later studies confirm our results, the WM-III strain is the type 3 virus of choice for production of attenuated poliovirus vaccine.

Summary. Development of a new type 3 attenuated poliovirus (WM-III) is described. Starting with the W-Fox strain, passage and clone selection at 23°C were employed to develop a strain with improved stability after human intestinal passage. The end result was a virus which had no capacity to grow at 40°C and was not virulent for monkeys injected intracerebrally. The results of tests of fecal virus strains isolated from infants fed the WM-III strain did not show the instability in relation to laboratory markers that has been reported with other attenuated type 3 viruses.

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Studies of Inhibitory Effect of Ammonium Ions in Several Virus-Tissue Culture Systems. (26770)

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It was observed(1) that ammonium ions in trace amounts caused a very marked suppression of influenza virus replication in a dog kidney tissue culture system. This suppression was accompanied by a complete protection of the cells from the cytopathic effects of the virus. Similar results have been observed independently by Eaton and Scala (2) with both influenza and Newcastle disease virus in Krebs 2 ascites tumor cells. Since this unique inhibition may have a significant effect on many types of virus-host

cell studied it was considered important to determine the range of viruses and cell systems affected by this inhibitor.

This report presents results obtained with ammonium ions in test systems which include 10 virus species and 10 cell lines. In addition, ammonia was tested for inhibition against influenza virus in suspended allantoic membranes, embryonated eggs and in mice.

Materials and methods. The dog kidney cell culture has been described(1). Primary roller tube cultures of monkey, bovine, hamster and porcine kidney cells, and established cell lines, L-929, HeLa, Chang conjunctiva, human amnion (FL) and KB cells were obtained from a commercial laboratory.* Suspended allantoic membrane cultures were prepared by cutting the membrane from 11-day-old chick embryos into one cm square sections; the sections were washed several times in Hanks' balanced salt solution, pooled and distributed at random into T-30 flasks. Each flask contained 8 sections in 10 ml of BSS, and the membranes were maintained in suspension during incubation by a gentle action on a reciprocating shaker.

Viruses employed in this study were influenza, strains PR8, A′ and A_2, herpes simplex strain HF, Adenovirus type 4, Enders strain of mumps, Edmonston strain of measles, ECHO type 9, Parainfluenza type III, poliomyelitis type II (Lansing), vaccinia and Newcastle disease virus (NDV) Beaudette strain. All cultures were maintained in Eagle's minimum essential medium(3) from which glutamine was omitted and which was supplemented by 2.5% calf serum. Glutamine was omitted in order to eliminate, as much as possible, the presence of endogenous ammonia. Ammonia, in the form of NH_4Cl, was tested in each system by a tube dilution method. Two-fold dilutions of the compound in maintenance medium ranging from 1024 to 4.0 μg/ml were added to each culture and held one hour at 37°C. Virus was then added and the tubes were incubated at 37°C. The virus inoculum in each system was sufficient to give a 3-4+ cytopathic effect in 48 to 72 hours. Tubes were observed at the time of maximum CPE production in control tubes and, with influenza virus in allantoic membranes, hemagglutination (HA) titrations were conducted according to the method of Salk(4).

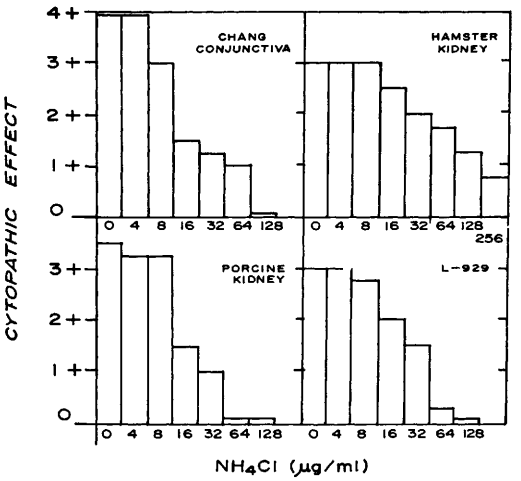

FIG. 1. Inhibition of influenza virus CPE by ammonia in four cell cultures.

Results. The inhibitory effect of ammonium ions on influenza virus in the dog kidney cell systems has been described(1). Inhibition of PR8 virus in 4 other cell systems is shown in Fig. 1. The inhibition of viral CPE was essetially the same in each cell line except hamster kidney cells which required higher concentrations of NH_4Cl for significant inhibition. The degree of protection afforded by ammonium ions may be seen in Fig. 2-4. These phase contrast photomicrographs of strain L-929 mouse fibroblasts clearly show the inhibition of influenza virus CPE by 40 μg/ml of NH_4Cl. Although a slight viral effect is apparent in the treated cells, in the form of vacuolization in the Golgi region, the cells are generally protected from the destructive effect of the virus.

The protective effect of ammonia was also shown in monkey kidney cells infected with influenza virus. Fig. 5 shows the effect of virus inoculum size on the inhibitory effect of NH_4Cl. The results indicate that a 100-fold change in inoculum size had little effect upon the amount of ammonia required for protection. From this experiment it can be concluded that inhibition is a result of the action of ammonia in the host cell and not upon the virus. This supports observations made in

* Microbiological Associates, Inc., Bethesda, Md.

the previous study where no direct inactivation of virus by NH_4Cl was demonstrated (1). Two other strains of influenza virus, A′ and A_2, were also studied in the monkey kidney cell system and essentially similar results were obtained.

To determine the effect of ammonia on influenza virus in more complex systems, tests were conducted in suspended chick allantoic membrane cultures, intact chick embryos and in mice. In the suspended membrane cultures virus production was measured by HA titrations and the effect of ammonia is shown in Fig. 6. Ammonium chloride appeared to

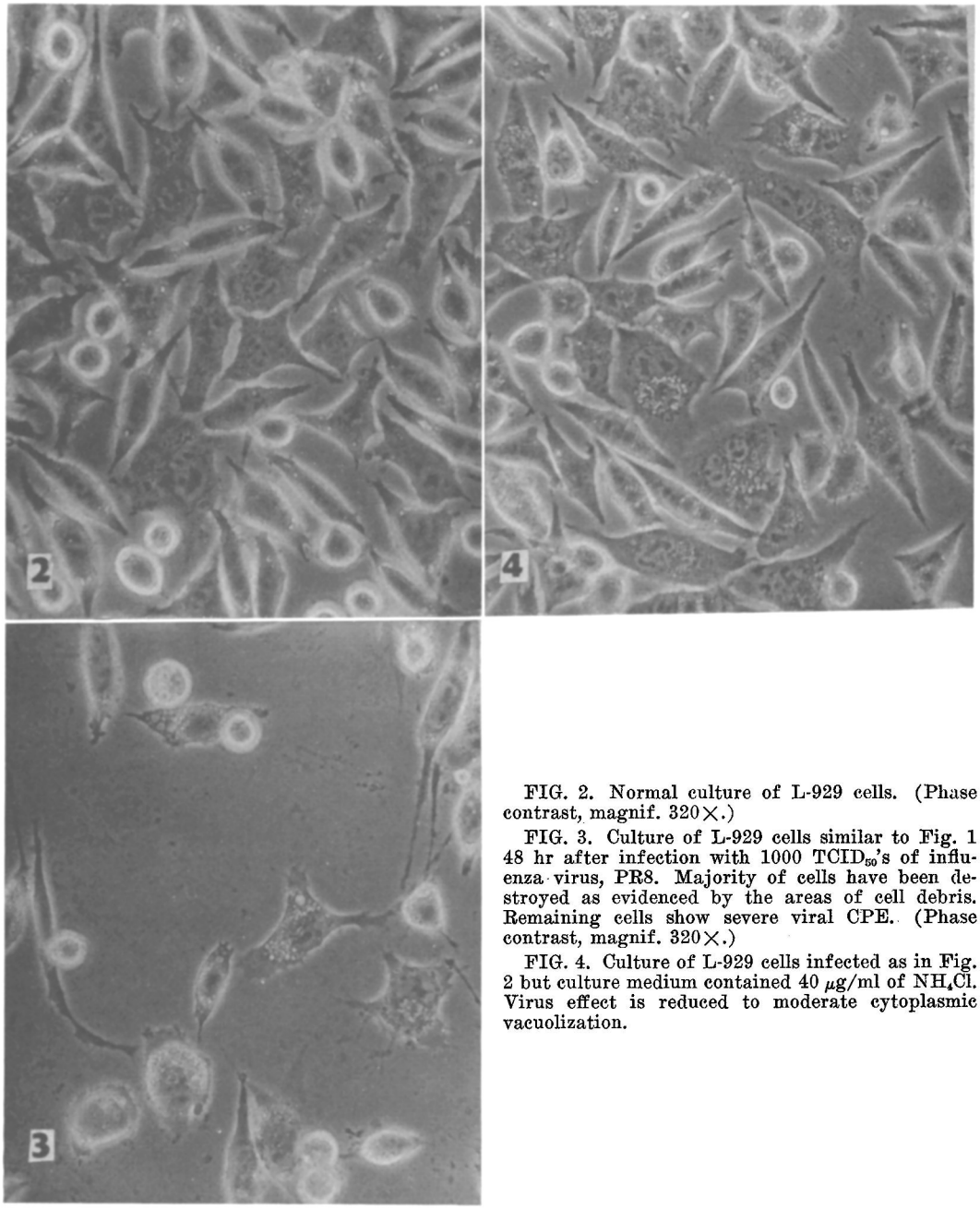

FIG. 2. Normal culture of L-929 cells. (Phase contrast, magnif. 320×.)

FIG. 3. Culture of L-929 cells similar to Fig. 1 48 hr after infection with 1000 $TCID_{50}$'s of influenza virus, PR8. Majority of cells have been destroyed as evidenced by the areas of cell debris. Remaining cells show severe viral CPE. (Phase contrast, magnif. 320×.)

FIG. 4. Culture of L-929 cells infected as in Fig. 2 but culture medium contained 40 μg/ml of NH_4Cl. Virus effect is reduced to moderate cytoplasmic vacuolization.

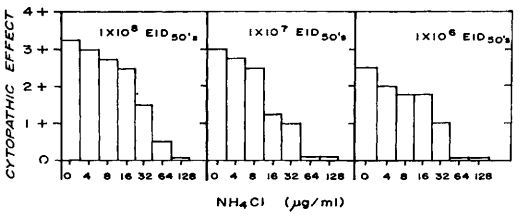

FIG. 5. Effect of virus inoculum size on inhibition by ammonia. Influenza virus PR8, in monkey kidney cell.

be toxic at levels between 500 and 1000 μg/ml, but significant virus inhibition was observed at levels as low as 16 μg/ml. Virus inhibition appears to be directly proportional to the amount of compound used.

In 10-day-old embryonated eggs a marginal and sporadic protective effect was observed when the eggs were treated with high concentrations of NH_4Cl. The compound is toxic at 40 mg/egg, but 4 to 8-fold reductions in HA titers at 24 hours were observed at levels as low as 5 mg/egg. An increase of 20 to 40% in survival rate was also observed. This phenomenon has been reported by Fauconnier(5) who attributes the effect to an acidification of the allantoic fluid. It is not known whether the inhibition observed in chick embryos is related to that observed in tissue culture.

Survival studies conducted in mice infected with approximately 10 LD_{50}'s of influenza virus, PR8, and treated with ammonium chloride administered orally, intraperitoneally or intranasally showed no protective effect.

Ammonium ions appeared to have no significant protective effects against the other viruses tested in this study. Only virus-cell line systems which produce an observable CPE were employed. In the case of herpes simplex virus indications of protection were observed in Chang conjunctiva, bovine and hamster kidney cells, but these effects were marginal and occurred only at near-toxic levels. Herpes simplex virus was not inhibited in dog kidney, HeLa or rabbit kidney cultures. Adenovirus type 4 was tested in dog kidney, HeLa, Chang conjunctiva and bovine kidney. Other systems studied include mumps in HeLa and Chang conjunctiva; measles in human amnion and KB; NDV in HeLa; ECHO-9, vaccinia and poliomyelitis in monkey kidney; and Parainfluenza type III in HeLa, Chang conjunctiva and bovine kidney cells.

Discussion. Although only a small number of viruses have been tested in this study, it appears that the inhibitory effect of ammonium ions is limited to influenza viruses. This is not in agreement with results reported by Eaton and Scala(2) on suppression of NDV in Krebs 2 cells. This discrepancy may be due to the difference in host cells or viruses used. We previously showed(1) that the effect cannot be explained in terms of direct virus inactivation or of interference with virus adsorption to the host cell. Thus, it was postulated that ammonia may interfere with some intracellular synthetic mechanism. The present study shows that cell protection is independent of virus inoculum size, which reinforces the concept of a compound-host cell reaction. If ammonia is interfering with some step in the synthesis of influenza virus, it may be concluded that this step is specific for influenza, and possibly NDV, since other viruses in the same cell line are not affected.

The marginal inhibition of influenza virus by NH_4Cl sometimes observed in the intact chick embryo and the definite suppression observed in suspended allantoic membrane are of interest. Although rather high concentrations are required in the intact embryonated egg, and Fauconnier(5) has attributed this

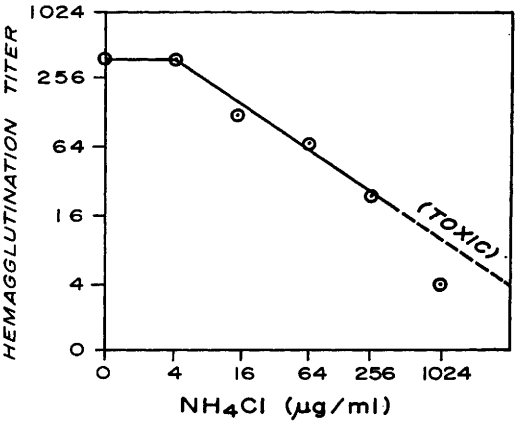

FIG. 6. Inhibition of influenza virus replication in suspended chorioallantoic membranes 48 hr after infection.

effect to a change in pH, it is conceivable that the tissue culture and the intact embryo results are related. Further studies in both systems may yield new information concerning the mechanism of action of the ammonia inhibition.

Summary. The antiviral effect of ammonium ions, previously shown in dog kidney cells against influenza virus, has been reexamined with 10 viruses in 10 tissue culture cell lines. Only influenza virus appeared to be affected by the inhibitor, which functioned in a number of cell systems. The effect was shown to be independent of virus inoculum size and therefore apparently on the host cell. The inhibition was demonstrated in suspended allantoic membrane cultures, and a marginal effect was also observed in intact chick embryos.

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Growth Characteristics of Ascitic Tumor Cells in the Heparinized Peritoneal Cavity of the Mouse.* (26771)

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Six years ago, at a conference on ascites tumors(1), we mentioned the occurrence of fluctuations in the amount of serous fluid and in its cellular content in mice bearing ascites tumor and given heparin intraperitoneally. Recently, we attempted to study the mechanism of this phenomenon by varying the method of administration, specifically by injecting heparin to some groups of animals at various intervals before tumor inoculation and to other groups after inoculation. In each experiment, the amount of ascitic fluid, its concentration of tumor cells, frequency of mitoses in these cells and extent of their implantation into the peritoneum were estimated, at requisite intervals (3 or 7 days) after inoculation. The results are reported and tentatively interpreted below.

Materials and methods. a) *Mouse and tumor strains.* Swiss Albino mice (Albino Farms, Red Bank, N. J.) of 24-27 g weight, mostly females, were used. As female mice with ascites tumors show a uniform and distinctive pattern of periuterine implant growth(1), they were preferred for our purposes. Krebs-2 ascites tumor was carried in our laboratory in serial intraperitoneal transfers. b) *Experimental technics.* Technics of tumor inoculation with requisite numbers of ascitic cells, of tumor cell counts, etc., have been described(2,3,4,5). Doses of approximately 10^5 tumor cells from ascitic fluid diluted (with .85% solution of NaCl) to a volume of .5 ml were injected intraperitoneally into lower *left* quadrant of the abdomen; the fluid subsequently accumulated in these mice was withdrawn by puncturing the *right* lower abdominal quadrant, thus avoiding trauma to granulating tissue and abundant bleeding (in heparinized animals) at sites of inoculation and of heparin injections (upper left quadrant). c) *Injections of heparin.* A standardized and commercially available solution of heparin (Panheparine Abbott) was used in our investigation. This solution, containing 1000 units (1 mg) per ml was diluted 1:20 with saline solution immediately before injections. Doses of .5 ml or 1.0 ml (25 to 50 units) were given before inoculation, but

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