PROPERTIES OF A BACTERIOPHAGE DERIVED FROM ESCHERICHIA COLI K235*

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PLATES 7 AND 8

Many strains of Enterobacteriaceae elaborate colicines, substances which kill bacteria of the same or related species (1, 2). Some colicinogenic strains produce still other agents, the bacteriophages (3, 4). These remarkable products of bacterial synthesis exhibit similarities in their mode of action as well as differences. For example, when the two attack sensitive bacteria they first combine with specific receptors present upon the surface of the bacterial cell and change the metabolism of the host. Cells which have come in contact with colicines cease their metabolic processes altogether. They die without producing additional bacteriocine. On the other hand, microorganisms which have been infected with phage synthesize new viral particles or become lysogenized (5–7).

The capacity to produce colicines can be transmitted from one bacterial strain to another by mating. The genetic determinants which control this property have been termed colicinogenic factors, and are independent of the normal genetic structure of the cell (8). It has been suggested that the colicinogenic factor might be regarded as a bacterial virus distinct from phage, but related to it through common ancestry (1).

In the study to be presented here we have undertaken to determine whether the bacteriocine and virus produced by the same microorganism have certain properties in common. A strain of *Escherichia coli* which has been designated as K235 was chosen for study because it is lysogenic and elaborates colicine K (3). It has been shown that the colicine is an integral part of the somatic antigen of *E. coli* K235 and that its bacteriocidal activity is associated with the protein component of the antigenic complex (9–11). It has also been demonstrated that the host specificity of the bacteriophage carried by this bacillus differs from that of colicine K, although the virus has not been further characterized (3). Its isolation and properties will be described in this communication. It will be shown that this virus which we have named PK is a temperate dysentery-coli phage related to P2 virus both morphologically and serologically. In addition, it will

be seen that no relationship of any kind exists between PK virus and either colicine K or the T-even phages. Finally, it will be demonstrated that PK prophage does not induce colicinogenesis in its host and that the formation of PK phage and colicine K are probably controlled by independent genetic determinants.

Materials and Methods

Bacteria and Bacterial Substances.—The colicinogenic strain of *E. coli* K235 and *Shigella sonnei* E90 used in these studies were originally obtained from Dr. P. Frédéricq of the University of Liége. Two variants of *E. coli* K235, termed L^+OC^+ and L^-OC^-, were employed (11). Phage and colicine-resistant mutants of *Sh. sonnei* E90 were isolated during the course of this study. *E. coli* B and its phage-resistant mutants were originally obtained from the late Dr. M. H. Adams of New York University. A bacteriocine-resistant mutant, B/K, was isolated in our laboratory. *E. coli* Cullen was obtained from Dr. I. N. Asheshov of the Bronx Botanical Gardens, New York, and *E. coli* C-1 was sent to us by Dr. J. F. Anderson of the University of Pennsylvania. *E. coli* K12 (λ) and *Salmonella typhimurium* LT2 were supplied by Dr. N. Zinder of The Rockefeller Institute. Purified colicine K was obtained from Dr. W. F. Goebel (10).

Bacteriophages.—Coli-dysentery phages T1 to T7 were originally obtained from Dr. M. H. Adams. The viruses were maintained by passage on *E. coli* B. The temperate P2 phage was supplied by Dr. T. F. Anderson; its virulent mutant P2 *vir* was isolated here. Both phages were propagated on *E. coli* C-1.

Viral Stocks and Assays.—The strains of PK and PK *vir* phages to be described were derived from single plaques in the following manner. An aliquot of a phage-containing solution was plated on *Sh. sonnei* E90 or on E90/K and the plates were incubated at 37°C. A single plaque was then removed, suspended in nutrient broth, and 0.1 ml portions were plated on *Sh. sonnei* E90. After incubation, the soft agar layer of the plate was suspended in 10 ml of nutrient broth and 0.2 ml chloroform was added to kill the bacteria. The suspension was centrifuged and the supernatant, containing the phage, was aerated to remove chloroform. The stocks of PK phages so obtained contained 10^6 to 10^8 viral particles per ml. The *titer* of the phage stocks was determined by plating on *Sh. sonnei* E90 using 18-hour-old agar slant cultures (12). The platings were made on neopeptone-meat infusion-agar. The *plating efficiency* of the phages on a given bacterial strain was determined by assaying them on this strain and on *Sh. sonnei* E90. The ratio of the two titers represents the efficiency of plating. The *host range* of the phages (P) was determined by spotting 0.02 ml aliquots containing approximately 10^7 P/ml on the surface of plates seeded with the appropriate indicator. The *serological specificity* of various phage strains was determined in the following manner: 0.3 ml of an antiserum to a known phage and 0.05 ml of a slant suspension of *Sh. sonnei* E90 were added to 2.5 ml of 0.5 per cent nutrient agar maintained at 45-50°C and the mixture was plated. Droplets of dilutions of the phage in question were deposited on the surface of the plates and the latter were incubated at 37°C. If the phage under consideration was serologically related to the known virus, it was neutralized by the antiserum, and the areas where the phage had been deposited would be overgrown with bacteria. In those instances where phage and standard virus were serologically different, the phage lysed the host cells and the areas remained clear.

Antisera.—Antisera to T2, T4, P2, and PK *vir* phages were obtained from rabbits which had been injected intravenously on alternate days over a 2 week interval with graded doses of virus suspensions containing 10^9 to 10^{11} P/ml. Two courses of injections were administered before bleeding. Antisera to purified colicine K were prepared as previously described (10).

Production of Bacteriophage by E. coli K235.—If *E. coli* K235 L⁺OC⁺ is grown
in an appropriate medium it releases both phage and colicine K (3, 13). The
amount of bacteriocine in the cell-free medium can be determined by spotting
serial dilutions of the latter on plates seeded with an appropriate sensitive bac-
terial strain (10, 15). The phage is detected by plating aliquots of the medium
on colicine K resistant mutants of these same bacteria. In our experiments, *E.
coli* B was used as the test microorganism for colicine assays whereas
the colicine-resistant strain *Sh. sonnei* E90/K was employed as the indicator for
the phage. The latter was selected because the phage liberated by *E. coli* K235
forms over a hundred times as many plaques on this microorganism as it does
on *E. coli* B/K.

The production of colicine K and the production of the phage liberated during
the growth of *E. coli* K235 were followed quantitatively as described below:

1 liter of casamino acid medium (10) was seeded with 10^8 cells of *E. coli* K235 L⁺OC⁺
and the culture incubated for 12 hours at 37°C. 30 ml of 50 per cent glucose solution was then
added and aeration was begun. The pH of the culture was maintained at 7.0. At various
intervals 10 ml samples were removed and the titer of viable cells was determined. Each
sample was then sterilized by adding a few drops of chloroform and spun to remove the
bacteria. The cell-free supernatant was assayed for its colicine content (10) and for phage.
The results of a typical experiment are shown in Text-fig. 1.

As will be seen, the titer of bacilli (B) increased from 2×10^8 to 3×10^{10}
B/ml during the logarithmic phase of growth. This rise was accompanied by an
increase of colicine K titer from 40 to 800 units and of phage from 30 to 12,000
P/ml. When bacterial growth ceased the colicine titer decreased whereas that of
the phage did not change significantly.

A photograph of the plaques formed by the phage present in the supernatant
of the K235 culture on *Sh. sonnei* E90/K is shown in Text-fig. 2 *a*. The plaques
are turbid and have a clear area in their center. Their diameter varies from 0.2
to 1.2 mm. Since plaques of different sizes might harbor different phages, ten
of the smallest and ten of the largest were isolated and replated on *Sh. sonnei*
E90/K. It was found that each of the isolates formed plaques of varying size, a
fact which indicated that the small and large plaques contained the same virus.
It appears that *E. coli* K235 liberates but one phage capable of multiplying on
Sh. sonnei E90/K. This virus we have termed PK phage.

Isolation of PK Bacteriophage.—In order to learn whether the virus which one
obtains from different plaques of PK have the same characteristics, a number of
stocks were prepared and their properties compared.

An aliquot of a cell-free supernatant of *E. coli* K235 culture was plated on *Sh. sonnei*
E90/K. Five plaques were isolated and stocks (Nos. 1 to 5) were prepared using *Sh. sonnei*
E90 as host. To determine whether the individual viral particles in these stocks would yield

subcultures having the same plating characteristics, an aliquot of stock 4 was plated on the Sonne bacillus and new stocks, designated 6 to 10, were prepared from single plaques. Three plaques were again isolated from a plate seeded with stock 6 and stocks 11 to 13 prepared. All of the stocks were now assayed on *Sh. sonnei* E90 and on *E. coli* B and their efficiency of plating was calculated. The data are summarized in Table I.
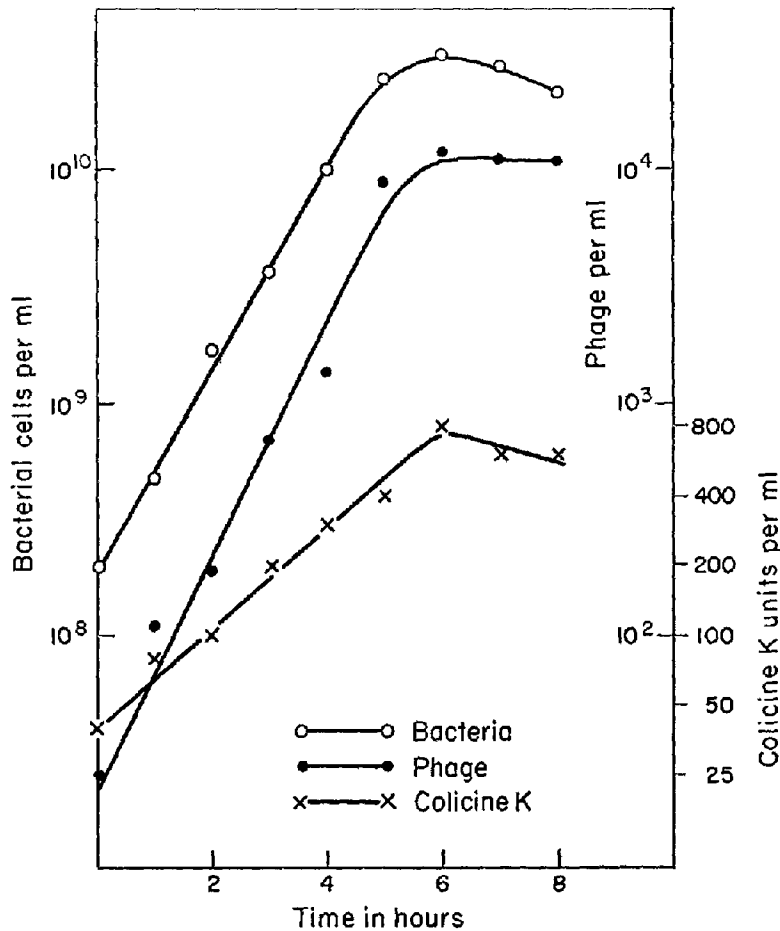
TEXT-FIG. 1. Production of colicine K and bacteriophage in growing culture of *E. coli* K235 L⁺OC⁺.

It will be seen that the various PK stocks contained from 1.5 × 10⁵ to 1.4 × 10⁶ plaque forming particles per ml, when assayed on *Sh. sonnei* E90. The phage in each formed plaques which were turbid and which showed a minute clearing in the center. The diameter of the plaques varied between 0.2 to 1.2 mm. On *E. coli* B, these phages formed turbid plaques 0.1 to 0.5 mm in diameter, yet the titers on this bacillus were lower than on *Sh. sonnei* E90. It is evident, therefore,

that PK phage in each of the stocks had similar plating characteristics and that these properties were heritable. It may also be seen that phage in stocks 1 to 5 plated with a higher efficiency on *E. coli* B, than did those of stocks 6 to 10. The latter, in turn, plated more efficiently than did the viruses of stocks 11 to 13.

TABLE I

Titers of Single Plaque Stocks of PK Bacteriophage

Stock No.	Titer on *Sh. sonnei* E90	Titer on *E. coli* B	Efficiency of plating*
1–5	$1.5 \times 10^5 - 1.4 \times 10^6$	$6.2 \times 10^3 - 9.1 \times 10^4$	0.015–0.22
6–10	$1.3 \times 10^5 - 1.3 \times 10^6$	$1.0 \times 10^3 - 1.3 \times 10^4$	0.008–0.029
11–13	$1.2 \times 10^5 - 4.7 \times 10^5$	$8.5 \times 10^1 - 6.5 \times 10^2$	0.0007–0.0025

* Ratio of phage titer on *E. coli* B to that on *Sh. sonnei* E90.
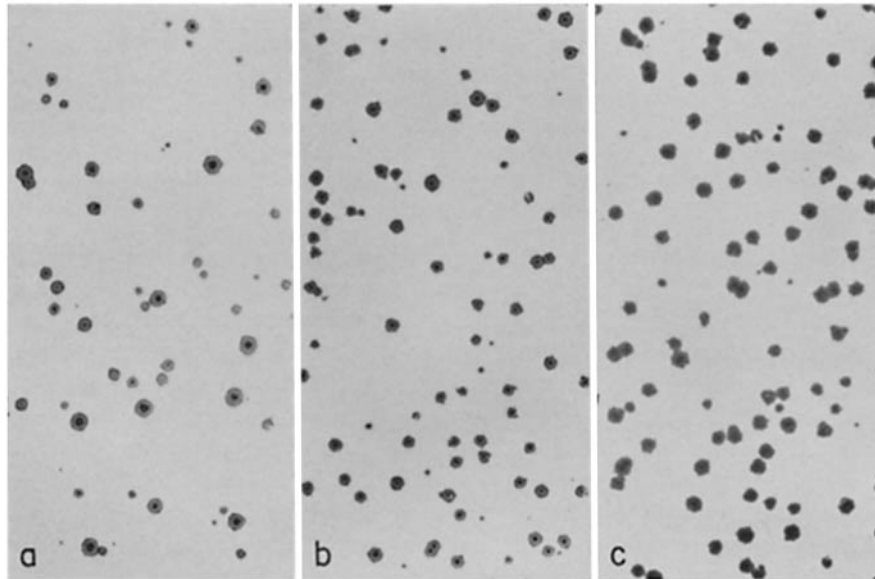
TEXT-FIG. 2. Plaques produced by the bacteriophages of *E. coli* K235. *a*, phage present in the supernatant of *E. coli* K235 L^+OC^+ culture; *b*, PK phage; *c*, PK *vir* phage. $\times$ 2.

The capacity of PK phage to plate on the colon bacillus decreased during passages on *Sh. sonnei* E90, a fact which indicates that this property may be controlled by the bacterial host.

A high titer stock of PK virus was prepared on the Sonne bacillus as follows:

A culture of *Sh. sonnei* E90 in 15 ml of nutrient broth, containing 10^8 cells per ml, was infected with 1 ml of PK phage (4×10^5 P/ml) and aerated for 3 hours at 37°C. After killing

the bacteria with chloroform, the culture was spun and the supernatant assayed on *Sh. sonnei* E90. The stock so obtained contained 1.8×10^6 P/ml. To increase the phage titer, 0.5 ml of a culture of *Sh. sonnei* E90 (1×10^8 B/ml) was added to 10 ml of the phage stock (multiplicity of infection 0.36). The mixture was aerated for 90 minutes at 37°C and processed as described above. The stock so obtained now contained 6.8×10^8 P/ml. Finally, 100 ml of a culture of the Sonne bacillus (10^8 B/ml) was infected with 4 ml of the second stock. The culture was processed and the final stock of PK phage contained 2.9×10^{10} P/ml.

The plaques produced by this PK phage on *Sh. sonnei* E90 are shown in Text-fig. 2 *b*. They are turbid, with a small clearing in the center and resemble those produced by the phage liberated from K235 bacillus on the colicine-resistant strain of *Sh. sonnei* E90 (Text-fig. 2 *a*). As one would expect the virus formed turbid plaques on *E. coli* B and plated with a low efficiency (0.012).

Lysogenization of Sh. sonnei E90 by PK Phage.- -The facts that the PK phage forms turbid plaques and produces low titered stocks when bacterial cultures are infected at low multiplicity suggest that the virus is temperate. To ascertain whether this is indeed so, the following experiment was performed.

A culture of *Sh. sonnei* E90 (2×10^3 B/ml) was infected with PK phage at a multiplicity of 8.7, and incubated for 20 minutes at 37°C. Upon assaying, it was found that some 25 per cent of the bacteria still produced colonies. Ten of these were subcultured and their sensitivity to the PK and to the seven T-phages was determined. Their lysogenicity was ascertained by growing each bacterial strain in nutrient broth and testing the cell-free medium for the presence of free virus.

The results of this experiment revealed that all of the strains were sensitive to T2, T3, T4, T6, and T7 phages and resistant to T1 and T5. In addition, two strains were sensitive to PK virus, whereas eight were resistant. Since *Sh. sonnei* E90 itself is sensitive to all of these phages, save T1 and T5, it is apparent that the majority of the bacteria which survived the infection acquired resistance to PK virus. When the cell-free supernates of the ten bacterial cultures were assayed on *Sh. sonnei* E90, it was found that the PK-resistant strains liberated 10^4 to 10^6 viral particles per ml and that the sensitive strains failed to produce virus. The liberated phage formed plaques which resembled those of PK virus. Thus, it is apparent that the strains of *Sh. sonnei* E90 which became resistant to PK phage were also lysogenic. Hence, it is evident that this virus is temperate for the Sonne bacillus.

Isolation of a Virulent Mutant of PK Phage.- -When PK phage is grown on *Sh. sonnei* E90, part of the bacterial population becomes lysogenized and the yield of phage is therefore relatively low. Since it was desirable to obtain higher yields of virus, it was necessary to isolate a virulent mutant. To select for such a mutant, the PK virus was grown as follows:

100 ml of a culture of *Sh. sonnei* E90 in nutrient broth containing 10^8 B/ml was infected with 10^6 particles of PK phage. The culture was aerated overnight, sterilized with 1 ml of chloroform, centrifuged, and the supernatant collected. Nine additional passages were made

by infecting cultures of the Sonne bacillus with 1 ml of phage stock obtained from the preceding transfer. In this manner ten stocks (Nos. 1 to 10) were obtained. Each was then assayed on *Sh. sonnei* E90 and the titers of the temperate and virulent phages were estimated by counting the turbid and clear plaques.

The first two stocks had low titers (5×10^5 P/ml) and contained only temperate PK phage. The virulent mutant of the latter appeared on the third passage and it replaced the temperate phage in the viral population on the fifth transfer. Upon the emergence of the virulent mutant the titer of the stocks increased and reached a value of 8.0×10^9 P/ml on the eighth passage. Since two additional transfers failed to increase the titer further, it is apparent that the virulent mutant was stable and did not mutate to a still more virulent variant.

A pure culture of the virulent virus, termed PK *vir*, was now prepared.

An aliquot of stock 10 was plated on *Sh. sonnei* E90, a single plaque was isolated, replated, and a new single plaque obtained. The suspension of the latter was then plated so as to produce confluently lysed plates and from these a stock was prepared (9×10^6 P/ml). In order to increase its titer the virus was passed two times in liquid culture of *Sh. sonnei* E90. A stock was finally obtained containing 8.3×10^9 P/ml.

A photograph of the plaques produced by PK *vir* phage on *Sh. sonnei* E90 is shown in Text-fig. 2 *c*. It will be seen that the viral population is homogeneous for it forms clear, sharp edged plaques 0.5 to 1.0 mm in diameter. When assayed on *E. coli* B, the virus produced clear plaques which were slightly smaller. As in the case of the temperate virus, the virulent PK phage plated with a low efficiency (0.006) on *E. coli* B.

The Effect of PK vir on Sh. sonnei E90.—In order to determine whether PK *vir* is indeed virulent for *Sh. sonnei* E90, a culture of the latter was infected with the phage at multiplicity of 8 particles per bacterium, incubated for 10 minutes and 0.1 ml aliquots were plated. A colony count indicated approximately one bacterium in a million survived. Six colonies were isolated, freed of virus by streaking on nutrient agar, and subcultured on agar slants. These strains were then tested for sensitivity to various phages and for lysogenicity.

It was found that all bacterial strains were sensitive to T2 and T6 phages and resistant to PK, PK *vir*, T1, and T5. In addition, one strain was resistant to T4, another to T3 and T4, and the remaining four strains were not attacked by T3, T4, and T7. Since *Sh. sonnei* E90 itself is sensitive to all of these phages except T1 and T5, it is apparent that the six strains were resistant mutants. When cultures of these were grown and their supernatants tested for phage it was found that they were free of virus. It is evident that PK *vir* differs from PK in that it is incapable of lysogenizing the Sonne bacillus and hence must be considered a virulent mutant.

The Morphology of PK Phage.—The temperate PK phage and its virulent variant was now examined in the electron microscope. Phage stocks were prepared as follows:

2 liters glucose-phosphate medium enriched with 0.2 per cent Difco nutrient broth (14) was infected with *Sh. sonnei* E90 and the culture grown until it contained 2×10^8 B/ml. The suspension was then infected with PK phage at a multiplicity of 0.4 P/B. The culture was aerated for 2 hours and then killed with chloroform. After standing overnight, the lysate was spun to remove debris and the phage was precipitated from the supernatant by adding ammonium sulfate (2.5 moles/liter). The precipitate was collected by centrifugation, suspended in 20 ml of saline (pH 7.5), and spun at low speed to remove insoluble material. The phage was now sedimented by spinning for 2 hours at 30,000 g. The phage pellets were resuspended in 4 ml of 0.02 M ammonium acetate at pH 6.8. Finally, the viral suspension was spun at low speed and the supernatant assayed. It was found to contain 1.07×10^{11} P/ml. A sample of PK *vir* phage was prepared in an identical manner except that the bacteria were infected with virus at a multiplicity of 0.1. The suspension contained 1.7×10^{12} P/ml.

The phages were then examined in electron microscope (Siemens elmiscope I). The specimens were prepared by depositing droplets of phage suspension in 0.02 M ammonium acetate (10^{11} P/ml) on carbon-coated grids and by embedding the adsorbed phage in sodium phosphotungstate (16).[1]

The electron micrographs of PK and PK *vir* phages are shown in Figs. 1 and 2, and their structural components are shown in Figs. 3 to 15. The micrographs reveal that the temperate and virulent PK phages are tadpole-shaped microorganisms which are morphologically identical. Their heads are 48 to 52 mμ in diameter and, in some instances, they appear polyhedral. They consist of a membrane 2 to 3 mμ thick which is filled with a substance possibly nucleic acid. Their tails are about 104 to 110 mμ long and 15 to 17 mμ wide. They consist of a sheath and core. The former is contractile, for many of the phage particles show a partially exposed core. The contracted sheath is a hollow cylinder 38 to 42 mμ long and 16 to 18 mμ wide. Since the surface of the intact phage tail contains ten diagonal *striae*, it would appear that the sheath is composed of several fibers which are wound helically around the core. The core itself is about 105 mμ long and 6 to 7 mμ wide, and is hollow; at the proximal end there are two platelet-like structures which may serve to attach the core to the head membrane. Finally, several thin fibers can be seen attached to the tip of the tail of the viruses.

A comparison of the electronmicrographs of PK phage with those of phages of T-series or P1, P2, and λ viruses, reveals that PK and P2 phages have a similar structure (17–19). Both viruses have polyhedral heads and rigid, striated tails which are equipped with a contractile sheath and tail fibers. They differ, however, in that the PK virus is about 1.3 times smaller than P2.

Host Range Specificity of PK Phages.—It has been shown that colicine K inhibits the growth of bacteria which are sensitive to T6 phage (20). It is now evident that PK phage, also produced by *E. coli* K235, is morphologically similar to P2 virus. In order to determine whether PK and P2 phages have any

other common characteristics or whether they are related to colicine K or to the T-even viruses, a comparison was made of the ability of these agents to attack various microorganisms.

Stocks of the various phages were diluted in nutrient broth (10^7 P/ml) and were tested on the eighteen bacterial strains listed in the Table II. The action of colicine K on these microorganisms was also tested using techniques described earlier (10).

TABLE II

Host Range of Bacteriophages and of Colicine K

No.	Bacterial strain	Phage						Colicine K
		PK	PK *vir*	P2	T2	T4	T6	
1.	*Sh. sonnei* E90	+	+	+	+	+	+	±
2.	*Sh. sonnei* E90/2	+	+	+	0	+	+	±
3.	*Sh. sonnei* E90/4	+	+	0	+	0	+	±
4.	*Sh. sonnei* E90/3,4,7	0	0	0	+	0	+	±
5.	*Sh. sonnei* E90/6	+	+	+	+	+	0	0
6.	*Sh. sonnei* E90/K	+	+	+	+	+	+	0
7.	*Sh. sonnei* E90/PK *vir*	0	0	0	+	0	+	±
8.	*Sh. sonnei* E90/P2	+	+	0	+	0	+	±
9.	*Sh. sonnei* E90 (PK)	0	0	0	+	+	+	±
10.	*Sh. sonnei* E90(P2)	0	0	0	0	+	+	±
11.	*E. coli* B	±	±	±	+	+	+	+
12.	*E. coli* B/3,4,7	0	0	0	+	0	+	+
13.	*E. coli* B/K	±	±	0	+	+	0	0
14.	*E. coli* C1	+	+	+	+	±	+	+
15.	*E. coli* Cullen	0	0	0	0	0	0	+
16.	*E. coli* K12(λ)	±	±	0	+	+	+	+
17.	*E. coli* K235 L+OC+	0	0	0	0	±	0	0
18.	*S. typhimurium* LT2	0	0	0	0	0	0	0

⁺ Bacteria are lysed by the phage or their growth is inhibited by 1 unit of colicine K.

± Phage plates on the bacterial strain with a low efficiency (0.01 to 0.0001) or the growth of bacteria is inhibited by 32 units of colicine K.

⁰ Bacterial strain is resistant to phage or to colicine K.

The results of these experiments are shown in Table II. It will be seen that PK and PK *vir* phages have an identical host range. Both attack the same *Sh. sonnei* and *E. coli* strains, they plate with low efficiency on *E. coli* strains B, B/K, and K12 (λ), and both fail to form plaques on *S. typhimurium* and on *E. coli* Cullen and K235. Furthermore the host range of the PK and PK *vir* phages is similar to those of P2 and T4, for bacteria which are sensitive to the former are, in most instances, attacked by the latter. The PK viruses differ in that they plate on *Sh. sonnei* strains which are specifically resistant to P2 and T4. In addition, T4 phage differs from PK in that it attacks *Sh. sonnei* strains carry-

ing PK or P2 prophage. Finally, the host range of PK phages was found to differ greatly from that of T2 and T6 viruses or from that of colicine K. PK and PK *vir* can therefore be classified as viruses of the coli-dysentery group having host specificity which is similar but not identical with that of P2 and T4 phages. It may also be seen from the table that colicine K and T6 have similar, but not identical, host specificities, for *Sh. sonnei* E90/K and *E. coli* Cullen are sensitive to one of these agents but resistant to the other.

Effect of Host Bacterium upon PK Phages.—It has been shown that PK and PK *vir* phages plate on *E. coli* B with a lower efficiency than on *Sh. sonnei* E90. To determine whether those phage particles which form plaques on the colon bacillus are host-range variants or whether they are host-modified phage, the virus was passed through colon and Sonne bacilli and the plating characteristics of the progeny were studied.

TABLE III

The Effect of Host Bacterium on Plating Properties of PK Phages

Phage type	Original stock	Stocks grown on ECB	Stocks grown first on ECB, then on E90
PK	0.006	1.2–7.4	0.004–0.018
PK *vir*	0.002	1.0–4.0	0.001–0.011

Figures indicate the ratio of phage titer on *E. coli* B to that on *Sh. sonnei* E90.

An aliquot of an appropriate dilution of PK stock, which had been prepared by growing the virus on *Sh. sonnei* E90, was plated on *E. coli* B. After incubation the bacteria were killed with chloroform vapor and five plaques were cut from the plate. Each plaque was then suspended in 1 ml nutrient broth and the suspensions (clonal stocks) were assayed on *E. coli* B and on *Sh. sonnei* E90. Then, an aliquot of one of these stocks was plated on *Sh. sonnei* E90 and five new clonal stocks were prepared in the manner described above. These were also assayed on both the colon and Sonne bacilli. An identical experiment was also performed with PK *vir* phage. The results of the two experiments are shown in Table III.

It can be seen from the table that the original stocks of PK and PK *vir* phages, which had been grown on *Sh. sonnei* E90, plated with much lower efficiency on *E. coli* B than on the Sonne bacillus. When these phages were passed through the colon bacillus however, their progeny formed more plaques on *E. coli* B than on *Sh. sonnei* E90. Thus the passage of both PK phages through a different host changed their plating characteristics. This change is reversible, however, for upon passing the phage which had been grown on *E. coli* B through the Sonne bacillus the progeny again plated with a low efficiency on the colon bacillus. It is evident therefore that both PK phages show a host-controlled variation, and in this respect they resemble P2 virus (21).

The Serological Specificity of PK Phages.—In order to determine whether

there is an immunological relationship between PK, P2, and T4 phages, and colicine K, their neutralization reactions in the homologous and heterologous antisera were investigated.

The stocks of PK, PK vir, P2, and T4 phages were diluted in nutrient broth to contain approximately 4 × 10⁵ particles per ml. The antisera to PK vir, P2, T4, and colicine K were also diluted appropriately in broth. One ml of the virus under study was mixed with 1 ml of each antiserum dilution and the mixture was incubated at 37°C. Equal volumes of virus and broth served as a control. 0.1 ml aliquots of each mixture were then removed at various time intervals and assayed on Sh. sonnei E90 or, in the case of P2 phage, on E. coli C-1. From the results of these assays the neutralization constants of each phage in each of the antisera were calculated (12). The results of these experiments are presented in Table IV.

It may be seen from the table that the antiserum to PK vir phage neutralized both PK and PK vir phage at similar rates but reacted more slowly with P2.

TABLE IV

Neutralization of Bacteriophages by Antisera

Antiserum	Phage			
	PK	PK vir	P2	T4
PK vir	329	417	79	<0.1
P2	164	277	501	<0.1
T4	<0.1	<0.1	0.1	1680
Colicine K	0.1	<0.1	<0.1	<0.1

Figures in the table indicate neutralization constant (K/minutes).

The antiserum to P2 virus also reacted with the homologous phage and with the two PK viruses. In this instance, however, P2 was neutralized more rapidly than the two PK viruses. From this it is apparent that PK and PK vir have essentially the same serological specificity and are immunologically related to P2. Furthermore, the PK vir and P2 antisera did not inactivate T4 virus and the T4 antiserum failed to react with PK and P2 phages. It is evident, therefore, that there is no immunological relationship between the PK and T4 phage. Finally, the data revealed that the colicine K antiserum did not significantly neutralize any of the phages tested, a fact which indicates that the viruses and the bacteriocine have different serological specificities.

The action of the antisera on colicine K was tested in the following manner.

0.3 ml of the various antiserum was added to 2.5 ml of soft agar containing 5 × 10⁷ cells of E. coli B, and the mixtures were plated. 0.02 ml aliquots of serial dilutions of colicine K containing between 20 and 0.6 μg per ml were placed on the surface of the agar and the plates incubated at 37°C. After 6 hours the plates were examined for inhibition of bacterial growth. The data so obtained are given in Table V.

It can be seen that colicine K, at concentrations of up to 20 μg/ml, was completely neutralized by the homologous antiserum. In contrast to this, the antisera to PK *vir*, P2, and T4 phages failed to neutralize colicine K even at a lowest concentration of the bacteriocine. These results strengthen the previous conclusion that there is no serological relationship between colicine K, and PK, P2, or T4 phages.

Mixed Infections with PK, P2, and T4 Phages.—It is evident from the data presented that PK and P2 phages resemble each other morphologically and have similar host ranges and serological specificities. Furthermore, it is apparent that PK and T4 phages also have similar host ranges yet they differ in the other two characteristics. In order to learn something of the genetic relationship of the phages in question, bacteria were infected with either PK *vir* and T4

TABLE V

Neutralization of Colicine K by Antisera

Antiserum	Concentration of colicine K (μg/ml)					
	20	10	5	2.5	1.25	0.6
PK *vir*	4	4	4	4	4	3
P2	4	4	4	4	4	3
T4	4	4	4	4	4	3
Colicine K	1	0	0	0	0	0

4 = complete inhibition of bacterial growth; 1 to 3 = partial inhibition; 0 = no inhibition.

or PK *vir* and P2 *vir* viruses and the progeny of single infected cells was investigated. *Sh. sonnei* E90 was used as the host for the first two phages; *E. coli* C-1 was employed in the experiments with the other two viruses. The latter was selected because P2 *vir* plates on the Sonne bacillus with a lower efficiency (0.05 to 0.1) than on the colon bacillus.

First, the latent period of PK *vir* phages in the two bacterial strains was determined by a one-step growth technique (12). It was found that this virus is released from *Sh. sonnei* E90 after 23 to 24 minutes with an average burst size of 150 particles per bacterium. *E. coli* C-1 liberated the phage more slowly. In this host, the latent period of the virus was found to be 35 to 36 minutes and the average burst size 90 phages per infected cell. Thus, it is apparent that PK *vir* grows in the Sonne bacillus as fast as does T4 in *Sh. sonnei* Phase II (latent period 23.5 to 24.5 minutes) and that it multiplies in *E. coli* C-1 at a rate similar to that of P2 (latent period 30 to 32 minutes) (22, 7).

The mixed infection of bacteria with PK *vir* and T4 or PK *vir* and P2 *vir* phages was carried out as follows:

A culture of *Sh. sonnei* E90 or *E. coli* C-1 was grown in nutrient broth until it contained about 2 × 10⁸ cells/ml. The growth of bacteria was stopped by adding KCN (to 0.02 M)

and the number of viable cells was determined by plating. An aliquot was now infected with an equal volume of a mixture of the phages under study at a multiplicity 11 to 21 P/B and the infected culture was incubated for 10 minutes at 37°C. It was then diluted tenfold in an appropriate mixture of antiphage sera and incubated for 5 additional minutes to neutralize unabsorbed phage. Finally, the culture was diluted in broth to contain 0.5 to 1 B/ml and the viral progeny of the individual cells was isolated by the means of single burst technique (12). *Sh. sonnei* E90 and E90/4 were used as a mixed indicator for PK *vir* and T4, whereas *E. coli* C-1 and *Sh. sonnei* E90/P2 were employed to distinguish PK *vir* and P2 *vir*. The results of these experiments are presented in Table VI.

The table reveals that bacterial cells infected with PK *vir* and T4 phages produced progeny containing either one or the other of the parental viruses, but never both. The two viruses exclude each other in mixed infections. Since

TABLE VI

Mixed Infections of Bacteria with Phages

	PK *vir* and T4	PK *vir* and P2 *vir*
1. Phages used for infection	PK *vir* and T4	PK *vir* and P2 *vir*
2. No. of experiments	2	4
3. Multiplicity of infection (P/B)	11.5–12.6	12.6–21.2
4. Phage ratio (PK *vir*/P2 *vir* or T4)	0.83–0.85	0.36–1.25
5. No. of bacteria per dilution tube	0.12–0.13	0.08–0.21
6. No. of dilution tubes plated	160	318
7. No. of bursts expected	20	40
8. No. of bursts observed	10	28
9. No. of bursts containing:		
a. Mixed progeny	0	9
b. PK *vir*	5	10
c. P2 *vir* or T4	5	9

they differ in many other characteristics as well, it is obvious that they are not related genetically.

The results of mixed infections with PK *vir* and P2 *vir* phages are different. In this instance, nearly a third of the infected cells formed progeny containing both parental viruses, whereas the remaining portion formed but one of the phages. These facts indicate that the two phages are able to multiply simultaneously in the same bacterial cell and that they exhibit a strong tendency to exclude one another.

Whether PK and P2 phages are genetically related cannot yet be answered with certainty for there have been reports that bacteria infected with unrelated phages produced mixed yields (23). Nevertheless, the facts that PK and P2 phages have similar host ranges, that they are morphologically and serologically related, and that their virulent mutants are capable of growing in the same bacterial cell, strongly indicate that a genetic relationship between these phages may exist.

The Relationship between PK Prophage and Colicinogenic Factor of E. coli K235.—From the data which have been presented, it is quite clear that PK phage and colicine K differ in host range and in serological specificity. Despite this, it is still possible that PK prophage may play a role in colicinogenesis for it is known that the formation of toxin in *Corynebacterium diphtheriae* is induced by a phage which bears no serological relationship to the toxin itself (24). Furthermore, certain *Salmonellae* phages are capable of inducing the formation of antigenic components in their host (25). It was necessary, therefore, to determine whether PK phage might not be capable of eliciting colicine synthesis in the host bacterium upon lysogenization of the latter.

Sh. sonnei E90 was lysogenized with PK phage as described earlier and five strains were derived from single colonies. Each of these was found to be resistant to PK phage and to liberate the virus spontaneously. A colony of each strain was then grown on nutrient agar plates and the bacteria killed with chloroform vapor. 2.5 ml of soft agar containing 5×10^7 cells of *E. coli* B were poured on each and the plates were incubated for 6 hours.

Upon examining the plates it was found that in all instances there was no inhibition of growth of the test organism in the vicinity of the dead colonies of lysogenic *Sh. sonnei* E90. None of the strains tested was capable of producing the bacteriocine.

In order to ascertain whether the phage carried by lysogenic *Sh. sonnei* E90 is identical with PK, the virus was isolated and characterized.

A culture of lysogenic *Sh. sonnei* E90 in nutrient broth was grown for 5 hours and killed with chloroform. After removal of the cells the supernatant was plated on *Sh. sonnei* E90. As before, five single plaques were isolated and viral stocks prepared from each. The latter were assayed and their host range and serological specificity determined.

It was found that the viral stocks contained 8×10^5 to 3.1×10^6 P/ml which formed plaques on *Sh. sonnei* E90 and that the plaques were morphologically identical with those of PK virus. These phages attacked *Sh. sonnei* strains E90/2, E90/4, E90/6, and E90/K, plated on *E. coli* B with a low efficiency and failed to grow on *Sh. sonnei* E90/3,4,7 and E90/PK *vir*. The antiserum to PK *vir* phage neutralized the viruses in question, yet T4 antiserum had no effect upon their infectivity. Thus, it is apparent that the phage carried by lysogenic *Sh. sonnei* E90 has the same plating characteristics and serological specificity as PK virus. Since the *Sh. sonnei* E90 strains which liberate this virus contain prophage but do not produce bacteriocine, it is evident that PK prophage by itself does not induce colicinogenesis in this bacillus.

Finally, it was necessary to determine whether PK prophage might not induce colicine K synthesis in *E. coli* K235. An answer to this question might be had were it possible to lysogenize with PK phage a strain of this bacillus which is neither colicinogenic nor a carrier of phage. Since such a strain is unavailable, a study was made of the virus carried by the non-colicinogenic strain of *E. coli*

K235 L⁻OC⁻. The bacillus is a mutant of the colicinogenic *E. coli* K235 which has lost its ability to produce colicine K and yet is lysogenic (11).

A culture of *E. coli* K235 L⁻OC⁻ was grown in nutrient broth and killed with chloroform. Aliquots of the cell-free supernatant were plated on *Sh. sonnei* E90, five plaques were isolated and stocks were prepared as before. The phage stocks were then assayed on *Sh. sonnei* E90 and *E. coli* B and their host range and serological specificity were also determined.

Upon examining the plates it was found that the five phage strains formed plaques on *Sh. sonnei* E90 which resembled those produced by PK phage. The titer of these stocks varied between 1.0×10^5 and 1.8×10^6 particles per ml. When assayed on *E. coli* B, the phages plated with a low efficiency (0.01 to 0.06). They also grew on *Sh. sonnei* strains E90/2, E90/4, E90/6, and E90/K, but failed to attack the E90/3,4,7 and E90/PK *vir* strains. Finally, the serological tests revealed that these phages were neutralized by the PK *vir* antiserum and were unaffected either by T4 or by colicine K antisera. Because of this, the virus of the non-colicinogenic bacillus must be identical with PK and hence, *E. coli* K235 L⁻OC⁻ must carry PK prophage. Since neither this bacillus nor the lysogenic *Sh. sonnei* E90 (PK) produces colicine K, it must be concluded that PK prophage by itself does not elicit the production of the bacteriocine in its host. It is obvious, therefore, that the synthesis of colicine K and the elaboration of PK phage in the colicinogenic strain of *E. coli* K235 L⁺OC⁺ are controlled by independent genetic determinants.

DISCUSSION

The temperate bacteriophages of the coli-dysentery group have been studied extensively during the last decade. Those best characterized are the P1 and P2 phages derived from *E. coli* Lisbonne, and the λ virus isolated from *E. coli* K12. Because these three phages differ in many of their characteristics, they are regarded as members of three distinct viral species (7).

The study reported in this communication has revealed that the phage liberated by the colicinogenic bacillus *E. coli* K235, termed PK, and P2 virus have many properties in common, yet they exhibit differences. The two phages resemble each other morphologically, but differ in size. They have similar host-range specificities, for in most instances they attack the same *Sh. sonnei* and *E. coli* strains. However, their host ranges are not identical, for a mutant of *Sh. sonnei* has been isolated which is resistant to P2 phage yet sensitive to PK. The two phages are also similar in that they exhibit host-controlled variations. Furthermore their prophages confer upon their host immunity against infection with either of the viruses. In addition, the two viruses are serologically related, though this relationship is not close as evidenced by the fact that their antisera neutralize the homologous phage more rapidly than the heterologous virus. Thus, PK and P2 phages resemble each other in many respects. Because of

this and because the virulent mutants of these phages will grow simultaneously in the same bacterial cell, it is possible that the two are genetically related. It is not unlikely, therefore, that they are members of the same species of temperate coli-dysentery viruses.

Our investigations have also revealed that there is no relationship between the phage and bacteriocine produced by *E. coli* K235. The host range of PK virus and the action spectrum of colicine K were found to be different, as were their immunological specificities. In addition, *Sh. sonnei* E90, lysogenized with PK phage, failed to produce colicine K, and a mutant of *E. coli* K235 which had lost its ability to produce the colicine still liberated PK virus. It is evident, therefore, that colicine K and PK phage have no demonstrable characteristics in common and that PK prophage by itself is incapable of inducing colicinogenesis in its host. From this it can be concluded, that the formation of colicine K and of phage PK in *E. coli* K235 are independent processes controlled by different genetic determinants.

There is, however, a certain similarity between colicine K, PK virus, and T-even phages. The bacteriocine and T6, on the one hand, and PK and T4, on the other, frequently attack the same bacterial strains. It is tempting to speculate that these four agents might have a common precursor. However, a closer scrutiny of their properties has revealed that the host ranges of PK and T4 and those of T6 and colicine K are not identical, for certain mutants of *Sh. sonnei* E90 were resistant to T4 and sensitive to PK, whereas others were susceptible to T6 and immune to the action of the bacteriocine. Furthermore, since PK and T4 phages, as well as T6 and colicine K (26, 11) are not related immunologically their protein components must be dissimilar. Finally, our study has established the fact that PK and T4 phages are not related genetically. Since PK and T4 phages, as well as colicine K and T6, have different characteristics, their similarities in host-range specificity are in all probability fortuitous.

SUMMARY

A temperate bacteriophage was isolated from the colicinogenic strain of *Escherichia coli* K235 and characterized. This phage, termed PK, is related to P2 virus morphologically, serologically, and, possibly, genetically and it bears no relationship to the T-even phages. It was also demonstrated that PK virus and colicine K differ both in their host range and in their immunological specificity, and that PK prophage does not induce the colicinogenesis in its host bacterium. It was concluded that the formation of colicine K and PK phage in *E. coli* K235 are controlled by different genetic determinants.

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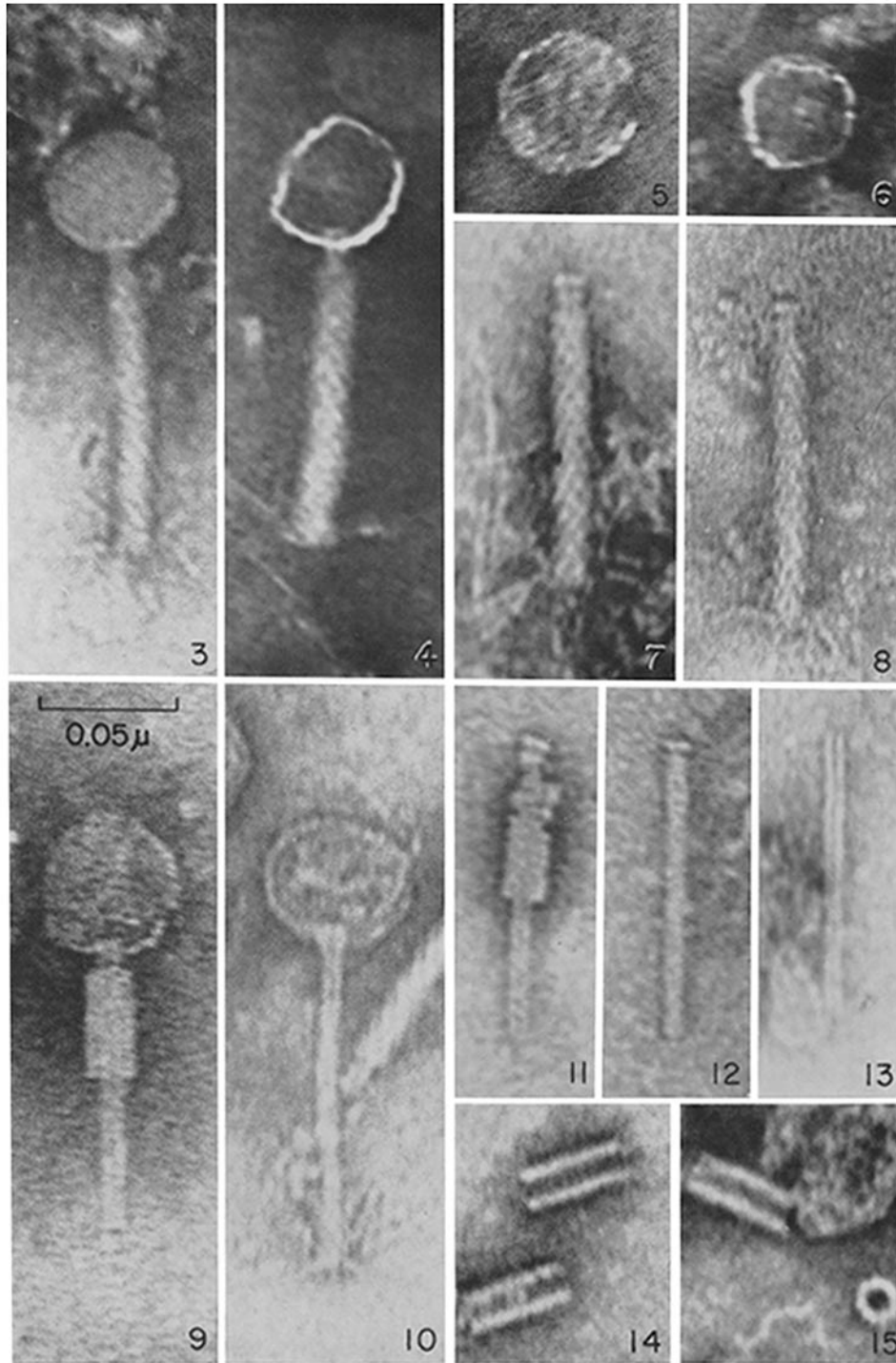
EXPLANATION OF PLATES

PLATE 7

FIGS. 1 and 2. PK and PK *vir* phages. The viruses were stained negatively with sodium phosphotungstate. × 230,000.
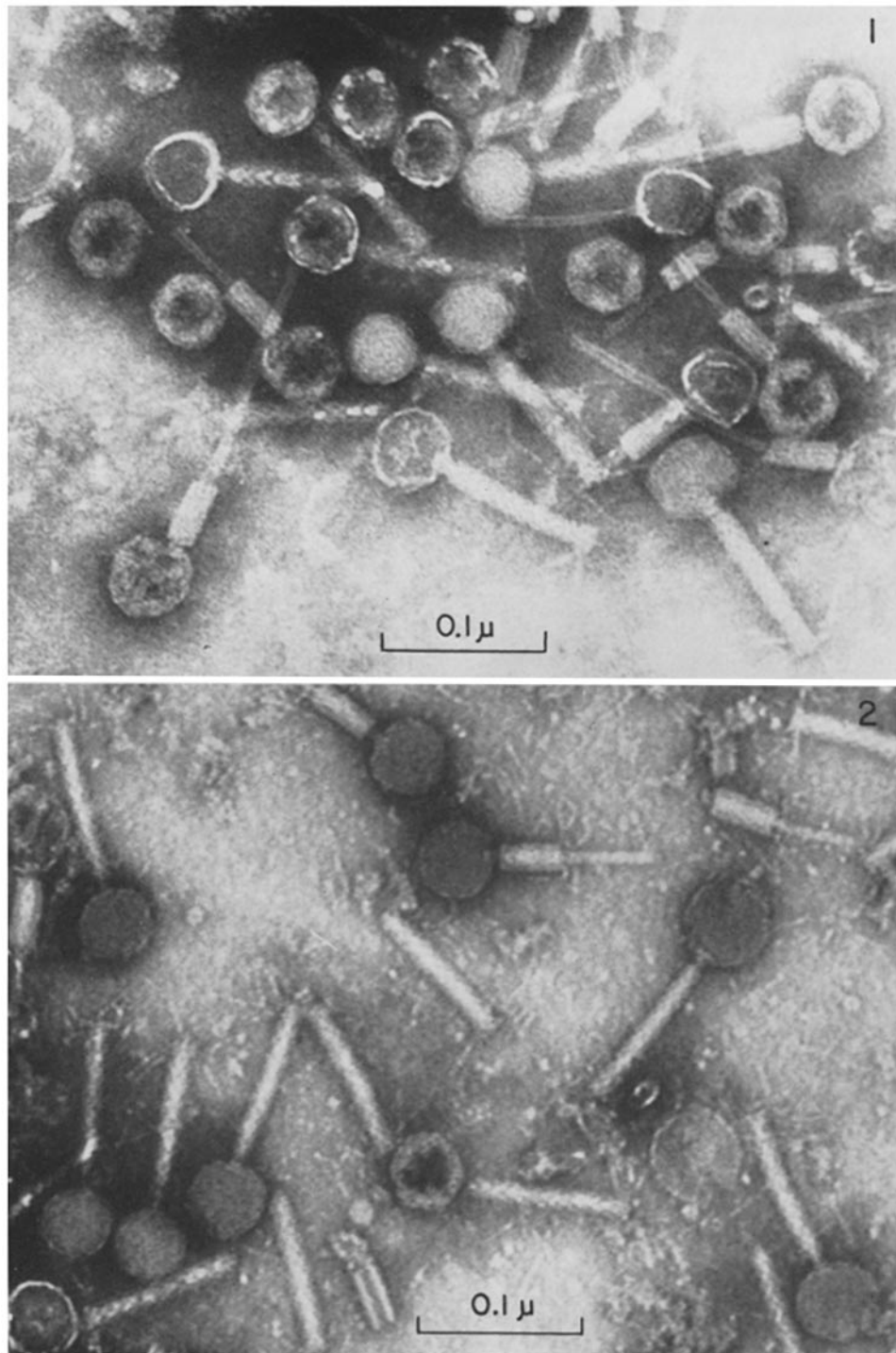
(Jesaitis and Hutton: Bacteriophage from *Escherichia coli* K235)

FIGS. 3 to 15. PK virus and its structural components. Fig. 3. Intact phage. Fig. 4. Phage "ghost". Figs. 5 and 6. Head membranes. Figs. 7 and 8. Phage tails. Fig. 9. Phage with contracted sheath. Fig. 10. Phage without sheath. Fig. 11. Phage tail with contracted sheath. Figs. 12 and 13. Tail cores. Figs. 14 and 15. Contracted sheaths. The specimens were stained negatively with sodium phosphotungstate. The micrographs presented in this communication were taken by Dr. S. Dales. × 380,000.

(Jesaitis and Hutton: Bacteriophage from *Escherichia coli* K235)