10. Vaux St. Cyr, C. de, *7th Colloquium Sint Jans Hospital-Brügge,* 1959.
11. Bezkorovainy, A., Winzler, R. J., *Biochim. Biophys. Acta,* 1961, v49, 559.
12. Warren, L., *J. Biol. Chem.,* 1959, v235, 1971.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J., *ibid.,* 1951, v193, 265.
14. Mayron, L. W., Robert, B., Winzler, R. J., Rafelson, M. E., Jr., *Arch. Biochem. Biophys.,* 1961, v92, 475.
15. Yamashima, I., *Acta Chem. Scand.,* 1956, v10, 1666.

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Inhibition of Arbor Viruses (Group A) by a Protein-Like Constituent of a Corynebacterium.* (27791)

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During the course of experiments in which Sindbis virus was being cultivated in primary human amnion cell cultures(1) it was observed that the expected cytopathic effects of this agent failed to occur in one preparation. This culture proved to be contaminated with a bacterium which was subsequently classified by Miss Elizabeth King of the U. S. Public Health Service Communicable Disease Center as a corynebacterium. The species, however, could not be identified. It was demonstrated that the bacterium regularly inhibited cytopathogenicity of Sindbis virus and the experiments described here were undertaken to determine the nature of the bacterial factor responsible for the viral inhibition and its mode of action.

Methods. It was noted that the bacterium grew better in bovine amniotic fluid (BAF) tissue culture medium(2) than in bacteriologic media such as beef broth and thioglycolate. BAF medium was therefore used as routine to propagate the organism. The original contaminated fluid from the tissue culture was diluted with 25 ml of BAF medium and stored at 6°C. In subsequent experiments 0.5-ml aliquots of this suspension which contained approximately 20,000 viable bacteria per ml were inoculated into 100-ml aliquots of BAF medium which were then incubated for 3 days at 37°C. Total counts at this time gave 50,000,000 viable organisms per ml of culture. The bacteria were removed by centrifugation at 800 rpm for 10 minutes, the supernate (medium supernate, MS) was passed through a Millipore filter (pore size: 0.45 mμ) and then assayed for viral inhibitory capacity. Bacteria were suspended in 5 ml of BAF medium and then sonically disrupted for 30 minutes. The suspension was frozen and thawed 3 times, and again sonicated for 30 minutes. The preparation was then filtered through a Millipore filter. The filtrate (bacterial concentrate, BC) was assayed for inhibitory capacity.

Except as otherwise noted, assay of bacterial inhibitory factor (BIF) was carried out in 3-4-week-old primary cultures of human amnion cells employing 100 $TCID_{50}$ of Sindbis virus as the test dose. In these assays materials to be tested for inhibitory capacity were diluted serially by a factor of 2 in BAF medium and 1-ml aliquots were added to each of 1-3 amnion cell cultures immediately before inoculation of virus.

Titer of a given preparation of BIF is expressed as the highest dilution which completely suppressed CPE (Cytopathic Effect) in human amnion cells on the day that in virus control cultures approximately 50% of the cells exhibited CPE. Unless specifically stated all preparations of BIF used were BC.

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Results. Association of BIF with bacterial cells. It was found that the titer of different lots of BC ranged from 1:64-1:512. In contrast MS prepared from the same bacterial cultures inhibited in only low dilutions (1:4 to 1:8). Thus, BIF appears to be closely associated with the bacterial cell itself.

Physical properties. BIF activity was not decreased by dialysis in a cellophane bag against BAF medium for 24 hours, heating at 37°C for 3 hours or heating at 56°C for 1 hour. It was completely destroyed by dialysis against a glycine hydrochloric acid buffer at pH 2.3 and heating in boiling water for 5 minutes.

Effect of trypsin and nucleases. BIF was inactivated by trypsin under the following conditions. BIF (titer 1:256) was diluted 1:10 in BAF medium. An equal volume of 0.25% trypsin‖ was added and the mixture kept at 37°C for 3 hours. The effects of DNAase and RNAase§ were also studied. A preparation of BIF (titer 1:64) was diluted 1:16 with BAF medium. DNAase and RNAase were respectively added to an aliquot of BIF to give a concentration of 1.0 μg/ml of the enzyme and the mixture was incubated at 37°C for 3 hours. Serial 2-fold dilutions of each preparation were then prepared and tested in amnion cell cultures. Results indicated a delayed and partial reduction in inhibitory capacity. Thus on the day on which the virus control became positive inhibition was apparent. Later, however, CPE became evident which appeared more rapidly and was more extensive in the highest concentrations of the inhibitor. Because of these equivocal reactions obtained with the nucleases, BIF was treated with an equal volume of 1% protamine solution and allowed to stand for 15 minutes at room temperature. The mixture was then tested for inhibitory activity which was found to be undiminished. This result together with the failure of the nucleases to rapidly destroy BIF would seem to indicate that its activity is not attributable to a nucleic acid component. Accordingly, taken as a whole all the properties of BIF so far defined strongly suggest that it belongs among the proteins.

Inhibition of viral cytopathogenicity and multiplication. As stated above, BIF was active in suppression of viral cytopathogenicity in dilutions as high as 1:512 when readings were made at the time when approximately 50% of the cells in the control cultures were affected. Subsequently, the endpoint of inhibitory effect was reduced approximately 4-fold. Experiments were carried out in which the infectivity of the fluids of cultures manifesting no CPE was assayed several days after treatment with BIF and inoculation of virus and in infected preparations without BIF. Infectivity titers of fluids from cultures without BIF in 2 such experiments were $10^{5.3}$ and $10^{5.8}$; in BIF treated cultures no virus was demonstrated. Thus it was shown that BIF interfered with viral infectivity as well as cytopathogenicity. In other experiments in which varying concentrations of virus were employed it was demonstrated that BIF interfered with cytopathogenicity when as much as 10^5 $TCID_{50}$ were added.

Mode of action of BIF. That the action of BIF did not consist in a generalized toxic effect on the cells which might render them incapable of supporting replication of all viruses was shown by results of the following experiment. BIF was titrated, employing Sindbis virus as the test agent. After 2 weeks CPE was not observed in the cultures that had received dilutions of BIF ranging from 1:4-1:64. At this time after replacing the BIF-containing fluid with BAF medium 100 TCD_{50} poliovirus Type I was added to those cultures that had been previously treated with 1:4 and 1:8 dilutions of BIF. Multiplication of poliovirus occurred in the usual manner after 1-2 days as indicated by appearance of typical cytopathic changes.

In addition to this evidence for lack of profound toxic effect of BIF, cells treated with the inhibitor in concentrations ranging from 1:16 to the endpoint exhibited no morphologically recognizable signs of injury when examined either in the natural state or in

‖Crystalline 2x salt free trypsin obtained from Nutritional Biochemicals Corp., Cleveland.

§ DNAase 1x crystalline obtained from Nutritional Biochemicals Corp. RNAase crystalline salt free obtained from Worthington Biochemical Co.

stained preparations. Moreover cells treated with dilutions of BIF ranging from 1:4 to the endpoint although exhibiting rounding and increasing granularity continued to metabolize in a normal manner as indicated by continuing acid production comparable to that of untreated control cultures.

Experiments were performed to determine whether the inhibitor acted by (a) directly inactivating the virus, (b) interfering with penetration of virus into the cell, or (c) by interfering with viral replication. (a) A mixture consisting of equal parts of BIF (diluted 1:10 in BAF medium) and a suspension of virus (100 TCD_{50}/0.1 ml) was allowed to stand at room temperature for 2 hours. Aliquots (0.2 ml) of the mixture were added to amnion cell cultures. After incubation for 2 hours at 37°C cells were washed 5 times with medium 199. BAF medium was then added and cultures incubated in the usual manner. Inhibition of CPE was not observed in cultures exposed to the mixture when compared with others without inhibitor that were inoculated with an equivalent dose of virus. BIF, therefore, does not appear to act directly upon the virus.

(b) Results of the following experiments indicate that penetration is not affected by the presence of BIF. To each of 9 amnion cell cultures from which medium was removed 1 ml of BIF (titer 1:256) diluted 1:30 in BAF medium was added and 100 TCD_{50} Sindbis virus. Nine control cultures without BIF were also inoculated with this dose of virus. Cultures were incubated at 37°C for 2 hours and washed 5× with 5-ml aliquots of medium 199. Pooled fluids of the final washing were tested for virus. None was demonstrated in washings from BIF treated cultures. In one of the washings from the 9 cultures without BIF virus was found. BAF medium was added to all cultures which then were incubated at 37°C. Subsequently no inhibition of CPE was observed in those treated with BIF as compared with cultures exposed only to virus. Further evidence that BIF does not interfere with penetration was obtained as follows. To each of 3 human amnion cell cultures aliquots of BIF (titer 1:256) diluted 1:30 and 10^5 TCD_{50} of virus were added. Three cultures without BIF were similarly inoculated. All preparations were incubated at 37°C for 2 hours. Each culture was then washed 5× with 5 ml medium 199. Cells were suspended with trypsin as described by Ho and Enders(3). Separate pools of cells from experimental and control tubes were prepared, and each was treated with homologous antibody diluted 1:20. Cells were then washed 2× with BAF medium and resuspended in 1 ml of BAF medium. No virus was demonstrated in the 2nd washings from either experimental or control suspensions. Cells in each preparation were counted and diluted in series by a factor of 10. Aliquots of each dilution were added to 3 human amnion cell cultures which were incubated at 37°C. CPE endpoints were recorded on the 4th day. Calculating the number of infected cells according to the method of Ho and Enders no significant difference was found between BIF treated and control cells; 1.3% of control cells and 1.2% of BIF treated cells were estimated to be infected.

In other experiments to determine whether BIF might inhibit when added *after* the virus it was shown that this factor was effective in suppressing CPE in cultures infected 1 hour previously with 100 TCD_{50} of virus. BIF, however, was ineffective when added 2 hours after the virus. Pfefferkorn(4) has found that by 1 hour following exposure of primary human amnion cells to Sindbis virus at least 70% was adsorbed. Although he employed somewhat different conditions (bottle cultures treated with a small volume of inoculum) his results suggest that in our experiments a significant proportion of virus was absorbed by 1 hour. Accordingly, the fact that BIF when added 1 hour after the virus inhibits CPE may be regarded as providing further evidence that BIF does not interfere with adsorption and penetration.

(c) The possibility that BIF prevents release of newly formed virus has also been investigated. Each of 3 amnion cell cultures from which medium was removed received 1 ml of BIF (titer 1:160) diluted 1:80 in BAF medium and 100 TCD_{50} of Sindbis virus. Three control cultures without BIF were also inoculated with the same dose of virus. Three

days after the inoculation of virus the control cultures showed the cytopathic effects of this virus in 50% of the cells while these changes were not present in the BIF treated cultures. At this time the medium in the BIF treated tubes was harvested and pooled. No virus was detected when this medium was inoculated into amnion cell cultures. The cells in the BIF treated cultures were washed 5× with 5-ml aliquots of medium 199 before being scraped down from the glass of the tubes and pooled in a total of 1 ml of BAF medium. This pool of cells was frozen and thawed 3× to release intracellular virus before it was inoculated into amnion cultures. No virus was detected.

Activity of BIF in chick embryo cell systems. Complete interference by BIF with Sindbis virus CPE and replication was also demonstrated in trypsinized cultures of chick embryo cells prepared according to the procedure of Katz and his associates(5). Inhibitory titers employing a viral test dose of 100 TCD_{50} for this system were comparable to those recorded in human amnion cell cultures.

Specificity of BIF. Capacity of BIF to inhibit agents unrelated to Sindbis virus was studied. It was found that in a dilution of 1:4 a preparation of BIF (titer *vs* Sindbis virus, 1:256) in human amnion cell cultures failed to suppress CPE of ECHO 11, vaccinia, Coxsackie B5 and Herpes simplex viruses. Another preparation (titer *vs* Sindbis virus, 1:512) when diluted 1:50 exhibited no inhibition of CPE when tested with poliovirus I, adenovirus 2 and ECHO 9.

In view of the failure of BIF to inhibit these unrelated viruses it became of interest to determine whether replication of other members of the Arbor group is suppressed by the factor. So far only Chikungunya virus (Group A) has been studied. In both human amnion and chick cultures BIF completely inhibited its cytopathogenicity. Experiments with other representatives that include members of Group B are in progress.

Discussion. The experimental data seem to eliminate the possibilities that BIF acts by inactivating the virus directly, by preventing penetration of virus or interfering with release of the agent. By exclusion, then, it may be tentatively concluded that BIF acts to prevent viral replication within the cell.

Among numerous substances that have been found to limit viral multiplication, those derived from bacterial sources consist, to our knowledge, only of single or conjugated polysaccharides. Horsfall and his coworkers(6) showed that the capsular polysaccharides of *K. pneumoniae* B and streptococcus MG as well as the somatic antigen of *Sh. paradysenteriae,* Type V inhibited proliferation *in vivo* of mouse pneumonia virus (PVM). Inhibition of mumps virus in chick embryos was also described by these workers(7). Others have reported limitation of pathological effects or survival following inoculation of materials derived from various bacteria(8,9,10) into animals infected with different viruses. No evidence that these substances interfered with viral replication was presented.

Our findings appear to be significant not only because they offer another instance of a factor present in a bacterium that inhibits viral multiplication, but also because this inhibitor appears to be a protein. Of interest, too, is the specificity of the inhibition.

With the exception of interferon, none of the viral inhibitors so far described are proteins. Many of them are of small molecular size and known chemical configuration. Although both BIF and interferon appear to be proteins they cannot be identical. The activity of BIF is destroyed in acid solutions whereas under these conditions interferon is unaffected. Interferon reduces viral multiplication whereas BIF may suppress it completely.

The sharply specific action of BIF in respect to the Arbor viruses is noteworthy since it suggests that certain metabolic pathways may be required for synthesis of some agents which are not essential in the replication of others. From the standpoint of the possible future development of effective antiviral compounds these observations encourage the search for substances which, like BIF, may specifically interfere with the multiplication of a given virus by affecting only a limited number of cellular processes that are essential

for replication of the agent but not crucial in the function of the cell.

Summary. A factor (BIF) has been demonstrated in a corynebacterium of unidentified species which interferes with the multiplication of 2 Group A Arbor viruses, Sindbis and Chikungunya, in cultures of human amnion and chick embryo cells. Cytopathogenicity of seven unrelated viruses was not inhibited by the factor. Evidence is presented that interference of the Arbor viruses depends upon inhibition of replication after virus has entered the cell. Evidence suggesting that the factor is a protein is presented.

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1. Milovanovic, M. V., Enders, J. F., Mitus, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 120.
2. Enders, J. F., *ibid.*, 1953, v82, 100.
3. Ho, M., Enders, J. F., *Virology,* 1959, v9, 446.
4. Personal communication from Dr. E. R. Pfefferkorn, Jr.
5. Katz, S. L., Milovanovic, M. V., Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 23.
6. Horsfall, F. L., McCarty, M., *J. Exp. Med.,* 1947, v85, 623.
7. Ginsberg, H. S., Goebel, W. F., Horsfall, F. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 99.
8. Groupé, V., Pugh, L. H., Levine, A. S., *ibid.,* 1952, v80, 710.
9. Cutting, W., Furst, A., Read, D., Grant, D., Cords, H., Megna, J., Butterworth, E., *Antibiot. and Chemother.,* 1960, v10, 623.
10. Florman, A. L., Airvender, E., Zepp, H., Scoma, J. L., Hodes, H. L., Abst., *Am. Pediatric Soc.,* 1962, 17.

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Effect of Periodate on Cell Wall Antigens of Streptococci.* (27792)

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The streptococci are divided into 17 serological groups on the basis of specific carbohydrate antigens present, with the exception of group D, in the cell wall(1). In the case of group A, the antigen is known to be composed of rhamnose and N-acetyl glucosamine (2), and in group C, rhamnose and N-acetyl galactosamine(3). Among the other groups of streptococci, however, the composition of the antigen appears to be more complex(1). In group K for example, glucose, galactose, glucosamine and galactosamine are present; rhamnose may or may not be present depending upon the strain(4). The same compounds have been found in the group antigen of group O and in the cell wall carbohydrate of a serologically unclassified streptococcus(4).

The sugars and amino sugars given above plus, in a few cases, arabinose and/or mannose, are most likely components of the streptococcal cell wall grouping antigens of all the serological groups(1). In addition, a number of type antigens of the streptococci are located in the cell wall and are carbohydrate in nature. As a consequence, many of the known grouping and typing antigens of the streptococci, and many others yet to be detected, depend for their specificity upon chemical structures composed of these substances.

To obtain further information on the properties of these cell wall antigens, a study has been made of the effect of periodate oxidation on the antigens as determined by ability of the antigen to combine with antibody. The results indicate that resistance or sensitivity to oxidation by periodate are properties which might aid in identification of antigenic determinants, as well as providing information on

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