Mr. John Blize, and Miss Jacqueline Ashby is greatly appreciated. We thank Dr. H. A. Wenner for advice and encouragement.

1. Lennette, E. H., Horsfall, F. L., Jr., *J. Exp. Med.*, 1941, v73, 581.
2. Henle, W., Wiener, M., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 176.
3. Hoyle, L., *J. Hyg.*, 1945, v44, 170.
4. Lief, F. S., Fabiyi, A., Henle, W., *J. Immun.*, 1958, v80, 53.
5. Fabiyi, A., Lief, F. S., Henle, W., *J. Immun.*, 1958, v81, 467.
6. Lief, F. S., Henle, W., *Bull. WHO,* 1959, v20, 411.
7. Lief, F. S., *J. Immun.*, 1963, v90, 172.
8. Henle, W., Lief, F. S., Fabiyi, A., *Lancet,* 1958, v1, 814.
9. Lief, F. S., Ostapiak, M., Fabiyi, A., Henle, W., *J. Immun.*, 1958, v81, 478.
10. Dubes, G. R., Chapin, M., *J. Gen. Microbiol.*, 1958, v18, 320.
11. Fabiyi, A., Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 81.
12. Hilleman, M. R., Mason, R. P., Buescher, E. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 829.

Received October 7, 1963. P.S.E.B.M., 1963, v114.

Influence of Acid Polysaccharides on Plaque Formation by Influenza A2 and B Viruses. (28806)

K. K. TAKEMOTO AND P. FABISCH (Introduced by S. Baron)
U. S. Department of HEW, Public Health Service, Nat. Inst. Health, National Institute of Allergy and Infectious Diseases, Laboratory of Biology of Viruses, Bethesda, Md.

Although a number of investigators have reported plaque production by certain strains of influenza viruses(1-6) plaque assays with these viruses in general have not been very successful. Simpson and Hirst(5) for example, examined a number of influenza A and B strains for ability to form plaques and most of these were either negative or formed faint plaques. It was recently reported from this laboratory(7) that plaque development and morphology for EMC virus was markedly influenced by the sulfated polysaccharide which is an inherent component of the agar used in most procedures involving the plaque technique. It has subsequently been shown that this polyanion is active against a number of different animal viruses(8,9,10). The studies reported here were undertaken to determine the influence of sulfated polysaccharides on plaque production by a strain of influenza A2 and a recently isolated B strain, utilizing the procedure for removal of the inhibitor with polycations(11).

Materials and methods. Viruses. The influenza A2 and B strains used in this study were isolated by Dr. Robert M. Chanock in rhesus monkey kidney cultures (MK) and were received as the third passage in these cells, without cultivation in other hosts. Up to 4 additional passages were made in this laboratory in MK cultures and these virus stocks were used throughout these experiments. Virus titers ranged from $10^{5.5}$ to 10^{7} pfu/ml when plated under conditions described below.

Cell cultures and media. MK cultures were prepared by a modification of Youngner's trypsinization procedure(12) and grown in 60 mm Petri plates in a medium of Earle's salt solution, 5% fetal bovine serum, and 0.5% lactalbumin hydrolysate. The cultures were grown in a humidified incubator containing 4% CO_2. The overlay medium for plaque assays consisted of the following: 0.1% yeast extract (Difco), 0.1% bovine albumin (Armour Pharmaceutical Co.), Earle's salt solution, 1% fetal bovine serum, and 0.9% Noble agar (Difco). DEAE-dextran (Pharmacia, Uppsala, Sweden) was dissolved in sterile distilled water at a concentration of 10 mg/ml and the desired concentrations were added to melted 1.8% agar at 44°C. An equal volume of 2 × concentrated nutrient medium was then added to the agar solution prior to pouring into inoculated plates. For staining of cultures, the same

TABLE I. Effect of DEAE-Dextran on Influenza A2 and B Plaque Formation.

Virus	DEAE-dextran (mg/ml agar)	Plaque count*
Influenza A2	0	7
	.3	23
	.6	28
	1.0	53
Influenza B	0	22
	.3	28
	.6	31
	1.0	63

* Avg of 2 plates after 8 days' incubation.

overlay medium (without DEAE-dextran) containing 1/40,000 neutral red was added on the 5th day.

Results. Effect of DEAE-dextran on influenza A2 and B plaque formation. Attempts to demonstrate plaque formation with both strains of influenza viruses without addition of DEAE-dextran resulted in highly variable and inconsistent results. After incubation periods of 6 or more days, faint, diffuse plaques could be observed when plates were held up to a light source. Addition of DEAE-dextran to the agar at varying concentrations resulted in more rapid and clearer plaque development as well as an increase in counts. These results are summarized in Table I. An optimal DEAE-dextran concentration of 1.0 mg/ml agar was necessary to achieve the highest counts as well as clearest plaques, and higher concentrations of DEAE-dextran did not improve plaque morphology or increase the number of plaques. A difference in type of plaques was noted with the 2 viruses. The B strain produced clear plaques with completely lysed cells within the plaque area, while plaques of the A2 strain were frequently turbid due to a mixture of living and dead cells. A slight difference in the diameter of the plaques was also noted, the A2 strain usually averaging 4-6 mm at 6 days, while the B strain measured from 6-8 mm. Both are illustrated in Fig. 1. DEAE-dextran was used at a concentration of 1 mg/ml agar in all the experiments described below where plaque assays were employed.

Effect of sulfated polysaccharides on hemagglutination and infectivity. The experiments described above clearly showed that the sulfated polysaccharide influenced the growth of both viruses and therefore their plaque development. Attempts were made to determine the mechanism involved in inhibition of viral growth. In previous studies with EMC virus(7), it was found that the acid polysaccharide obtained from agar inhibited the hemagglutination of sheep erythrocytes by EMC virus. Sulfated agar polysaccharide was prepared as previously described(7) and hemagglutination-inhibition tests were performed as follows: serial 2-fold dilutions of virus were made in phosphate buffered saline (pH 7.2) to which an equal volume of 0.5% guinea pig erythrocytes suspended in 100 μg/ml of the agar polysaccharide was added. The test was read after 90 minutes' incubation at room temperature. With both strains of influenza viruses, the hemagglutination reaction was unaffected by the sulfated polysaccharide, and the hemagglutination titer was the same as that of controls without the inhibitor.

To test whether there was direct inactivation of virus by the sulfated polysaccharide, between 10^5 to 10^6 pfu of each virus was mixed with 2 mg/ml of inhibitor dissolved in phosphate buffered saline, pH 7.2. Controls consisted of the same concentration of virus mixed with buffer. After 1 hour incubation at room temperature, the mixtures were diluted and assayed for infectivity. The infectivity of both viruses mixed with the inhibitor remained the same as the controls. These experiments indicated that the virus was not irreversibly inactivated by the inhibitor.

Inhibition of adsorption of virus to MK

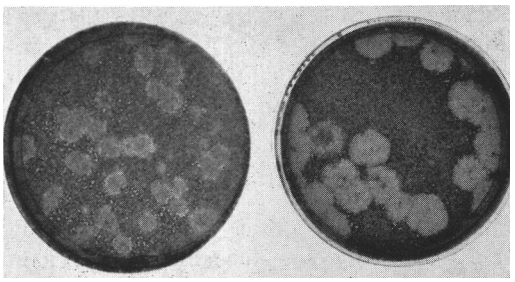

FIG. 1. Diffuse plaques of influenza A2 (left) and influenza B (right). DEAE-dextran added at 1 mg/ml agar. Photographed at 9 days.

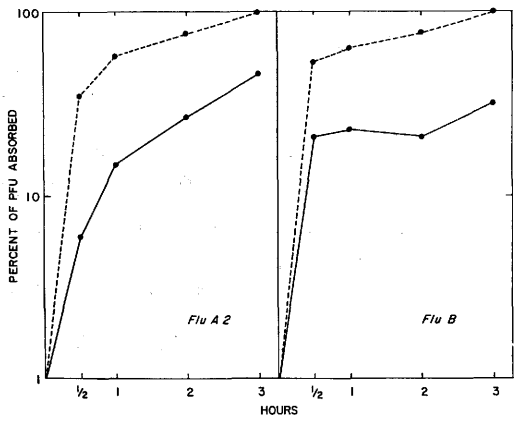

FIG. 2. Inhibition of adsorption of influenza A2 and B viruses by sulfated agar polysaccharide. ●——● = adsorption in presence of 80 μg inhibitor. ●-----● = normal adsorption curve without inhibitor.

cells. Experiments to determine the effect of the sulfated polysaccharide on adsorption of virus to cells was conducted in the following manner: MK cultures in 60 mm petri plates were inoculated with a mixture of approximately 50 pfu of virus and 80 μg of the inhibitor. Control plates received a mixture of the same amount of virus and phosphate buffered saline, pH 7.2. At intervals up to 4 hours, 2 plates from each group were washed 3 times to remove unadsorbed virus and inhibitor, then overlay medium was added. Plaques were counted on the 6th day and the results are shown in Fig. 2. The adsorption of both the influenza A2 and B viruses was inhibited by the sulfated polysaccharide, the degree of inhibition at 3 hours being greater with the B strain (67%) than with the A2 strain (55%).

Discussion. In view of previous reports on inhibition of influenza viruses by acidic polymers(13,14), it is not surprising that the sulfated agar polysaccharide would influence plaque formation by these viruses. The studies by Gerber *et al*(14) are especially pertinent here, since these workers found a protective effect against influenza B and mumps virus in embryonated eggs treated with extracts from *Gelidium cartilagenium,* the usual source of commercial agar, as well as from carrageenan. From their extraction procedures, it appears likely that their active material resided in the sulfated polysaccharide. The sulfate content of carrageenan, for example, has been reported to be between 23 to 33%(15,16). In the experiments of Gerber *et al,* no effect was noted with 2 influenza A strains as well as with Newcastle disease virus. However, their criterion of effectiveness depended on either complete survival of the infected embryos or an increase in survival times.

In contrast to previously described mucopolysaccharide or mucoprotein inhibitors of influenza viruses, no inhibition of hemagglutination was observed nor was infectivity affected by the sulfated polysaccharide. These results are similar to those found by Gerber *et al*(14). Whether interference with adsorption is the sole mechanism of inhibition remains to be determined. In addition, it should be stated that the inhibition of adsorption could have been due to an interaction of the inhibitor with either cell receptors or virus.

Whether the observations reported here are applicable to other strains of influenza or to other myxoviruses remain to be determined. Strains which have already been reported to produce plaques in untreated agar overlay may be resistant to the effects of the inhibitor. It is also conceivable that inhibitor-resistant mutants may be developed by growing the virus in the presence of inhibitor; development of resistance to agar polysaccharide by a strain of influenza B virus has been reported by Gerber and Adams(17).

Summary. 1. The inhibitory effect of the sulfated agar polysaccharide on strains of influenza A2 and B viruses was investigated. Complexing of the inhibitor from agar through the use of DEAE-dextran resulted in an improvement in plaque development as well as an increase in number of plaques formed. An optimum DEAE-dextran concentration of 1.0 mg/ml agar was found. 2. The inhibitor did not inactivate virus and had no effect on the hemagglutinating activity of both viruses, but it did interfere with virus adsorption to MK monolayers.

1. Ledinko, N., *Nature,* 1955, v175, 999.
2. Granoff, A., *Virology,* 1955, v1, 252.

3. Henry, C., Youngner, J. S., *J. Immunol.*, 1956, v78, 273.
4. Wright, B. S., Sagik, B. P., *Virology*, 1958, v5, 573.
5. Simpson, R. W., Hirst, G. K., *ibid.*, 1961, v15, 436.
6. Choppin, P. W., *ibid.*, 1962, v18, 332.
7. Takemoto, K. K., Liebhaber, H., *ibid.*, 1961, v14, 456.
8. ———, *Fed. Proc.*, 1962, v21, 461.
9. Choppin, P. W., Eggers, H. J., *Virology*, 1962, v18, 470.
10. Schulze, I. T., Schlesinger, R. W., *ibid.*, 1963, v19, 49.
11. Liebhaber, H., Takemoto, K. K., *ibid.*, 1961, v14, 502.
12. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 203.
13. Heymann, H., Gulick, Z. R., De Boer, C. J., De Stevens, G., Mayer, R. I., *Arch. Biochem. Biophys.*, 1958, v73, 366.
14. Gerber, P., Dutcher, J. D., Adams, E. V., Sherman, J. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 590.
15. Smith, D. B., Cook, W. N., *Arch. Biochem. Biophys.*, 1953, v45, 232.
16. O'Neill, A. N., *J. Am. Chem. Soc.*, 1955, v77, 6324.
17. Gerber, P., Adams, E., *Science*, 1958, v128, 1571.

Received October 7, 1963. P.S.E.B.M., 1963, 114.

Cytopathic Effects of Duck Hepatitis Virus in Duck Embryo Kidney Cell Cultures.* (28807)

J. E. FITZGERALD, L. E. HANSON AND M. WINGARD
(Introduced by C. A. Brandly)
Department of Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana

Duck hepatitis has been the subject of periodic investigations since it was first described by Levine and Fabricant(1). The virus of duck hepatitis (DHV) has been successfully propagated in tissue cultures but cytopathic effects (CPE) associated with its multiplication in tissue cultures have not been described(2-4). This report describes the cellular alterations associated with the growth of DHV in duck embryo kidney (DEK) cell cultures.

Materials and methods. Virus. Duck hepatitis virus strain R85952(5) which had undergone 18 serial passages in embryonating chicken eggs, 16 passages in chicken embryo liver cells, 4 passages in duck embryo liver cells and 24 passages in duck embryo kidney cells was used.

A twenty-fourth DEK passage cell culture medium with a titer of $10^{5.0}$ chicken embryo infective doses 50 per 0.1 ml served as the source of virus.

Tissue culture. Primary monolayer cultures were obtained by placing trypsin-dispersed DEK cells in 15 × 175 mm rubber stoppered glass tubes containing 11 × 22 mm glass cover slips. Tubes were incubaated in a slanted position at 37°C. The growth medium consisted of 79.5% Hanks' balanced salt solution, 0.5% lactalbumin hydrolysate, 20% sheep serum, and 100 units of penicillin and 100 μg of streptomycin per ml. The same medium except containing 5% instead of 20% sheep serum was used for maintenance of the cultures. When a confluent cell sheet had formed, usually in 3 to 4 days, cultures were inoculated with virus.

Cytologic techniques. For determination of cellular alterations in infected monolayer cultures, cover slips from tubes containing infective and noninfective fluids were removed at 4, 8, 12, 16, 24, 36, 48, 72 and 96 hours after inoculation and were fixed in absolute methanol. Cells on cover slips were stained by the May-Grünwald-Giemsa technique and examined microscopically for evidence of CPE.

Results. Uninoculated control cultures con-

* This project supported by grants from NIH and Illinois Agri. Exp. Station.