culture infected same as that in Fig. 2 but culture medium contained 40 μg/ml of NH_4Cl. (Phase contrast, magnif. 269 ×.)

TABLE III. Effect of Ammonium Ions on Adsorption of Influenza Virus to Dog Kidney Cells.

Culture No.	NH_4Cl (μg/ml)	Virus inoculum* ($TCID_{50}$/ml)	Treatment of tissue culture	48 hr results CPE	HA/ml
1	—	1000	Not washed or refed	4+	2048
2	40	"	*Idem*	0	<4
3	—	"	Washed and refed 1 hr after infection	4+	4096
4	40	"	*Idem*	3-4+	2048

* Influenza virus, PR-8.

tures could not be infected if the medium was not first replaced with fresh maintenance medium. The following experiment was conducted to determine whether this non-susceptibility of old cultures could be attributed to the ammonia.

Maintenance medium was collected from several T-30 flask cultures after 3 days exposure to the cells. The medium was divided into 2 portions, one of which was saved unchanged. The other portion was adjusted to pH 10 with 1 N • NaOH and lyophilized to remove the ammonia. It was then reconstituted to its original volume with sterile distilled water and neutralized with 1 N • HCl. Three healthy T-30 flask cultures were then arranged as shown in Table IV. Culture 1 was refed with the untreated old medium. Culture 2 was refed with the lyophilized reconstituted medium (ammonia partially removed) and culture 3 was refed with fresh maintenance medium. All cultures were then infected with 1000 $TCID_{50}$'s of influenza virus and all 3 cultures were incubated at 37°C for 48 hours, at which time they were observed for CPE and the fluids were titrated for hemagglutinins and chick embryo infectivity. The medium from each culture was assayed for total free ammonia at time of infection.

The results given in Table IV show that although some viral synthesis occurred in the cells refed with untreated old medium, the yield was only a fraction of that produced in cells refed with old medium from which the ammonia had been partially removed, or with new medium. The viral effect in culture 1 was not apparent as a CPE; however, the cells did not appear as healthy as the control cultures, undoubtedly because of lack of nutrients and/or accumulation of other waste products. This probably also accounts for the difference in virus titers in cultures 2 and 3. Although cultures 2 and 3 were not free of ammonia at zero-time, the level was apparently not high enough completely to suppress virus production.

Discussion. The virus-host cell system employed in this study appears similar to systems reported by other workers(5,6) in that a large initial inoculum of virus is necessary for development of CPE. The infection results in a high yield of incomplete hemagglutinating virus but a very low yield of infective virus. Subpassage of the virus in this system results in its eventual disappearance.

The demonstration that a simple inorganic cation such as ammonia can cause a marked inhibition of viral synthesis and prevent subsequent destruction of the host cell is of considerable interest. As shown here, chemotherapeutic studies may yield misleading results due to trace amounts of ammonia present in the test compounds. It is conceivable also that some of the undefined inhibitors of viruses found in various organic materials,

TABLE IV. Inhibitory Effect of Old Tissue Culture Medium on Virus Synthesis and Its Relation to Ammonia Content.

Culture No.	Medium	CPE	HA/ml	EID_{50}
1	Old medium (22 μg NH_4^+/ml)*	0	32	$10^{4.66}$
2	Old medium - ammonia partially removed (9 μg NH_4^+/ml)	3+	256	$10^{5.33}$
3	New medium (11 μg NH_4^+/ml)*	3-+	2048	$10^{6.5}$

All cultures inoculated with 1000 $TCID_{50}$'s influenza virus and results read at 48 hr.

* Concentration of ammonia present at time of infection.

for example that of lactalbumin hydrolysate (7), may be due to presence of ammonia. This may also account for the apparent variations observed in susceptibility of tissue culture cell systems to virus infections. Since ammonia tends to accumulate in the medium during cell growth, the age of culture media becomes a significant factor in cell susceptibility to infection. Preliminary studies indicate that the primary source of ammonia in fresh medium is glutamine. Inasmuch as glutamine has been shown to be required for virus synthesis in some systems(8) and markedly to change the type of CPE observed in others(9) careful consideration must be given to incorporation or exclusion of this material in all systems.

Of particular interest is the mechanism by which this inhibition is effected. Since addition of the ammonium salts must be made prior to or simultaneously with the virus inoculum for maximum protection, one would suspect an interference with virus adsorption to the host cell or a direct virucidal effect. Experimental evidence seems to rule out these explanations and consequently some intracellular mechanism must be suspected. An understanding of this inhibition may shed new light on the process of virus replication with possible applications to viral chemotherapy. Studies are now in progress to explore the mechanism involved as well as the effect of ammonium ions on other virus-cell systems. The effect of various other inorganic ions and the smaller organic amines is also being investigated.

Summary. Ammonium ions in trace amounts were shown to exert a very marked protective effect upon a tissue culture system infected with influenza virus. The ions inhibited viral synthesis as well as viral CPE and the effect was apparently not due to interference with virus adsorption or to a direct inactivation of the virus. It was also shown that the tissue culture system produced sufficient ammonia during growth to render itself insusceptible to the virus.

Addendum. Since the preparation of this manuscript a paper has appeared, Eaton, M. D. and Scala, A. R., *Virology*, 1961, v13, 300, in which inhibition of both influenza and Newcastle disease viruses by ammonia in Krebs 2 cells was reported. The results and conclusions given by these authors are very similar to those presented here.

1. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

2. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

3. Eagle, H., Oyama, V. I., Levy, M., Freeman, A. E., *J. Biol. Chem.*, 1957, v226, 191.

4. Conway, E. J., Byrne, A., *Biochem. J.*, 1933, v27, 419.

5. Henle, G., Girardi, A., Henle, W., *J. Exp. Med.*, 1955, v101, 25.

6. Mason, E. J., Kaufman, N., *J. Inf. Dis.*, 1960, v107, 245.

7. Holper, J. C., Marquis, G. S., Jr., *ibid.*, 1959, v105, 288.

8. Eagle, H., Habel, K., *J. Exp. Med.*, 1956, v104, 271.

9. Reissig, M., Black, F. L., Melnick, J. L., *Virology*, 1956, v2, 836.

Received April 27, 1961. P.S.E.B.M., 1961, v107.

Structure-Activity Relationships of Adrenocorticoids. (26654)

I. RINGLER, S. MAUER AND E. HEYDER (Introduced by Earl H. Dearborn)
Experimental Therapeutics Research Section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The past 8 years have heralded significant advances in syntheses of cortisone and hydrocortisone analogs possessing quantitative and qualitative differences in metabolic activities. Though it is not possible to predict accurately the qualitative metabolic effects of chemical substituents on hydrocortisone, this communication describes the influence of 1,2 unsaturation, 6α-methylation, 9α-fluorination, 16α-hydroxylation and 16α,17α-ketalization, alone and in combination, on liver glycogen deposition and thymus involution in