STUDIES OF A BACTERIOPHAGE ACTIVE AGAINST A CHROMOGENIC NEISSERIA

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A previous report from these laboratories (Stone *et al.*, 1953) described a bacteriophage active against one of the chromogenic nasopharyngeal neisseriae. The purpose of this paper is to describe further studies of the bacteriophage and the host organism.

MATERIALS AND METHODS

Cultures and media. Of the 36 strains of *Neisseria* employed in this study, two strains (155B and 157) were obtained from Dr. S. E. Branham, National Institutes of Health; one strain (10555) from the American Type Culture Collection; one strain (W) from Dr. D. Walcher, Riley Memorial Hospital, Indianapolis, Indiana; and one (B) from Dr. W. Burrows, University of Chicago. Eight strains (1707, 1711, 1712, 1713, 681, 682, 683, and 684) were Lilly stock strains. The remaining strains were recent isolates from human nasopharyngeal washings. These latter strains were classified according to pigment production and carbohydrate fermentation (Breed *et al.*, 1948) with the exception of four atypical strains (K1, B3, GB1, and K4), which were designated *Neisseria species*.

The cultures were maintained on a glucose starch agar (Difco) to which had been added 0.1 per cent cysteine hydrochloride and 0.5 per cent yeast extract (NM V). Two basal liquid media were used with or without the addition of one or more salts. A concentrated nutrient broth (NB) contained 1 per cent dehydrated nutrient broth (Difco). The second liquid medium (NM XX) contained cysteine hydrochloride, 1 g; proteose peptone #3 (Difco), 15g; glucose, 2g; NaCl, 5 g; $Na_2HPO_4 \cdot 7H_2O$, 5.6 g; yeast extract (Difco), 5 g; and soluble starch, 10 g, in 1 L of distilled water, and was adjusted to pH 7.2. Additions of salts to the basal media were made by adding those with univalent cations as the dry salts prior to sterilization and those with divalent cations aseptically from concentrated aqueous solutions.

Electron microscopy. Plates of NM V were covered with a thin formvar film. The surface of each film was lightly inoculated with a mixture of actively growing host organisms and diluted bacteriophage lysate. Following 8- to 16-hr incubation at 37 C, portions of the films were floated onto water, transferred to grids, washed with a drop of distilled water, and dried over $CaCl_2$. The preparations were shadow cast and examined in the electron microscope at 10,000 × magnification.

Bacteria and bacteriophage counting. Bacterial counts of cultures of the host organism were made by determination of optical density and reference to appropriate optical density-viable cell count curves. Measurements were made with a Cenco photelometer type C5 using a 525 mμ filter. Bacteriophage titers were determined by a modification of the agar layer method of Gratia (1936). Seed suspensions were prepared by diluting 24-hr NM XX cultures of the host organism in NM XX so as to contain 10^8 organisms per ml. Serial decimal dilutions of the filtrate or lysate to be titered were made in distilled water or broth. Base layers of 40 ml of NM V in 9-cm petri plates were each overlaid with a seed layer composed of 2 ml of melted NM V at 52 C, 0.5 ml of seed suspension, and 0.5 ml of a phage dilution. The plates were incubated at 37 C for 18 hours and the plaques counted. Titers were computed from counts of duplicate plates.

Effects of cations. The effects of various cations on bacteriophage multiplication were determined by incorporating appropriate salts into NB, inoculating the media with the bacteriophage and host bacteria, and determining the content of bacteriophage in the supernatant following incubation. The media were tubed in 5-ml amounts and inoculated with 0.1 ml of a 24-hour culture of the host organism in NB and 0.1 ml of an aqueous dilution of phage lysate containing approximately 5 × 10^2 phage particles. Duplicate

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series were inoculated with host only. Following 24 hours' incubation at 37 C the growth of the host was evaluated. The series inoculated with phage were shaken to disperse the growth and placed at 5 C for 20 hours, following which the supernatant fluids were decanted and titered for phage.

Host range determination. The agar layer procedure was employed in determining the susceptibility of various strains of *Neisseria* to neisseriaphage A. Approximately 10^3 phage particles in aqueous suspensions and 0.5 ml of a 24-hr NM XX culture of the test organism were included in the seed layer and the plates were examined for plaques at 6-hour intervals during 72 hours' incubation at 37 C.

Tests for lysogenicity. Test organisms were seeded into NM XX containing 10^{-3} M $CaCl_2$ and incubated at 37 C for 24 hours, then at 5 C for 20 hours. The supernatant fluids were then decanted and titered for phage by the agar layer procedure.

RESULTS

Characteristics of the host organism. The host organism, designated strain 7A, produced smooth, circular, 2.0 to 2.5-mm, slightly raised colonies when streaked onto NM V and incubated 48 hours at 37 C. The growth was semi-opaque, yellowish-gray, glistening, and had a butyrous consistency. Gram-stained preparations showed gram negative diplococci 0.6–0.8 μ in diameter and the colonies gave a positive oxidase reaction with *p*-aminodimethylaniline HCl. The organism fermented glucose, maltose, sucrose, and fructose, but not mannite, when tested in phenol red broth media containing 0.5 per cent of these carbohydrates. A polysaccharide of the starch-glycogen class was produced on sucrose media (Hehre and Hamilton, 1948). A greenish-yellow pigment was produced on Loeffler's medium. Antisera prepared in rabbits against strain 7A and against ATCC strain 10555 of *Neisseria perflava* agglutinated the two organisms in similar dilutions.

With the exception of the failure to ferment mannite, strain 7A conformed with the description of *N. perflava* in *Bergey's Manual* (Breed *et al.*, 1948). None of the neisseriae studied in this laboratory to date have fermented mannite. This absence of mannite fermentation in strains otherwise typical of *N. perflava* has been noted elsewhere (Hajek *et al.*, 1950; Wax, 1950), raising some doubts as to the value of mannite in identification procedures. Therefore, the strain 7A and similar strains described in this study have been assigned the species name *N. perflava.*

Morphology of the phage particle. The bacteriophage, designated neisseriaphage A, was examined by electron microscopy. Formvar films from plates upon which the infected host cells had been growing showed typical tadpole-shaped particles. The oval head portion of the phage was approximately 50 × 70 mμ and the straight tail measured approxmately 20 × 160 mμ.

Effect of cations on phage multiplication. Since it was observed that cultures of strain 7A in NB containing 0.85 per cent NaCl failed to support multiplication of the neisseriaphage, attention was directed to the effects of metallic ions upon multiplication of the phage. The addition of $CaCl_2$ was found to reverse the action of the NaCl. Table 1 shows the effect of sodium and calcium ions on neisseriaphage multiplication in NB. The presence of 0.1 M or more of sodium chloride was sufficient to prevent multiplication of the phage in the medium; however, the addition of 0.001 M calcium chloride allowed phage multiplication in the presence of 0.2 M sodium chloride.

The effect of a univalent cation other than sodium ion on neisseria-phage multiplication in NB was determined. The effect of potassium ion is shown in table 2. Potassium chloride in a concentration of 0.1 M suppressed the multiplication of neisseriaphage A. The addition of 0.001 M calcium chloride reversed the effect of potassium ion. That the reversal effect was due to the

TABLE 1

The effect of Na+ and Ca++ on neisseriaphage A multiplication

NaCl concentration	Phage titer in media containing NaCl	
	NB	NB + 10^{-3} M $CaCl_2$
1 M*	1.0×10^8	4.8×10^1
2×10^{-1} M	0	9.7×10^7
10^{-1} M	8.9×10^1	2.7×10^8
10^{-2} M	2.2×10^8	6.2×10^7
0.0	2.1×10^8	4.2×10^7

* No growth of the host organism at this concentration. In lower NaCl concentrations control cultures (w/o phage) show optimal growth.

TABLE 2

The effect of K+ and Ca++ on neisseriaphage A multiplication

KCl concentration	Phage titer in media containing KCl			
	NB	NB + 10^{-3} M $CaCl_2$	NB + 10^{-3} M $CaSO_4$	NB + 10^{-3} M Ca acetate
10^{-1}M	9.6×10^{5}	3.4×10^{8}	1.2×10^{8}	2.9×10^{7}
10^{-2}M	5.0×10^{8}			
0.0	1.9×10^{8}			

calcium ion, rather than to the anion present, was shown by the fact that three salts of calcium were almost equally effective.

Visual observation of the growth of the host organism in media containing added NaCl or KCl suggested that the addition of concentrations of salts sufficient to suppress bacteriophage multiplication did not quantitatively affect the growth of the host. However, the possibility that the univalent salts were only indirectly interfering with phage multiplication through a primary effect of reducing the host population was investigated. The comparative effect of univalent salts on bacterial growth and phage multiplication was determined. Bacterial cell counts (based on turbidimetric data) indicated that concentrations of NaCl or KCl ranging from 2.5×10^{-2} M to 2.0×10^{-1} M had no significant effect on bacterial growth. However, the higher concentrations of either salt completely suppressed bacteriophage multiplication (table 3).

Divalent cations other than Ca^{++} were found to reverse the effect of univalent cations on phage multiplication. The relative amounts of Ca^{++} and other divalent cations required to reverse the action of Na^{+} in NB containing $1.5 \times$ 10^{-1} M NaCl are shown in table 4. Fairly high concentrations of some salts were required and the effect of the divalent ions on the host organism became a limiting factor. Concentrations tenfold higher than those shown in the table resulted in inhibition of bacterial growth.

TABLE 3

The effect of Na+ and K+ on neisseriaphage A multiplication in NB

Salt added	Phage count/ml
NaCl	
2×10^{-1} M	2.8×10^{2}
1×10^{-1} M	5.9×10^{1}
5×10^{-2} M	6.8×10^{5}
2.5×10^{-2} M	1.4×10^{8}
KCl	
2×10^{-1} M	3.0×10^{2}
1×10^{-1} M	6.4×10^{1}
5×10^{-2} M	2.0×10^{7}
2.5×10^{-2} M	5.6×10^{8}
None	1.9×10^{8}

TABLE 4

Reversal of Na+ action on neisseriaphage A multiplication by divalent cations

Divalent cation added	Phage titer in NaCl NB	Divalent cation added	Phage titer in NaCl NB
$CaCl_2$		$BaCl_2$	
10^{-2} M	7.5×10^{7}	10^{-2} M	1.1×10^{9}
10^{-3} M	5.0×10^{8}	10^{-3} M	4.3×10^{8}
10^{-4} M	5.0×10^{4}	10^{-4} M	1.3×10^{5}
10^{-5} M	2.5×10^{3}	10^{-5} M	2.6×10^{2}
10^{-6} M	4.8×10^{2}		
10^{-7} M	2.5×10^{2}	$MnCl_2$	
$MgCl_2$		10^{-3} M	3.2×10^{5}
10^{-2} M	9.0×10^{5}	10^{-4} M	1.0×10^{4}
10^{-3} M	1.4×10^{5}	10^{-5} M	2.4×10^{2}
10^{-4} M	2.9×10^{2}	None	6.3×10^{1}

Host range and lysogenicity study. A series of strains of *N. perflava* (H1, KP3R, P5-2, 10555, J1, J3, K2, K3, L1, L2, C1, C2, C3, K5, P1, and P2), several strains of the closely related species in the "pharyngis" group (*N. subflava* L3; *N. sicca* M2, P4; *N. species* K1, B3, GB1, K4), and strains of *N. flavescens* (155B, 157), *N. catarrhalis* (B, 1706, 1711, 1712, 1713), and *N. meningitidis* (W) were tested for susceptibility to neisseriaphage A by the agar layer technique. None of the strains were susceptible to the bacteriophage. Due to the stringent growth requirements of *N. gonorrhoeae*, the phage susceptibility of this organism could not be tested by the agar layer technique. There was, however, no evidence of multiplication of neisseriaphage A when it was added to cultures of these strains (681, 682, 683, and 685) growing in NM XX containing 10^{-3} M $CaCl_2$. Phage assays of the supernatants of such cultures after 24 hours' incubation showed 61 to 68 per cent of the input phage, while in control tubes incubated with no bacterial inoculum 74 per cent of the input phage was detectable.

All of the above Neisseria strains were tested for lysogenicity for the host strain 7A. Two strains of *N. perflava*, P1 and P2, were found to carry phage active against this strain. None of the other species were lysogenic for strain 7A. NM XX cultures filtrates of strains P1 and P2 were plated with strain 7A and single plaque

isolations of the phages were made. These two phages, A1 (from P1) and A2 (from P2), were then compared with neisseriaphage A. The plaques produced by the three phages on strain 7A were identical. When seeded with strain 7A into tubes of NB and NM XX + 10^{-3} M $CaCl_2$ the three phages produced lysates with similar phage titers. Since the two bacterial strains P1 and P2 were isolated (at a later date) from the same individual as were the host organism 7A and the phage A, it is highly probable that P1 and P2 are lysogenic substrains of 7A and that the phages are identical to phage A.

In order to determine whether strains P1 and P2 were true lysogenic strains or were merely carrier strains—a mixture of resistant (nonlysogenic) cells, sensitive cells, and phage—the release of phage by the two strains was studied during serial transfers in NB and in NB containing 1.5×10^{-1} M NaCl. Six transfers were made at 24-hour intervals by inoculating 9.9 ml of fresh medium with 0.1 ml of the previous culture. Thus a 10^{-12} dilution of the original inoculum (containing 10^2 to 10^3 phage particles) was effected. If maintenance of the phage in P1 or P2 depended upon lysis of sensitive organisms in the culture then, since phage multiplication in sensitive organisms was blocked in the medium containing added NaCl, the phage content of these cultures would be expected to decline and eventually be eliminated by dilution. The amount of phage released by both P1 and P2 remained markedly constant during serial transfer and was unaffected by the presence of high concentrations of NaCl. Approximately 3×10^3 phage particles per ml were detectable in cultures of P1 after 24-hour incubation of either medium following each of the six transfers. Strain P2 released a larger number (8.4×10^4 to 1.7×10^5 particles per ml of supernatant) following each transfer in either of the media. It was therefore concluded that the two strains were lysogenic rather than carrier strains.

Lysogenic substrains of 7A were also developed by inoculating the organism onto NM V agar layers, containing 10^8 neisseriaphage A particles per 2 ml agar overlay, and incubating the plates 48 hours at 37 C. Colonies were picked from these plates and subcultured on similar plates. Colonies picked from the second series of plates were subcultured in NB containing 1.5×10^{-1} M NaCl. Six 1:100 dilution transfers were made at 24-hour intervals in this medium to free the culture of any extracellular phage which might have been mechanically transferred from the plates. The phage-resistant substrains isolated in this manner were found to be of two types—lysogenic and nonlysogenic. Twenty-four-hour NB supernatants of the lysogenic substrains contained 10^4 to 10^5 phage particles per ml while culture supernatants of the remaining substrains contained no detectable phage.

Phage-resistant substrains of *N. perflava* 7A were also isolated in the absence of phage A. Old cultures of the parent strain were occasionally found to contain resistant forms and several such substrains were isolated. These substrains did not regain phage sensitivity on subculture in fresh media and were not lysogenic for the parent strain.

DISCUSSION

The host organism, isolated from throat washings from a human subject and designated strain 7A, is one of the chromogenic neisseriae. The classification of the organisms in this group is in dispute (Wilson and Miles, 1946). Breed *et al.* (1948) recognize four species, defining these according to colony characteristics, pigment production and sugar fermentation. Many strains of chromogenic neisseriae—including the present one—do not conform to the description of any of the recognized species; some occupy intermediate positions. Furthermore, the reliability of one of the fermentation reactions (mannite fermentation) upon which this classification is based, has been questioned (Hajek *et al.*, 1950). Since the host organism differed from the description of *N. perflava* only in its failure to ferment this carbohydrate, it has been designated *N. perflava.*

The structure of the bacteriophage particle as revealed by electron micrography conforms to that of many previously described bacteriophage particles. In size the particle approximates that of the smaller, tailed coliphages of the T series.

The effects of divalent cations on phage multiplication have been described by other workers; a summary of this literature has been presented by Fildes *et al.* (1953). These reports have generally described the promotion of phage activity by Ca^{++} and other divalent metallic ions, without reference to the effects of any univalent cations present in the same system. From the results of our experiments it would appear that divalent cations are required for neisseriaphage A activity only in the presence of a

certain minimum level of univalent cations. Nutrient broth, containing no added Na^+ or K^+, would support phage multiplication without the addition of Ca^{++} or other divalent cations. Whether this indicates that there is no absolute requirement for divalent cation is difficult to ascertain. Trace amounts of both univalent and divalent cations are present in the basal medium. Fildes *et al.* (1953) have described the difficulties involved in obtaining systems free of Ca^{++} In order to determine whether the dependence of bacteriophage multiplication on divalent cations was an absolute or a relative one, a system free of both types of cations would be required, unless some indirect method were devised. The results of the present experiments would indicate, however, that any measure of the effect of divalent cations must take into account the presence of univalent cations. Ion antagonism phenomena, shown by Burnet and McKie (1930) to affect bacteriophage inactivation, may be exerting an effect on bacteriophage multiplication.

The failure to find other hosts for neisseriaphage A among the strains of *N. perflava* and related species tested is not surprising in view of the small number of strains tested. However, it might be interpreted as indicating that the bacteriophage does not have a particularly wide host range.

The isolation of lysogenic strains of *N. perflava* from throat washings and the production *in vitro* of lysogenic substrains of strain 7A demonstrate that neisseriaphage A conforms to the description of a temperate phage (Lwoff, 1953). Conditions restricting sensitive cell growth (i. e., presence of phage or culture aging) also permitted the isolation of phage-resistant substrains which are nonlysogenic. These two types of resistant cells, lysogenic and non-lysogenic, may increase the utility of this host-virus system in studies of bacteriophage phenomena.

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SUMMARY

Some characteristics of a bacteriophage and of its host, one of the chromogenic nasopharyngeal neisseriae, are described.

The host organism was provisionally identified as *Neisseria perflava*.

Certain metallic ions were shown to have a pronounced effect on multiplication of the bacteriophage. Concentrations of 0.1 M or higher of Na^+ or K^+ suppressed bacteriophage multiplication. Ca^{++} and Ba^{++} in 0.001 M concentrations completely reversed this effect; similar concentrations of Mg^{++} and Mn^{++} partially reversed the suppressive effect.

The host range of the bacteriophage was investigated and a series of neisserian species was tested for lysogenicity for the host organism. Lysogenic and nonlysogenic phage-resistant substrains of the host organism were described.

REFERENCES

BREED, R. S., MURRAY, E. G. D. AND HITCHENS, A. P. 1948 *Bergey's manual of determinative bacteriology*. 6th ed. The Williams & Wilkins Company, Baltimore, Md.

BURNET, F. M. AND MCKIE, M. 1930 Balanced salt action as manifested in bacteriophage phenomena. Australian J. Exptl. Biol. Med. Sci., **7**, 183–198.

FILDES, P., KAY, D. AND JOKLIK, W. K. 1953 Divalent metals in phage production. In *The nature of virus multiplication*. Cambridge University Press, Oxford, England.

GRATIA, A. 1936 Numerical relations between lysogenic bacteria and particles of bacteriophage. Ann. inst. Pasteur, **57**, 652–676.

HAJEK, J. P., PELCZAR, M. J., JR. AND FABER, J. E., JR. 1950 Variations in the fermentative capacity of neisseriae. Am. J. Clin. Pathol., **20**, 630–636.

HEHRE, E. J. AND HAMILTON, D. M. 1948 The conversion of sucrose to a polysaccharide of the starch-glycogen class by *Neisseria* from the pharynx. J. Bacteriol., **55**, 197–208.

LWOFF, A. L. 1953 The nature of phage reproduction. In *The nature of virus multiplication*. Cambridge University Press, Oxford, England.

STONE, R. L., CULBERTSON, C. G. AND POWELL, H. M. 1953 A bacteriophage active against a member of the genus *Neisseria*. Bacteriol. Proc. **1953**, 39.

WAX, L. 1950 The identity of *Neisseria* other than the gonococcus isolated from the genitourinary tract. J. Venereal Disease Inform., **31**, 208–213.

WILSON, G. S. AND MILES, A. A. 1946 Topley and Wilson's *Principles of bacteriology and immunity*. 3rd. ed. The Williams & Wilkins Company, Baltimore, Md.