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Inhibition of Sindbis Virus Plaque Formation by Extracts of *Escherichia coli*

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ABSTRACT

VILČEK, JAN (New York University School of Medicine, New York, N.Y.), AND JOHN H. FREER. Inhibition of Sindbis virus plaque formation by extracts of *Escherichia coli*. J. Bacteriol. **92**:1716–1722. 1966.—Extracts prepared from washed cells of *Escherichia coli* B by sonic treatment and subsequent filtration through a 0.45-μ membrane filter significantly inhibited plaque formation with Sindbis virus in cultures or primary chick embryo cells up to a dilution of 1:20,000. The inhibitor acted on the cells rather than directly on the virus. The inhibiting substance was nondialyzable. Treatment of crude extracts with nucleases, trypsin, chymotrypsin, pepsin, or ether had no effect on the activity. Treatment with pronase destroyed the virus-inhibiting effect. Extracts prepared from two strains of *E. coli* B and one strain of *E. coli* K-12 all showed inhibitory activity against Sindbis virus. The inhibitor was present in the cytoplasmic fraction of bacteria. It was also active against Sindbis virus in human cells and showed some activity against vesicular stomatitis and vaccinia viruses in different types of cells. Interferon was not shown to be involved in the inhibition, although actinomycin D partially reversed the inhibitory activity of the extracts.

Of the numerous microbial products exerting some kind of selective antiviral activity, relatively few have been shown to act intracellularly and to be active in tissue culture. Among these, some act specifically against one group of viruses (1, 5, 11), and others are active against several unrelated viruses. Substances of the latter group, including the penicillium mold products statolon (9) and helenine (3, 14), are known to exhibit their antiviral activity through their ability to induce interferon formation.

Our experiments, originally concerned with the capacity of bacteriophage to induce interferon formation in cell culture, led to the finding that coliphage T2 lysates of *Escherichia coli* B, although not inducing demonstrable interferon formation in chick embryo cell cultures, inhibited plaque formation with Sindbis virus in the same system. However, control experiments revealed a similar inhibitory activity in sonically treated suspensions of uninfected *E. coli*, indicating that the presence of inhibitor was independent of infection with coliphage T2. The mode of antiviral activity and some properties of the inhibitor found in *E. coli* extracts are described in the present paper.

MATERIALS AND METHODS

Chemicals. The following enzymes were used: trypsin 1:250 (Difco); chymotrypsin, bovine (Armour and Co., Chicago, Ill.); pepsin, three times crystallized, B grade (Calbiochem); pronase, B grade (Calbiochem); ribonuclease (Boehringer and Sons, Mannheim, Germany); and deoxyribonuclease, B grade (Calbiochem). Purified lipopolysaccharide from *E. coli* O111:B4 was obtained from Difco. Actinomycin D (Dactinomycin) was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. All other chemicals were purchased from commonly available commercial sources.

Cell cultures. Primary chick embryo cells were prepared by trypsinization of 11-day-old chick embryos. They were grown in Eagle's minimal essential medium (MEM) with 200 units of penicillin per ml, 100 μg of streptomycin per ml, and 5% fetal calf serum. Petri plates (6 cm; Falcon Plastic, Los Angeles, Calif.) were seeded with 5×10^6 to 6×10^6 cells in 5 ml of medium. Plastic flasks (250 ml: Falcon Plastic) were seeded with 25×10^6 cells in 25 ml of medium. Screw-cap tubes (16 by 150 mm) were seeded with 10^6 cells in 1.5 ml of medium. The cultures were used after 2 days, at which time a confluent monolayer had been formed. The overlay medium was prepared by mixing equal parts of a 2% agar (Difco) and double-strength MEM. Fetal calf serum (2%) and neutral red (final concentration, 1:30,000) were included in the overlay. Dish

cultures were incubated at 36 C in an incubator provided with a humidified atmosphere of 5% CO_2 in air. Closed culture vessels were incubated in a dry incubator at 36 C.

L cells were obtained from W. K. Joklik, Albert Einstein College of Medicine, Bronx, N.Y. They were grown in MEM with 10% γ globulin-free calf serum and were maintained in the same medium with 2% γ globulin-free calf serum. Plastic petri plates were seeded with 10^6 cells and used when confluent monolayers were formed. In the first overlay medium (without neutral red), 2% γ globulin-free serum was used. A second overlay with neutral red (1:20,000) was added 2 days after inoculation.

Human embryonic lung cells, grown in continuous culture, were obtained from E. F. Wheelock, Western Reserve University, Cleveland, Ohio. In our laboratory, they were grown and maintained in the same media as those used for the primary chick embryo cells.

All sera and dried powdered MEM were purchased from Grand Island Biological Co., Grand Island, N.Y.

Viruses. Sindbis virus, strain SAAR 86, was obtained from J. Casals, Yale University, New Haven, Conn. The material used had undergone 66 to 68 passages in mouse brain. Stock 10% suspensions were prepared from brains of intracerebrally inoculated suckling Swiss Webster mice. The Indiana type of vesicular stomatitis virus (VSV) was grown in primary chick embryo cells. It was obtained from G. Todaro of the New York University School of Medicine and was the same strain used for interferon assay by Levy et al. (10). Commercial vaccine (Wyeth Laboratories, Philadelphia, Pa.) was used as the source of vaccinia virus. All virus stocks were kept frozen at -75 C. All viruses were titrated by the plaque method in chick embryo cell cultures. Plaques of Sindbis and VSV viruses were counted 2 days after inoculation, in agar-overlaid dish cultures. Plaques of vaccinia virus were produced in dish cultures with fluid maintenance medium; cells were stained with crystal violet 40 hr after inoculation. A photographic enlarger was used for plaque counts of vaccinia virus.

Bacteria. *E. coli* B Flanders strain was used for preparation of the extracts unless otherwise stated. Two other strains of *E. coli*, B and K-12, were employed. *E. coli* K-12 (strain W 3828) was kindly supplied by E. McFall of this department. The two strains of *E. coli* B were obtained from the departmental collection.

Preparation of extracts from E. coli. A 500-ml amount of nutrient broth was inoculated with a logarithmically growing culture in the same medium and grown for 20 hr in a New Brunswick incubator-shaker at 30 C. Cells were harvested by centrifugation, and the sedimented bacteria were washed three times in phosphate-buffered saline (PBS; 0.13 M NaCl, 0.007 M phosphate) in a centrifuge and finally resuspended in 15 ml of PBS. Amounts of 5 ml of the suspension were sonically treated in an MSE ultrasonic disintegrator until almost complete breakage of cells was achieved (usually 6 min). The sonically treated suspension was centrifuged for 30 min at 30,000 $\times$ g. The supernatant fluid was filtered through a sterile 0.45 μ membrane filter (Millipore Filter Corp., Bedford, Mass.). This product was referred to as "coli extract" and was stored in the frozen state at -20 C until used.

Testing of plaque inhibition with coli extract. Tenfold dilutions of coli extract were prepared in MEM. Cultures in petri plates were treated with 1 ml of the dilution tested. At least two cultures were used for each dilution. Unless otherwise indicated, dilutions of the coli extract were kept in contact with cells for 2 hr. The test fluid was removed and cultures were inoculated with approximately 50 plaque-forming units (PFU) of virus. The virus was adsorbed for 1 hr. Plaques were counted 2 days after inoculation, unless otherwise specified. For measurements of average plaque size, only isolated plaques were considered, and care was taken that a representative sample of plaques in each group was measured. Plaque sizes under 1.0 mm are approximate.

Interferon assay. Fluids tested for the presence of interferon were diluted in MEM by twofold steps. Dish cultures were treated overnight with 2 ml of the dilutions and inoculated with approximately 50 PFU of Sindbis virus. At least two plates were used per dilution. Plaques were counted 2 days after inoculation.

RESULTS

Nature of antiviral activity. Groups of chick embryo cell cultures in 6-cm petri dishes were exposed to the action of 10-fold dilutions of coli extract for 2 or 20 hr. The dilutions of extract were removed at the end of treatment. Cultures treated for 2 hr, and part of those treated for 20 hr, were inoculated with Sindbis virus immediately after the removal of the coli extract. Another group, treated for 2 hr, was washed five times with PBS prior to inoculation with virus. Still another group of cultures, treated for 2 hr, received the same dilutions of coli extract in the agar overlay. Finally, one group of cultures that had not been treated before inoculation with virus received the same final concentrations of the extract in the agar overlay after the adsorption of virus. Plaques were counted and measured 48 hr after inoculation. The results of this experiment are shown in Table 1.

A significant inhibition of plaque formation occurred in all groups. Cultures that had coli extract only in the overlay showed the least degree of inhibition. Treatment for 20 hr seemed to be less effective than the shorter exposure to coli extract. Inhibition of plaque size more or less paralleled the inhibition of the number of plaques, except in the group that received the extract after virus adsorption, where a more marked inhibition of plaque size than of the number of plaques was evident.

The inhibition was partially reversed with increased incubation time. Figure 1 shows the kinetics of the inhibition of plaque formation, read on three consecutive days, in plates treated

TABLE 1. *Mode of action of coli extract*

Dilution of coli extract	Avg no. of plaques/dish (and avg plaque diam in mm)[a]				
	Pretreatment 20 hr	Pretreatment 2 hr	Pretreatment 2 hr, 5 × washed	Pretreatment 2 hr. coli extract in overlay	No pretreatment, coli extract in overlay only
1/20	21 (1.5)	0	2 (~0.5)	0	28 (~0.8)
1/200	1 (~0.5)	0	0.5 (~0.5)	0	20 (~0.7)
1/2,000	12 (~0.7)	13.5 (~0.6)	10.5 (~0.6)	6.5 (~0.5)	34.5 (1.0)
1/20,000	50.5 (1.5)	33.5 (1.0)	22.5 (1.1)	32.5 (1.1)	48.5 (1.6)
1/200,000	48.5 (1.6)	34 (1.4)	32 (1.2)	39 (1.5)	37 (1.7)

[a] In the control, the plaque count was 46.7 and the average plaque diameter was 1.6 mm.

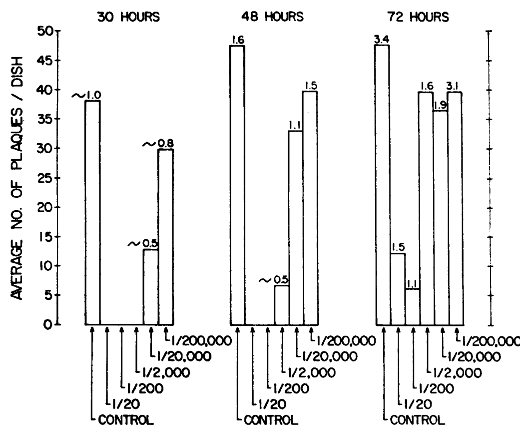

FIG. 1. *Kinetics of the inhibition of Sindbis virus plaque formation by different dilutions of the coli extract. Numbers on top of columns designate average plaque diameter in millimeters.*

with different dilutions of the coli extract for 2 hr prior to inoculation with Sindbis virus.

It was observed, on different occasions, that the rate of inhibition of both the number and size of plaques was not linear over the entire range of dilutions. In fact, some batches of the extract tended to be less active in the 1:20 dilution than at a dilution of 1:200. This can be seen from some of the groups in Table 1 and from the 72-hr reading in Fig. 1. Although the reason for this apparent irregularity is not known, it could be due to the presence of some substance which suppresses the activity of the virus inhibitor in the crude extracts. A similar observation was recently described by Cheng and Shope (3) working with helenine, an antiviral agent from *Penicillium funiculosum.*

Possible relationship to interferon. To test whether extracts of *E. coli* are able to induce interferon formation in tissue culture, several experiments were performed in which chick embryo cell cultures were treated with the coli extract, washed, and replenished with fresh medium. Samples of the culture fluid were taken and were tested for the presence of interferon.

In a typical experiment, monolayers of chick embryo cells in 250-ml plastic flasks were treated for 1 hr with 5 ml of coli extract at a dilution of 1:5 in MEM. The cultures were then washed five times with PBS and replenished with 25 ml of maintenance medium. Two flasks were placed at 36 C and two others at 2 C. Samples of medium were taken at intervals from each group of flasks and were tested separately for the presence of inhibiting activity against Sindbis virus.

Cultures were kept at both 36 and 2 C to distinguish between passive release of coli inhibitor and the active production of interferon. However, neither of the media caused significant inhibition of plaque formation (Table 2).

Similarly, no interferon was detected when cultures of chick embryo cells were tested with the coli extract diluted 1:100, a dilution which proved more active than the 1:20 dilution in inhibiting plaque formation with Sindbis virus in several experiments.

The possibility that the coli extract may increase the rate of interferon formation was tested by use of monolayers of chick embryo cells in 250-ml flasks. The cells were treated for 2 hr either with 5 ml of coli extract at a 1:100 dilution or with plain MEM. The treatment was followed by thorough washing and inoculation with Sindbis virus at a multiplicity of 1.5 PFU per cell. Media were collected after 24 hr and tested for interferon. Under these conditions, cultures treated with coli extract produced somewhat less interferon than control cultures.

The effect of actinomycin D on the inhibition of Sindbis virus plaque formation by the coli extract was tested by diluting and adsorbing the extract on chick embryo cells in MEM with or without actinomycin D. After the usual treatment for 2 hr, the cultures were washed three times with buffered saline, inoculated with Sindbis virus, and

TABLE 2. *Lack of detectable interferon in media from chick embryo cells after treatment with coli extract*

Dilution of fluid tested	Avg no. of plaques/dish[a] treated with fluids harvested after incubation at respective temperatures						
	0 time	3 hr		6 hr		24 hr	
		37 C	4 C	37 C	4 C	37 C	4 C
1/2	42.5	35.5	44	33.5	31	39	30.5
1/4	36	43	40	30.5	32.5	36	36
1/8	38.5	32	35	40.5	40	34.5	32
1/16	ND	ND	ND	36	ND	40	ND

[a] The number of plaques per dish in the control was 40.4.
[b] Not done.

overlaid with the agar medium. Control cultures received the same concentrations of actinomycin D without the coli extract or the pure MEM.

In otherwise untreated cultures, both concentrations of actinomycin used increased the diameter of plaques, without affecting the number of plaques formed. The same effect of actinomycin D on Sindbis virus plaque formation was described previously (4). In cultures treated with coli extract, actinomycin partially reversed the inhibitory action of the extract. Both the number and diameter of plaques were larger in cultures treated simultaneously with the extract and actinomycin, as compared with cultures treated with active dilutions of coli extract alone (Table 3). Two other experiments yielded similar results.

Properties of the inhibitor. Preliminary experiments revealed that the inhibiting activity resided in a nondialyzable fraction that was precipitated with ammonium sulfate. This suggests a macromolecular nature.

The inhibitory activity was not affected by treatment with three different proteolytic enzymes or treatment with nucleases, all used at fairly high concentrations. The inhibitor was also quite stable at *p*H 2 for at least 2 hr (Table 4). Overnight dialysis against *p*H 2 buffer at 2 C had no effect on the virus-inhibiting activiy of the coli extracts.

The inhibitor was quite stable to heat. Exposure to 60 C for 1 hr produced little or no effect, and activity was only decreased slightly after the crude material had been boiled for 5 min. Sensitivity of the extract to ultraviolet irradiation was tested by use of 5 ml of extract, diluted 1:10 in PBS, in a 10-cm petri dish. Exposure for 20 min at 15 cm from the source (15-w lamp) showed no effect on the inhibitory activity. Treatment with ether (4 volumes of undiluted coli extract plus one volume of ethyl ether, kept overnight at 2 C, followed by evaporation of the ether under vacuum) also had no measurable effect on inhibitory activity.

Inhibitory activity of the extract was destroyed by pronase (Table 5). The lowest tested dilution of the extract-pronase mixture proved toxic for the cells, though at higher dilutions the inhibitory activity of the extract was completely destroyed.

Distribution of the inhibitor in the bacterial cell and its presence in other strains of E. coli. To obtain separate wall and cytoplasmic fractions of bacterial cells, washed cells of *E. coli* B Flanders were ruptured in a French press (Carver Laboratory Press; Fred S. Carver, Summit, N.J.). After passage through the press, the resulting mixture of ruptured and whole cells was treated with deoxyribonuclease to reduce viscosity and then was centrifuged at 1,200 × *g* for 20 min to remove intact bacteria. The supernatant fluid was centrifuged at 10,000 × *g* for 30 min; the resulting supernatant fluid represented the cytoplasmic fraction. The sediment was resuspended in the same volume of PBS and washed three times in the centrifuge at 10,000 × *g*. The final suspension was used as the wall fraction. Both materials were subjected to sonic treatment in the same way as were intact cells for the preparation of extracts. After sonic treatment, both fractions were passed through a 0.45-μ membrane filter. The cytoplasmic fraction was slightly yellow and turbid, whereas the wall fraction appeared clear after filtration. Both products were tested in the usual way in chick embryo cell cultures against Sindbis virus. All the inhibiting activity was found in the cytoplasmic fraction, causing significant inhibition of plaque formation up to a dilution of 1:2,000. The filtered wall fraction caused no inhibition at a dilution of 1:20.

To determine whether the inhibitor was also present in other strains of *E. coli*, extracts were prepared from whole cells of another strain of *E. coli* B and *E. coli* K-12, as well as from the previously employed *E. coli* B Flanders. All strains were grown overnight in an incubator-shaker at 30 C, washed, and centrifuged three times in buff-

TABLE 3. *Effect of actinomycin D on the inhibition of plaque formation by coli extract*

Dilution of coli extract	Avg no. of plaques/dish (and avg plaque diameter in mm)		
	Without actinomycin D	0.1 μg of actinomycin D/ml	0.05 μg of actinomycin D/ml
1/20	0	12 (~0.8)	5.5 (~0.8)
1/200	7 (~0.6)	20 (1.2)	18.5 (~1.0)
1/2,000	20.5 (~0.8)	34.5 (1.7)	43.5 (1.2)
1/20,000	33 (1.2)	48 (1.8)	36 (1.4)
1/200,000	39.5 (1.3)	49 (1.8)	40 (1.5)
Control	42.5 (1.2)	42 (1.9)	41.4 (1.5)

TABLE 4. *Effect of nucleases and proteases on the inhibition of plaque formation by coli extract*[a]

Dilution of coli extract	Avg no. of plaques/dish[b] in group						
	Ribonuclease[c]	Deoxyribonuclease[c]	Trypsin[d]	Chymotrypsin[d]	PBS, pH 7.4 (control)	Pepsin[d]	HCl-KCl buffer, pH 2 (control)
1/20	1.5	4.5	5	0	5.5	0	2
1/200	1	3.5	0	0	1.5	1	2
1/2,000	15	13	8.5	4.5	17	3	6
1/20,000	40.5	40	40	47	39.5	19	27
1/200,000	37	49	47.5	40.5	42.5	35.5	37

[a] Conditions of enzyme treatment: 2 hr at 37 C. For pepsin treatment, 1 part of undiluted coli extract was mixed with 9 parts of pepsin diluted in HCl-KCl buffer, pH 2. For all other enzyme treatments, coli extract was mixed with an equal part of respective enzyme diluted in PBS, pH 7.5.
[b] The number of plaques in the control was 38.4.
[c] Final concentration, 50 μg/ml.
[d] Final concentration, 1.25 mg/ml.

TABLE 5. *Effect of pronase on the inhibition of plaque formation by coli extract*

Dilution of coli extract	Avg no. of plaques/plate[a] in group	
	Pronase[b]	PBS, pH 7.4 (control)
1/20	Toxic	2.5
1/200	51	6
1/2,000	36	13
1/20,000	35.5	18
1/200,000	41	40

[a] The number of plaques in the control was 41.
[b] Final concentration, 1.25 mg/ml. Conditions as for Table 4.

TABLE 6. *Comparison of the inhibiting activity of extracts from different strains of Escherichia coli and of purified endotoxin*

Dilution of extract or endotoxin solution	Avg no. of plaques/dish[a] treated with			
	E. coli B extract	*E. coli* K-12 extract	*E. coli* B (strain Flanders) extract	Lipopolysaccharide *E. coli* O111:B4[b]
1/2	ND[c]	ND	ND	27.5
1/20	9	2	4	33.5
1/200	20	10.5	5	34.5
1/2,000	30	30	15	32.5
1/20,000	ND	29	28	ND
1/200,000	ND	ND	31.5	ND

[a] The number of plaques in the control was 32.
[b] 2 mg/ml (in PBS) was used as undiluted material.
[c] Not done.

ered saline. The cells were then resuspended to the same optical density, centrifuged, and resuspended in one-tenth of the volume before centrifugation. All were subjected to sonic treatment followed by centrifugation at 30,000 × *g* for 30 min and filtration through a 0.45-μ membrane filter. All materials were simultaneously tested for virus-inhibiting activity. In the same experiment, purified lipopolysaccharide from *E. coli* B O111: B4 was tested for inhibitory effect on plaque formation with Sindbis virus.

The results (Table 6) showed that extracts prepared from all three strains of *E. coli* had an inhibitory effect on plaque formation. In terms of overall activity, the Flanders extract seemed to cause most inhibition, but the experiments do not allow any definite conclusions about quantitative differences in the concentration of inhibitor among the tested strains. Purified lipopolysaccharide showed no significant effect on plaque formation with Sindbis virus.

Activity of inhibitor in other types of cell cultures and against other types of viruses. E. coli extracts caused inhibition of the cytopathic effect of Sindbis virus in tube cultures of a strain of human embryonic lung cells when 10-fold dilutions of the extract were added to cells 2 hr before inoculation with about 5,000 PFU (for chick cells) of virus. No inhibition was observed beyond the 1:200 dilution of extract. Since human embryonic lung cells were considerably less sensitive to the cytopathic effect of Sindbis virus than chick embryo cells, a direct quantitative comparison of the activity of the extract in the two types of cells was not possible.

Coli extract had some inhibitory effect on plaque formation with VSV in chick embryo cells and L cells, but the degree of inhibition was much lower than against Sindbis virus. In both types of culture, extracts showing significant inhibitory activity against Sindbis virus plaque formation at a 1:2,000 dilution only slightly reduced the number and size of VSV plaques at a 1:20 dilution.

Higher dilutions of the coli extract had no effect on either plaque number or plaque size.

Both the number and size of plaques of vaccinia virus were significantly inhibited in chick embryo cells by dilutions of coli extract not exceeding 1:20.

Unlike the inhibition of plaque formation with Sindbis virus, the inhibition of both VSV and vaccinia virus plaques was destroyed by exposing the crude extracts to the action of trypsin. This indicates that the inhibition of the latter viruses is probably caused by a different substance(s).

DISCUSSION

Carver and Naficy (1) found virus-inhibiting activity, measured by the inhibition of cytopathic effect, in extracts of several bacteria. In their hands, extracts from different strains of *E. coli* and from *Salmonella typhimurium* inhibited vaccinia virus, but had no effect on the cytopathic effect of Sindbis virus in human amnion cell cultures. Sindbis virus and other arboviruses were found to be inhibited by extracts from an unidentified corynebacterium, and other corynebacteria yielded inhibitors of echo 2 and echo 11 viruses. All inhibitors were sensitive to the action of trypsin and were destroyed at *p*H 2.3. Another trypsin-sensitive inhibitor of virus multiplication, acting selectively on arboviruses, was found by Naficy and Carver (12) in extracts of *Penicillium cyclopium*.

Trypsin-resistant inhibitory activity against Sindbis virus and other arboviruses was found in extracts of a staphylococcus by Gresser and Grogan (5). Their extract, although highly active in inhibiting the cytopathic effect of Sindbis virus in human amnion cells, was almost devoid of protective effect when assayed in primary cultures of chick embryo and mouse embryo cells.

Centifanto (2) described an inhibitor of plaque formation, active in chick embryo cells against vaccinia virus and herpesvirus, in λ phage lysates of *E. coli* K-12. This inhibitor was not present in phage-free cells and was therefore named phagicin. We were unable to find any data on the sensitivity of phagicin to proteolytic enzymes.

The penicillium mold products statolon (9) and helenine (3, 14) are known to inhibit the growth of different viruses in vivo as well as in vitro. The virus-inhibiting activity of both substances is apparently due to their ability to induce interferon formation. Whereas statolon is a "complex polysaccharide" (9), the active principle of helenine seems to be of ribonucleoprotein nature (11).

It was shown that both purified bacterial lipopolysaccharide and endotoxin-containing gram-negative bacteria, injected into the blood stream of mice, caused a rapid release into the circulation of what appeared to be preformed interferon, but did not induce interferon formation in tissue culture (7, 13, 15). This explains why endotoxin, although exerting an antiviral effect in the intact organism, has not been found active in tissue culture (18).

The results of our experiments on the mode of inhibition of Sindbis virus plaque formation by crude extracts of *E. coli* have shown that the active substance in the extract acts on the cell rather than directly on the virus particle. This could be shown by the fact that washing the cells free from extract before inoculation with virus did not reduce the antiviral effect. However, the present experiments do not allow a definite conclusion on whether the effect takes place inside the cell or on the cell surface.

It seems to be quite unlikely that the antiviral activity against Sindbis virus found in our experiments was due to the presence of a provirus in the tested bacteria, particularly since two apparently nonlysogenic strains of *E. coli* B were found to contain the inhibitor. Moreover, an attempt to induce lysis by growing the bacteria in the presence of different concentrations of mitomycin C proved unsuccessful.

We were unable to detect interferon in chick embryo cell cultures treated with different dilutions of the coli extract. The observation that plaque formation with VSV was only very slightly inhibited by the lowest tested dilution of extracts active up to the dilution of 1:20,000 against Sindbis virus does not support the involvement of the interferon mechanism. Although independent experiments showed that the strain of Sindbis virus used is two to four times more sensitive to the action of chick interferon than the Indiana type of VSV (in terms of interferon titers measured by 50% plaque inhibition), one would still expect a higher degree of inhibition of VSV than that observed if interferon were involved. It was therefore surprising that actinomycin D appeared to reverse partially the inhibition of Sindbis virus plaque formation. Actinomycin D, an inhibitor of deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis, is known to interfere with both interferon formation and action (6, 16, 17).

In view of the results of experiments with actinomycin D, the possibility that interferon is somehow involved in the observed inhibition still cannot be completely rejected. The coli inhibitor might possibly induce the formation of very small quantities of interferon, even if present at a high concentration, or could perhaps act by in-

ducing, in a rather inefficient way, the antiviral protein which is believed to be formed in cells treated with interferon (8, 16). On the other hand, the antiviral effect of the coli inhibitor might be quite independent from that of interferon but could still require DNA-dependent RNA synthesis. Or, the plaque inhibition may be the result of a synergistic action of the coli extract and interferon, the latter or both being inhibited by actinomycin D.

The physical and chemical properties of the active substance in the coli extracts have not yet been completely defined. Resistance to nucleases and ultraviolet irradiation virtually ruled out the possibility that it is a nucleic acid. Sensitivity to pronase suggests that the activity is associated with proteinaceous material, despite the surprising resistance to three other proteolytic enzymes, which, however, may be due to the protective action of other substances in the crude extracts. It seems to be rather unlikely that the activity is due to the presence of endotoxin.

It is hoped that more will be learned about the mechanism of action and chemical nature of the inhibitor after its purification from the crude extracts. Attempts at the purification of the active substance are in progress.

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