steps in the utilization of mannitol, were linked genetically into the third group.

1. Murphey, W. H., Rosenblum, E. D., *J. Bact.*, 1964, in press.
2. Murphey, W. H., *Ph.D. Dissertation,* Univ. of Texas Southwestern Medical School, 1964.
3. Wolff, J. B., Kaplan, N. O., *J. Biol. Chem.*, 1956, v218, 849.
4. Marmur, J., Hotchkiss, R. D., *ibid.*, 1955, v214, 383.
5. Arcus, A. C., Edson, N. L., *Biochem. J.*, 1956, v64, 385.
6. Marcus, L., Marr, A. G., *J. Bact.*, 1961, v82, 224.
7. Egan, J. B., Morse, M. L., *Bact. Proc.*, 1962, 42.
8. Garen, A., Zinder, N. D., *Virology,* 1955, v1, 347.
9. Hotchkiss, R. D., Marmur, J., *Proc. Nat. Acad. Sci. U. S.,* 1954, v40, 55.
10. Hayes, W., *Symp. Soc. Gen. Microbiol.,* 1960, v10, 12.
11. Morse, M. L., *Proc. Nat. Acad. Sci. U. S.,* 1959, v45, 722.

Received March 17, 1964. P.S.E.B.M., 1964, v116.

Studies on Viral Inhibitors of Biological Origin. I. Inhibition of Viral Replication by Protein-Like Constituents of Bacteria.* (29303)

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Experimental evidence has been presented demonstrating that a factor derived from a Corynebacterium of unidentified species interferes with the intracellular stages of replication of 2 Group A arboviruses, Sindbis and chickungunya(1). This bacterial inhibitory factor (henceforth abbreviated BIF) did not interfere with the cytopathogenicity of 7 unrelated viruses. Further experiments have been performed concerning the nature of the substance and the spectrum of its antiviral activity. In view of the very narrow spectrum of antiviral activity exhibited by the original Corynebacterium, inhibitory substances against other viruses were sought in other bacteria.

Materials and methods. In addition to the original unidentified Corynebacterium, inhibitory factors were obtained from *Corynebacterium diphtheriae* (both a non-toxin producing strain and an intermedius), *Corynebacterium xerosis, Escherichia coli* (B, K12 and 0111) and *Salmonella typhimurium.* In all cases other than the original Corynebacterium, 100 ml aliquots of beef heart infusion medium were used for growing the bacteria. The fastidious growth requirements of the original organism have been described(1). Cultures were incubated at 37°C for 3 days prior to sedimenting the bacteria by centrifugation for 20 minutes at 3000 rpm. Bacteria were then resuspended in 5 ml of bovine amniotic fluid (BAF) tissue culture medium (2) and disrupted by sonication in addition to freezing and thawing as decribed previously. Following disruption, the bacterial suspensions were filtered through a millipore filter with a pore size of 0.45 μ. The resulting filtrates were assayed for inhibitory activity.

Except where otherwise noted all tests were carried out in cultures of primary human amnion cells using 100 $TCID_{50}$ of the virus as test dose. Serial 2-fold dilutions were made of the materials to be tested for inhibitory capacity, and 1 ml aliquots were added to 1-3 cultures immediately before inoculation of virus. The titer of a given inhibitory factor is expressed as the highest dilution which completely suppressed cytopathic effect (CPE) on the day that approximately 50% of the cells in viral control cultures exhibited CPE.

Treatment of inhibitory factors with enzymes (trypsin, DNAase, RNAase) dialysis, and heating were all performed in the manner described for the original BIF(1).

* These investigations were supported by grant from U. S. Public Health Service.

TABLE I. Inhibition of Various Viruses by Factors of Bacterial Origin.

Inhibitor factor	Virus tested: Sindbis	ECHO 11	ECHO 2	Vaccinia
Original corynebacterium	+	0	0	0
C. diphtheria	0	+	+	0
C. xerosis	0	+	0	0
C. creatinovorans	0	0	0	0
Coli K_{12}	0	0	0	+
Coli B	0	0	0	+
Coli 0111	0	0	0	+
S. typhimurium	0	0	0	+

+ = indicates inhibition.
0 = no inhibition.

Results. Spectrum of antiviral activity. Table I demonstrates the marked specificity of inhibitory action exhibited by each of the bacterial factors. Testing the original Corynebacterium BIF with an attenuated strain of WEE† revealed that both the cytopathogenicity and infectivity of this Group A arbovirus were inhibited in human amnion and chick embryo fibroblasts (CEF). This occurred to the same extent as has been reported for the 2 other Group A arboviruses inhibited by original BIF. Tests in CEF demonstrated that West Nile virus, a Group B arbovirus, was also inhibited by the original BIF. Examination of CPE revealed an inhibiting titer greater than 1:160. Thus, the original Corynebacterium BIF appears to inhibit both Group A and B arboviruses without affecting the replication of unrelated viruses.

Each factor when diluted higher than 1:5 inhibited only a single virus except for *C. diphtheriae* (both non-toxigenic strain and intermedius strain) which inhibited both ECHO 11 and ECHO 2. All of the inhibitors were tested against Sindbis, ECHO 2, ECHO 11, polio type 1, Herpes simplex, vaccinia and adenovirus type 2, and no other viral inhibition was demonstrable except for the coli BIF which inhibited adenovirus 2 and Herpes simplex in high concentration (1:5) and the inhibition of polio 1, adeno 2, and vaccinia virus by high concentration (1:5) of the *Corynebacterium diphtheriae* BIF. It is of interest that both adenovirus and Herpes simplex are DNA viruses as is vaccinia which, as noted below, is inhibited by the coli BIF in much greater dilutions. When the original Corynebacterium BIF was tested with measles virus, viral inhibition was not demonstrated. Neither the original Corynebacterium BIF nor coli K12 BIF prevented the lysis of staphylococcus and *E. coli* by their respective phages (staph 80, 81 - Coli T 2, 3, 4, 6 and 7).

As noted earlier, the inhibitory titer of a given BIF was designated to be that dilution of BIF which completely suppressed viral CPE on the day that 50% of the cells in the virus control tubes exhibited CPE. Titers of the original BIF against Sindbis virus ranged from 1:64 to 1:512 when the bacteria were grown at 37°C. However, when bacteria were grown at room temperature the BIF titers were in the range of 1:40,000 although the number of bacteria grown was no greater. The bacteria grown at room temperature had a mucoid appearance instead of the granular form which occurs with 37°C incubation. Different preparations of *E. coli* BIF ranged in titer from 1:80 to 1:640. The *S. typhimurium* BIF had a titer of 1:64. With observation of the cultures over a longer period the end point of inhibitory effect was reduced approximately 4-fold for the original Corynebacterium BIF and 8-fold for the coli and Salmonella BIFs. Treating cells with *E. coli* 0111 BIF for 24 hours prior to viral inoculation and then allowing the BIF to remain in contact with the cells did not result in an inhibiting titer greater than when the *E. coli* 0111 BIF was used at time of inoculation. When cells were pretreated with the original Corynebacterium BIF the titer was 4 times greater than that found with the usual method of using BIF at the time of viral inoculation. When the high concentration (1:10) of inhibitor used to treat cells for 24 hours was washed off and replaced with regular media prior to viral inoculation, both of these BIFs gave only transient viral inhibition.

The *E. coli* 0111 BIF gave effective inhibition of viral CPE when added 18 hours after viral inoculation while the original Corynebacterium BIF prevented CPE when added as long as 20 hours after viral inoculation.

† Supplied by Dr. Harold N. Johnson.

TABLE II Titration of Infectious Virus from Virus Controls and Treated Cultures.

BIF	Virus	Titer virus control $\log_{10}$/ 0.1 ml	Titer BIF $\log_{10}$/ 0.1 ml
Corynebacterium (original)	Sindbis	$10^{-5.8}$	No detectable virus
	WEE	$10^{-7.5}$	" " "
	Chickungunya	$10^{-5.5}$	" " "
	W. Nile	$10^{-6.8}$	" " "
E. coli K12	Vaccinia	$10^{-5.5}$	$10^{-3.5}$
B	"	$10^{-4.0}$	$10^{-2.0}$
0111	"	$10^{-3.5}$	No detectable virus
S. typhimurium	"	$10^{-4.0}$	$10^{-1.5}$

W. Nile + Chickungunya tests were done in chick embryo fibroblasts.
All others were tested in primary human amnion.
All samples obtained between 4 and 8 days after viral inoculation with viral control and comparable BIF aliquots simultaneously harvested.

It is of interest that the CPE exhibited by vaccinia virus in tubes treated with concentrations of coli BIF that delayed CPE but did not prevent it, differed markedly from that observed in the virus control. The virus control tubes showed both rounding and spindling of affected cells while the BIF treated cells exhibited only spindling.

Table II lists the infectivity titers of fluids from virus controls and from cultures treated with the BIFs which specifically inhibited the respective viruses. In all cases the virus controls had higher infectivity titers than the BIF treated cultures when they were simultaneously sampled. The BIF treated cultures which were used for back titration revealed no CPE. While in cultures with Corynebacterium BIF no infective virus was detected, in fluids from cultures treated with coli or typhimurium BIF, low infectivity titers suggestive of some viral replication were recorded in most instances.

Hemadsorption and hemagglutination tests were performed using goose red cells, but the method of Buckley(3) and Casals(4), to determine whether non-infectious Sindbis antigen might appear in BIF (original) treated cells that had been inoculated with Sindbis virus. Hemadsorption was demonstrated in virus control cultures but was absent in the BIF treated cultures. The fluid from virus control cultures caused hemagglutination in dilution up to 1:128 while none occurred in a 1:4 dilution of the fluid from the BIF treated cells. Thus it appears that BIF suppressed not only the formation of infectious virus but also that of incomplete Sindbis virus.

Physicochemical characteristics. The original Corynebacterium BIF has been shown to retain its activity in a dialysis bag, after being treated at 37°C for 3 hours, and 56°C for 1 hour. DNAase and RNAase did not inactivate the native BIF, whereas both trypsin and heating at 100°C for 5 minutes completely destroyed it. BIF from Coli 0111 has the same characteristics. The BIF preparations from *E. coli* B, *E. coli* K12 and *S. typhimurium* were also inactivated by trypsin. *E. coli* K12 BIF and *S. typhimurium* were tested and shown to remain in a dialysis bag and to be inactivated at pH 2.3.

After spinning at 35,000 rpm for 3 hours in a 40 head of the model L ultracentrifuge, the inhibitory activity of both the original Corynebacterium BIF and the *E. coli* 0111 BIF were concentrated in the bottom of the tube.

Mode of action. The BIFs could act either by direct inactivation of the respective viruses or by interfering with one of the stages of viral replication (*i.e.*, adsorption, penetration, uncoupling, synthesis, coupling, and release).

The original Corynebacterium BIF as well as the 3 Coli BIFs (Coli 0111, Coli B and Coli K12) were tested to evaluate the possibility of direct viral inactivation by the bacterial factors. For each BIF a dilution was chosen which was at least 4 times more concentrated than its titer as defined above. These BIF dilutions were each mixed with an equal volume of BAF media containing

100 $TCID_{50}$/0.1 ml of the appropriate virus. The mixtures were allowed to stand for 2 hours at room temperature prior to inoculation of 0.2 cc aliquots into amnion cell cultures. The cultures were then incubated at 37°C for 2 hours. Following incubation the cultures were washed 5 times with mixture 199 and then incubated in the usual manner. The simultaneous appearance of the same degree of CPE in control cultures which had been inoculated with 100 $TCID_{50}$ of virus alone and test cultures which had received the mixture of virus with the appropriate BIFs indicated that the BIFs tested do not directly inactivate the viruses they affect.

The experiment testing for direct inactivation of virus described above was also performed for the *C. diphtheriae* and *C. xerosis* preparations. These BIFs appear to cause some direct inactivation of ECHO 11 (both preparations) and ECHO 2 (*C. diphtheriae* alone) since the cultures inoculated with virus that had been allowed to stand with the individual BIFs exhibited CPE at a later time than control cultures inoculated with virus alone. Further studies concerning the mode of action of these two preparations are described elsewhere(5).

Experiments were designed to learn whether the original BIF and Coli 0111 BIF interfered with the stages of adsorption and penetration. Amnion cell cultures were treated with 1 ml aliquots of BIF in concentrations at least 4 times greater than the titers as defined above. These cultures were then immediately inoculated with 100 $TCID_{50}$ of the appropriate virus. Following 2 hours of incubation at 37°C the cultures were washed 10× with medium 199. The final washings were tested for virus which was found to be absent. The tubes received regular BAF media and were then incubated at 37°C in the usual manner. Subsequently no inhibition of CPE was noted in the cultures when compared to similarly washed control cultures inoculated with the same amount of virus, and not treated with BIF. The above experiment was repeated for Coli 0111 with the additional step of placing a 1:10 dilution of neutralizing antibody against vaccinia in the tubes after the 10 washings as a means of neutralizing any virus which was adsorbed by the cell surface. This was followed by an additional 10 washings with medium 199 and addition of BAF medium prior to incubation at 37°C. Again the BIF treated cultures showed CPE at the same time and to the same degree as viral control tubes which had not been treated with BIF but were otherwise similarly handled. The original Corynebacterium BIF was used to treat cells which were immediately inoculated with Sindbis virus, then incubated for 2 hours prior to being washed, trypsinized, and treated with antibody in the manner described by Ho and Enders(6). Following this, the number of infected cells was calculated by inoculation of serial 10-fold dilution of the cell suspension into primary amnion human cultures. Calculating according to the method of Ho and Enders, infection occurred in 1.2% of these cells and in 1.3% of a suspension of control cells treated in the same manner without the use of BIF.

Experiments were performed to detect whether the original Corynebacterial BIF and Coli 0111 BIF prevented cellular release of formed virus. Inoculation of 100 $TCID_{50}$ of the viruses (vaccinia and sindbis) were made both in cultures containing the appropriate BIF preparations and in control cultures containing only BAF media. The cultures were incubated at 37°C until CPE was evident in 50% of the cells in the viral control cultures. The BIF treated cultures did not exhibit CPE at this time and were washed 5× with medium 199. For each of the inhibitors the cells were scraped from 3 culture tubes that had been treated with BIF and inoculated with virus. The cells were also scraped from virus control tubes after they were washed in the same manner. Each pool of cells was then frozen and thawed 3× before being tested for virus by inoculation into primary amnion cultures. No virus was detected in the cells treated with the original BIF. In the test with Coli 0111 the cells from the virus control had a titer of $10^{3.3}$ while the BIF treated cells had a titer of only $10^{0.5}$. Thus BIF does not appear to interfere with viral release from cells.

Using the criteria of histologic appearance,

continued acid production, and capacity to support the growth of viruses not specifically inhibited, the cells in tissue culture showed no signs of toxicity when in contact with all but the lowest (1:5) dilutions of BIF which were effective in viral inhibition. Preliminary tests using the original Corynebacterium BIF in attempts to protect suckling mice from death due to Sindbis virus have not yielded conclusive results. Further tests will be conducted using other arboviruses in adult mice.

Discussion. It has been shown that each bacterial factor studied inhibits only certain viruses and not others. Evidence has also been presented indicating that the factors under consideration act by preventing viral replication within cells. It is possible, therefore, that the factors may act by specifically blocking certain celllular metabolic pathways which are necessary for the replication of some viruses and not others. Thus, the only viruses affected by blocking a given pathway would be those for which it was essential. Another possibility to be considered is blockade of a new enzyme system induced in cells by a given virus and essential for its replication.

Since the factors were able to inhibit viral replication without producing toxic changes in the cell cultures, it appears likely that the aspects of cellular metabolism affected by the inhibiting factors are not those which are essential for the continued viability of the cells.

The original Corynebacterium BIF inhibited only arboviruses and did not inhibit the other RNA viruses tested. Thus, it seems unlikely that this factor acts by depressing RNA synthesis.

The BIF formed by *E. coli* was most effective in inhibiting the replication of vaccinia, a DNA virus. It is of interest that 2 other DNA viruses were inhibited by high concentrations of this factor, Herpes simplex and adenovirus 2. In view of these findings, interference with DNA synthesis can be considered a possible mode of action for the Coli BIF. Further evidence of its interference with DNA synthesis is presented elsewhere, reporting measurements of cellular uptake of radioactive 5-iodo-2-deoxyuridine(7).

Summary. Viral inhibitory factors were isolated from several different bacterial species including representatives of both gram negative and positive groups. Two of the factors were found to prevent viral cytopathogenicity even when added as late as 18 hours after viral inoculation. Trypsin sensitivity indicates that these inhibitors are probably proteins. Evidence is presented that viral inhibition appears to occur during the intracellular stages of viral replication. Each factor has a sharply specific spectrum of antiviral activity. This specificity of action may reflect blocking of cellular metabolic pathways necessary only for the replication of the virus affected.

The authors are grateful to Dr. John Enders for advice and help in this work and to Dr. Fred Rosen for aid in performing the experiments with the T phages. The authors also wish to acknowledge the skillful technical assistance of Mrs. T. Palmer.

1. Carver, D. H., Naficy, K., PROC. SOC. EXP. BIOL. AND MED., 1962, v111, 356.
2. Enders, J. F., *ibid.*, 1957, v95, 120.
3. Buckley, S. M., *Ann. N. Y. Acad. Sci.*, 1959, v81, 172.
4. Casals, J., Brown, L. V., *J. Exp. Med.*, 1954, v99, 429.
5. Naficy, K., Carver, D. H., in press.
6. Ho, M., Enders, J. F., *Virology*, 1959, v9, 446.
7. Carver, D. H., Rosen, F. S., in press.

Received September 3, 1963. P.S.E.B.M., 1964, v116.