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Antiviral Agent from λ-infected *Escherichia coli* K-12

I. Isolation

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Phagicin, which is an antiviral agent active against deoxyribonucleic acid (DNA) viruses such as vaccinia and herpes simplex, has been identified as a phage internal protein. It was found in infected *Escherichia coli* lysates, but could also be obtained by disruption of the purified infective particles after incubation with LiCl at 46 C for 15 min or by sonic treatment. After centrifugation at high speed, the antiviral activity was found in the DNA phase and could be separated by chromatography on Sephadex gels with 0.2 M phosphate buffer (*p*H 7.5) as the eluent. Phagicin present in lysates after removal of infective particles was nondialyzable and was bound to nucleic acids. It could be released during precipitation of nucleic acids by streptomycin sulfate, and in this form it could be easily dialyzed. The antiviral activity of phagicin was specific for herpes simplex and vaccinia viruses.

When *Escherichia coli* is infected with the temperate phage λ, a soluble protein which inhibits herpes simplex growth in vivo and in vitro is produced (3). This material does not interfere with adsorption of the virus but acts at the stage of intracellular replication. Because such material is not found in measurable amounts in uninfected *E. coli* culture and its production is closely associated with phage replication, the term "phagicin" was proposed for this antiviral agent. The initial problem was to isolate the material, define its chemical nature, and determine its relationship and specificity to λ phage replication. This communication describes the isolation and purification of this material from *E. coli* K-12 cultures after infection with λ and λ b2b5c coliphages.

MATERIALS AND METHODS

Bacterial strains. The procedures used for the propagation of *E. coli* K-12 have been described previously (3). The cultivation and induction of *E. coli* K-12 (λ) was performed by the method of Korn et al. (7).

Bacteriophages. The procedure for the cultivation of λ phage has been described (3). A virulent mutant strain, λ b2b5c, was propagated in the same medium without agar. Purified phage stocks were prepared in the following way. The bacteria and debris were removed from the lysates by centrifugation at 37,000 × *g* for 30 min. After filtration through a 0.45 μ filter (Millipore Corp., Bedford, Mass.), the supernatant fluid was centrifuged at 105,000 × *g* for 5 hr. The pellet was suspended in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-chloride buffer (*p*H 7.5) and was chromatographed in a Sagarose 2 gel column equilibrated with the same buffer. The fractions containing the bacteriophage were filtered and stored at 4 C.

The phage stock used for the preparation of rabbit antiserum was prepared by banding the phage in a CsCl gradient according to the method of Kaiser et al. (5). Intramuscular injections of phage mixed with Freund's adjuvant were given for 3 consecutive weeks, and blood was collected at the fourth week. The titer of the antiphage serum was determined by standard neutralization tests as described by Adams (1), and the "serum blocking" tests were conducted according to the method of Stent (10). Phage particles were disrupted either by sonic treatment in the cold with a Branson Instrument Sonifier or by the use of LiCl as described by Dyson (4).

Virus strains. The medium used for growth of herpes simplex strain GCA3 on chick embryo fibroblasts has been described elsewhere (3).

Antiviral assay. The antiviral activity of the crude lysate and the purified fractions was studied by the plaque inhibition assay as previously reported (3). Units of phagicin were expressed as the reciprocal of the dilution which inhibits the plaque count by one-half. The specific activity was expressed as units/μg of protein. The in vivo assay was performed according to the method of Kaufman (6) with rabbit corneas. Virus was inoculated into the rabbit corneas; 48 hr after infection, the animals were randomized into similar groups of 12. The material tested was applied every 2 hr, and the ulcers were evaluated at the third day of treatment. The scoring was done in the following manner: 1 = ulcers covering 25% of the cornea; 2 = ulcers covering 50% of cornea; 3 = ulcers covering 75% of cornea; 4 = ulcers covering entire cornea.

Preparation of the crude lysate. The agar overlay technique (3) was used to prepare *E. coli* lysates, as

it provided a method of initially concentrating the material. Liquid media were used for the preparation of *E. coli* lysates infected with λ phage b2b5c. The infective phage particles were removed by centrifugation at 105,000 × *g* for 2 hr. The supernatant fluid was then passed through 0.45-μ filters (Millipore Corp.) and stored at 4 C.

Gel filtration. Sephadex G-100 and Sephadex G-200 were swollen in an excess of 0.1 M phosphate buffer, *p*H 7.5. The G-100 Sephadex gel was packed into a 39.5 by 2.5 cm column. The void volume determined with blue dextran 2000 (Pharmacia Inc., New Market, N.J.) was 45 ml, corresponding to tube 6 in Fig. 5. Sephadex G-25 was packed into a 28 by 1.5 cm column. All columns were monitored by absorbency at 280 mμ.

Enzymatic digestion and protein determination. Pepsin (twice crystallized; Worthington Biochemical Corp., Freehold, N.J.) was dissolved in a 0.1 M KCl solution adjusted to *p*H 2, to a concentration of 10 μg/ml. The enzymatic digestion was carried out at 37 C for 1 hr, and the proteolytic activity was stopped by dialysis in phosphate buffer (*p*H 8). The enzymatic digests were then tested in tissue culture for antiviral activity. Trypsin was purchased from Difco. Incubation was at 37 C and *p*H 8. At the end of the incubation period, the residual trypsin was inactivated with 1 ml of di-isopropyl fluorophosphate (1%). Both treated and untreated samples were dialyzed and filtered prior to testing for antiviral activity in the rabbit eye.

Protein was measured by the method of Lowry et al. (9).

RESULTS

Several experiments were performed to define the chemical nature of phagicin. Initially, it was determined that the antiviral activity was not destroyed by deoxyribonuclease, ribonuclease, or ultraviolet irradiation, thus eliminating a nucleic acid macromolecule (3). The loss of antiviral activity after treatment with proteolytic enzymes indicated that the activity resided in a protein moiety.

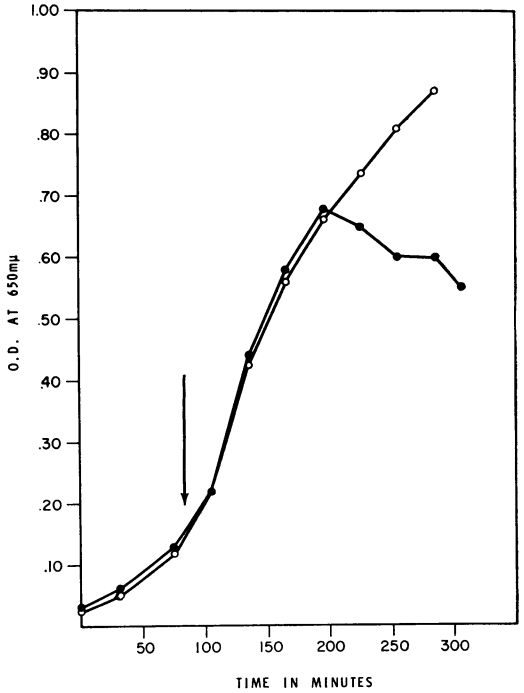

FIG. 1. *Induction of Escherichia coli K-12* (λ) *by mitomycin C. A sample of a culture of E. coli K-12* (λ), *grown overnight, was suspended in fresh tryptone broth. At the indicated time, mitomycin C (final concentration 1 μg/ml), Casamino Acids (final concentration 0.2%), and glucose (final concentration, 0.1%) were added to the culture* (●). *A duplicate culture without mitomycin* (○) *served as the control. Phage yields in the induced control were* $>10^8$*/ml at the completion of lysis.*

TABLE 1. *Effect of proteolytic enzymes on phagicin*

In vivo		In vitro	
System	Ulcer grade (0-4)	System	No. of herpes simplex plaques
Lysate + trypsin[a]	2.45	Fraction I + pepsin[b] 1:10 dilution	16
Trypsin control[c]	2.50	Fraction I control	
Lysate control[d]	1.43	1:10 dilution	0
		1:20 dilution	0
Saline control[e]	2.43	Herpes control	87

[a] Lysate, 42 ml (3 mg of protein/ml), plus 2.5 mg of trypsin.

[b] Fraction I refers to the first peak of G-100 Sephadex filtration.

[c] Represents a trypsin solution of equal concentration as in footnote *a*, and inactivated with equal concentration of di-isofluorophosphate as in *a*.

[d] Crude lysate with no additions.

[e] NaCl solution, 0.85%.

Trypsin. The crude lysate was incubated with trypsin for 18 hr, as described in Materials and Methods. Incubation with trypsin resulted in a total loss of antiviral activity (Table 1).

Pepsin. The effect of pepsin on fraction I (*see* Purification) was also investigated. It was observed that incubation of 5 ml of fraction I with 1 ml of pepsin solution resulted in partial loss of antiviral activity (Table 1). Similar experiments with longer incubation periods resulted in greater losses of activity. It can be concluded from these experiments that the antiviral activity resides in the protein moiety.

Production of phagicin. Phagicin is produced in *E. coli* upon infection with the bacteriophage λ, and it is released in the medium after lysis of the

cells. Noninfected controls have not been found to produce phagicin in any measurable amounts (3). It seemed necessary to establish the relationship between phage replication and the production of phagicin. It was first determined that phagicin is not a product of lysis of the cell per se. Lysis from without was achieved by superinfecting the cells with phage, and the lysate obtained was completely devoid of antiviral activity. Evidence that phagicin is indeed a phage-specific protein comes from several experimental facts.

In an induction experiment, *E. coli* K-12 (λ) was grown in a special Difco tryptone broth and was allowed to enter the logarithmic phase; then mitomycin C was added to a final concentration of 1 μg/ml. The culture continued to grow for another 90 min at which time lysis began (Fig. 1).

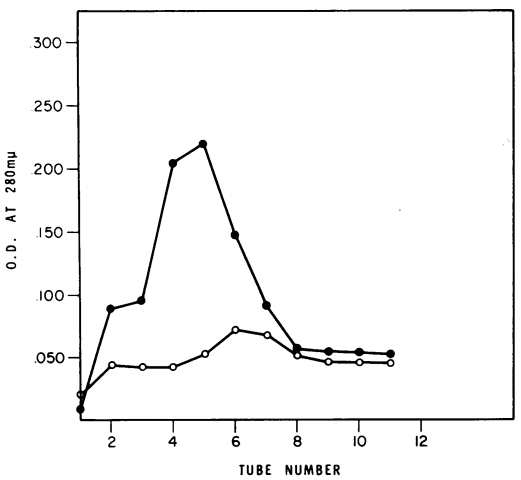

FIG. 2. *Elution pattern of gel filtration of samples of induced and noninduced lysates of Escherichia coli K-12 (λ) on Sephadex G-100. Samples applied to the column have equal protein content. Symbols: ○, control; ●, induced culture.*

TABLE 2. *Antiviral activity of lysates[a] of Escherichia coli K-12* (λ)

Final dilution in culture	No. of herpes simplex plaques
E. coli K-12 (λ) noninduced	
1:16	26
1:32	33
1:64	46
E. coli K-12 (λ) mitomycin induced	
1:16	11
1:32	13
1:64	33
Herpes control	43

[a] Each lysate contained 2,240 μg of protein/ml.

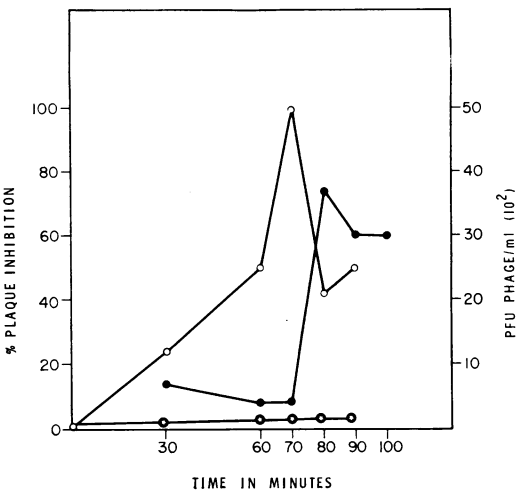

FIG. 3. *Production of phagicin in one cycle of replication of λ phage. A sample of washed Escherichia coli cells (10⁷/ml) was infected with λ phage at a multiplicity of 0.01. At intervals, samples were removed and tested for antiviral activity against herpes simplex virus in chick embryo monolayers. Symbols: ●, phage yields; ○, phagicin; ★, uninfected control. The phage counts were determined from a 2,000-ml final volume of the culture tube.*

Samples of noninduced and induced culture were taken and agitated with chloroform. Both samples were spun down to remove cell debris, and then were centrifuged at 30,000 rev/min for 1 hr. Gel filtration of both samples of equal protein content was done in Sephadex G-100. As shown in Fig. 2, peak 1 (*see* Antiviral activity) is present in the induced sample, in contrast to the small amount of material present in the noninduced control; this can be accounted for by the spontaneous replication of λ phage in the noninfected culture which is in the order of 10^2 plaque-forming units (PFU)/ml. Table 2 shows the results of the assay for antiviral activity in both induced and noninduced lysates. The activity is found mainly in the induced sample. The experiment above showed that phagicin is dependent on phage replication.

Since the preceding experiment shows that phagicin is dependent on phage replication, it seemed necessary to determine at what time during phage replication phagicin is produced.

In this experiment, 20 ml of *E. coli* (10^7 cells per ml) was mixed with 20 ml of phage inoculum (10^5 PFU/ml) and allowed to adsorb for 10 min at 37 C with agitation. After adsorption, the culture was diluted to 2,000 ml with broth. Samples of the culture were removed at different times and agitated with chloroform. A small

amount of each sample was saved for phage count. All samples were spun down to remove debris, passed through a filter (Millipore Corp.), concentrated two times, and tested in tissue culture for antiviral activity. The results (Fig. 3) indicate that phagicin is produced after infection, reaches a peak a few minutes prior to the first burst of phage particles, and decreases somewhat concomitantly with phage liberation. A similar bacterial inoculum not infected with λ phage and grown under the same conditions showed no antiviral activity.

An effort was made to find out whether phagicin could be obtained from whole infective phage. Phage was disrupted with LiCl, as described in Materials and Methods. The uncentrifuged shocked sample was then chromatographed in a G-25 column with water or 0.1 M phosphate buffer (*p*H 7.5) as the eluent. With the latter, a precipitate was observed indicating the position of Li in the column. The pattern obtained is shown in Fig. 4. All of the fractions obtained were tested in tissue culture and the antiviral activity was found in the last peak. The position of the last

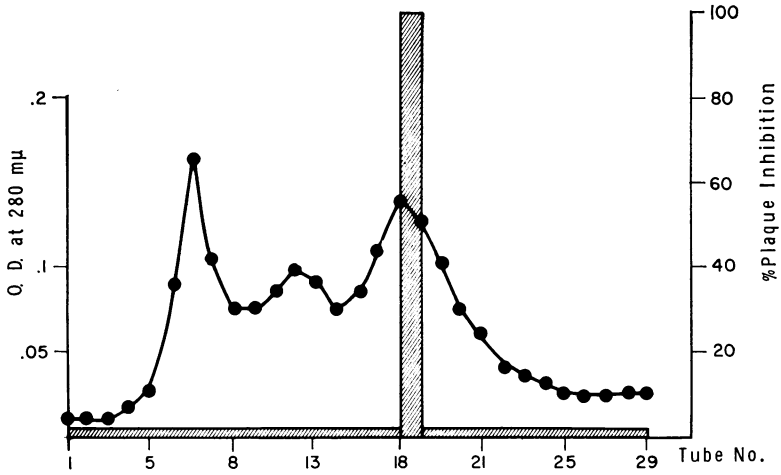

FIG. 4. *G-25 Sephadex column chromatography of disrupted λ b2b5c phage. Antiviral activity is expressed as the per cent inhibition of herpes simplex on chick embryo monolayers. Optical density at 280 mμ, ●; plaque inhibition, bar with diagonal lines.*

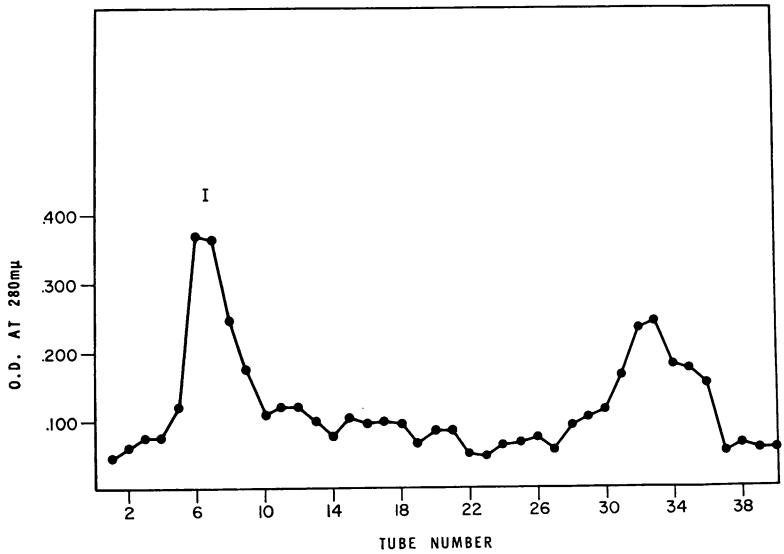

FIG. 5. *Representative elution diagram of crude lysate applied to a Sephadex G-100 column equilibrated with 0.1M phosphate buffer (pH 7.5). Peak I represents the fraction containing the antiviral activity.*

peak in the column would indicate a very low molecular weight, but the calibration measurements of the column indicated that the sample was really retarded by the column. Similar results were obtained when other Sephadex gels were used. Disruption of the phage by sonic treatment gave similar results.

The experiments above have indicated that phagicin is a product of phage replication. It is produced and can be detected before whole infective phage particles are released. It can be released from whole infective particles after disruption, indicating that it is an internal protein.

Purification. The initial step in the purification of phagicin is gel filtration of the crude lysate on Sephadex G-100. In most experiments, a sample of crude lysate containing 45 mg of protein was passed through the column and eluted with phosphate buffer. The elution pattern is shown in Fig. 5. All tubes were assayed for antiviral activity in tissue culture, and the activity was found repeatedly in the first peak, called fraction I. When this fraction was rechromatographed on other gels, a slow moving peak of apparently low molecular weight material occasionally appeared. Most of the antiviral activity was found in the first peak, and 15 to 20% of the activity was found in the low molecular weight material. It was then postulated that the appearance of a low molecular weight material could be due to dissociation or perhaps two separate entities were involved. For this reason, fraction I from Sephadex G-100 was again prepared with distilled water as the eluent. This fraction was lyophilized and suspended in various buffers prior to chromatography in Sagarose 4 gel columns. The best results were obtained when fraction I was chromatographed in 0.2 M phosphate buffer (*p*H 7.5). The column gave a good, distinct separation of both high molecular weight material, fraction A, and low molecular weight material, fraction B (Fig. 6). The total recovered antiviral activity was distributed between both fractions: 33.5% in fraction A and 66.5% in fraction B; four times more protein was needed in fraction B to attain similar antiviral activity results as in fraction A.

Chemical analysis of fraction I revealed protein and nucleic acid. Electron micrographs of this sample showed phage heads, tails, and deoxy-

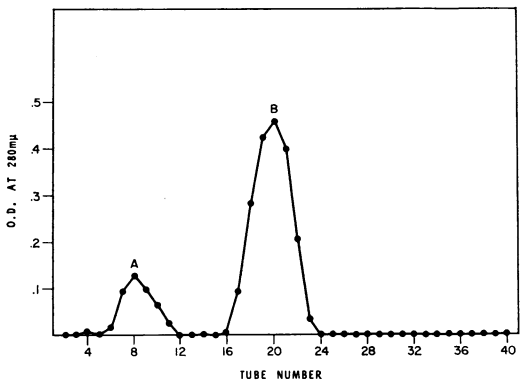

FIG. 6. *Rechromatography of fraction I on Sagarose 4 gel column equilibrated with 0.1 M phosphate buffer (pH 7.5). Fractions A and B were analyzed for antiviral activity on tissue culture, as described in Results.*

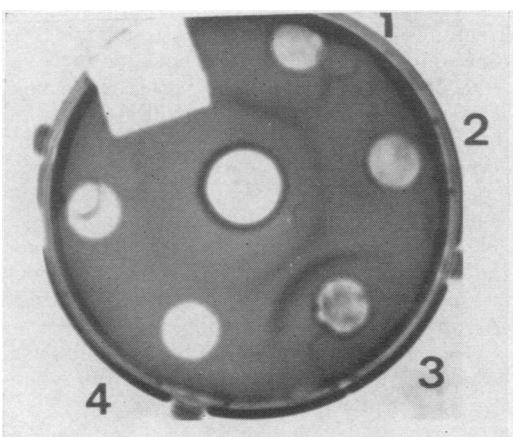

FIG. 7. *Agar immunodiffusion plate. Wells 1 and 2 represent different samples of fraction A; 3 represents phage lysates; and 4 represents fraction B. The center well contains λ phage antiserum.*

TABLE 3. *Effect of phage antiserum on phagicin*

Culture	No. of herpes simplex plaques
Phagicin + λ antiserum dilutions[a]	
1:10	0
1:20	3
1:40	12
Phagicin control dilutions	
1:10	4
1:20	12
1:40	22
Herpes control	98
Herpes control (λ serum)[b]	86
Herpes control (goat anti-rabbit serum)[b]	80

[a] All phagicin dilutions were made in tissue culture maintenance medium.

[b] Refers to herpes simplex control containing either goat anti-rabbit or λ serum in same concentrations as in the phagicin + λ antiserum dilutions.

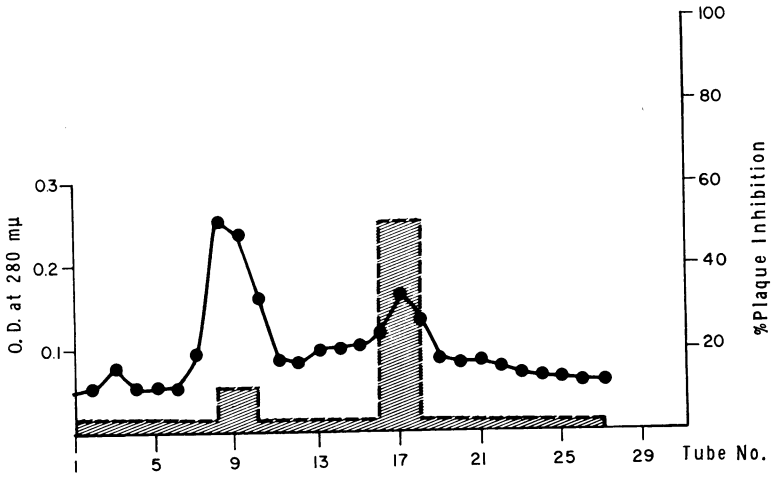

FIG. 8. *G-25 Sephadex column chromatography of fraction I after treatment with streptomycin sulfate. Optical density at 280 mμ, ●; plaque inhibition, bar with diagonal lines.*

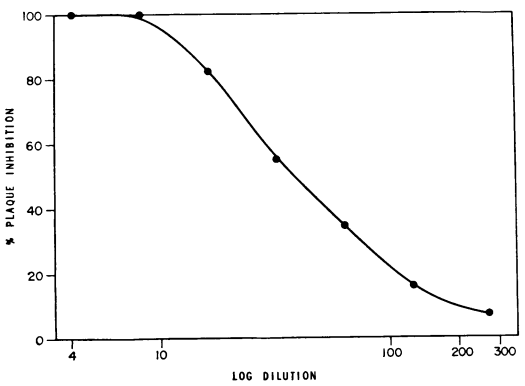

FIG. 9. *Representative dose response curve of the crude lysate. Dilutions of the crude lysate were tested for antiviral activity against herpes simplex virus by the plaque inhibition assay, as described in Materials and Methods.*

ribonucleic acid (DNA). Agar immunodiffusion plates also confirmed the presence of phage material. Antiserum, made against purified whole phage, was placed in the center well. Fraction A, whole phage lysate, and fraction B were placed in the outer wells. A line of identity was seen between fraction A and the phage antigens present in the phage lysate (Fig. 7). No precipitin line was seen with fraction B. Since it was shown that fraction A contained phage material, it seemed imperative to determine whether this material was involved in the antiviral activity.

In this experiment, a sample of fraction A was mixed with the appropriate amount of λ antisera and allowed to react for 10 min at 37 C. The antigen-antibody complex was precipitated by the addition of goat anti-rabbit immunoglobulins and removed by centrifugation. The supernatant fluid was filtered, diluted, and tested in tissue culture. Both rabbit and goat antisera were included in the controls. The results shown in Table 3 indicated that the removal of phage antigens by antiserum made against the whole phage did not diminish the antiviral activity of fraction A. Repeated experiments gave the same results.

Following this observation, fraction I was centrifuged at 105,000 × *g* for 7 hr to sediment the particulate material. The supernatant fluid which contained mainly DNA and nonsedimentable protein, retained all of the antiviral activity. The nucleic acids present in this sample were removed by the addition of 0.2 volumes of 5% streptomycin sulfate solution. The mixture was allowed to react for 1 hr in the cold and then was centrifuged prior to chromatography on a Sephadex G-25 column equilibrated with 0.05 M Tris-chloride buffer (pH 8.6). The results of this filtration are shown in Fig. 8. The antiviral activity resided mainly in the last peak. It could be concluded from this data that the antiviral activity is in the dialyzable low molecular weight fraction and that such material, which is associated with the high molecular weight fraction, is released after treatment with streptomycin sulfate.

Summary of purification. The experimental data show that phagicin can be obtained from lysates as well as from whole infective phage.

In view of this finding, phagicin can now be prepared by the following method: (i) gel filtration of the crude lysate to obtain fraction I; (ii) sonic treatment of fraction I to disrupt the infective phage, followed by centrifugation to remove

particulate material; (iii) precipitation of nucleic acids with streptomycin sulfate; and (iv) gel filtration of the supernatant fluid on Sephadex G-25 gels.

Antiviral activity. Although it has been shown that phagicin is effective in suppressing growth of vaccinia and herpes simplex viruses, all of the fractions obtained during the purification process were tested against herpes simplex virus in chick embryo monolayers, as described in Materials and Methods. A representative dose response curve of the antiviral activity of the crude lysate is shown in Fig. 9. The recovery of activity from the crude lysate was always more than 100%, perhaps indicating that the phagicin present in crude lysate is associated with or inhibited by other materials. The antiviral activity of phagicin is specific for herpes simplex and vaccinia viruses. No activity was observed against other viruses, such as poliovirus, Newcastle disease virus, influenza virus, Semliki Forest virus, vesicular stomatitis virus, or rhinovirus. With these viruses, the antiviral activity assays were done in tube cultures, which were observed for the presence or absence of cytopathic effects. Quantitative plaque reduction assays were not performed because phagicin does not readily diffuse through agar.

DISCUSSION

Lysogenic induction of *E. coli* K-12 (λ) or infection of the sensitive *E. coli* strain with the bacteriophage λ results in the appearance of an antiviral substance (phagicin) which suppresses herpes simplex growth both in vivo and in vitro. Phagicin is not found in measurable amounts in noninduced or noninfected bacteria. The role of phage is supported by the following evidence. (i) Phagicin is produced during the cycle of phage replication. The increasing concentration of this substance during a one-step growth experiment indicates that it is intimately related to the intracellular synthesis of the phage. (ii) Phagicin is obtained after induction of the lysogenic strain, and the antiviral activity is higher in the induced culture than in the noninduced culture. (iii) Phagicin can be obtained by disruption of purified intact infective phage, which in itself has no antiviral activity.

Thus, phagicin appears to be an internal protein of λ phage which is bound to the DNA of the phage, but can be released from the DNA by treatment with streptomycin sulfate.

Internal proteins of other coliphages have been described (8) but, to the best of my knowledge, this is the first time that an internal protein of phage has been shown to possess antiviral activity. It is possible that the antiviral activity occurs through the same metabolic inhibition that is found with T2 internal proteins which inhibit DNA-dependent ribonucleic acid polymerase (1).

Other types of viral inhibitors from *E. coli* extracts have been reported, and it is probable that *E. coli* harbors more than one kind of inhibitor. Carver and Rosen (2) described an extract of *E. coli* 0111 that inhibited vaccinia and herpes simplex in primary amnion cell cultures. The active component was sensitive to trypsin and was shown to impair the DNA metabolism of the cell. The antiviral compound described by Vilček (11) in *E. coli* is a protein of cytoplasmic origin. Because of the loss of viral inhibitory activity when actinomycin D is added to the infected cultures, it is possible that this cytoplasmic material acts by inducing interferon in the host. A comparison of these inhibitors and phagicin clearly shows that they are different entities. Phagicin is a phage internal protein and is obtained from purified phage particles which do not have antiviral activity. The activity is found after disruption of the intact particle. This agrees with the experimental finding that antiserum prepared against intact purified λ phage does not diminish the antiviral activity.

Phagicin is a potent antiviral agent for herpes simplex and vaccinia in vitro and in established corneal infections. The fact that this internal protein of phage λ has been shown to have antiviral activity opens a wide field in virus chemotherapy, since internal proteins of other phages may be effective in suppressing growth of viruses other than herpes simplex and vaccinia.

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