MYCOBACTERIOPHAGE

I. Physicochemical Characterization[1]

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Subsequent to the isolation of bacteriophages for particular bacterial genera, the physical and chemical characteristics and serological relationships of many of these viruses have been determined. Although extensive attempts have not been made to characterize mycobacteriophages other than for host range, one or more of the physicochemical characteristics of a mycobacteriophage at the time of isolation have been determined.

Gardner and Weiser (1947), by the soil enrichment technique using *Mycobacterium smegmatis*, were the first to isolate a bacteriophage for mycobacteria. They found that this phage, which was active only against *M. smegmatis*, was completely inactivated at 75 C after 10 min but only partially inactivated at 72 C. Penso and Ortali (1949) isolated 5 phages which were active against different saprophytic mycobacterial species. These phages were classified on the basis of host specificity, morphology as shown by the electron microscope, and antigenic differences. Two phages for *M. smegmatis* isolated by Whittaker (1950) were found to have identical thermal death points but not the same morphology as shown by electron microscopy; one of the isolates was identical with the Gardner and Weiser phage with respect to morphology and thermal inactivation. Hnatko (1953) studied 11 mycobacteriophages which he was able to place in four groups with some overlapping on the basis of plaque type, cross-resistance tests, and serological analysis.

Froman, Will, and Bogen (1954) reported the first isolations of phages active against both saprophytic and virulent mycobacteria. They described the characteristic plaque morphology and host range specificity of mycobacteriophages D28, D29, D32, and D34; Sellers et al. (1956) determined the shape and size of these phages by electron microscopy. The present paper deals with the further characterization of D28, D29, and D32. Serological relationships, pH optima, thermal inactivation rates, and the effects of certain cations on phage survival are presented.

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MATERIALS AND METHODS

In general, standard procedures developed by numerous investigators for phage studies and summarized by Adams (1950) were employed. The phages, D28, D29, and D32, were propagated on *Mycobacterium lacticola*, *Mycobacterium tuberculosis* ATCC 607, and *Mycobacterium* sp. ATCC 9033, respectively. Stock cultures of mycobacteria were maintained on Lowenstein-Jensen agar.

For phage propagation, subcultures were made on tubes or plates of Leventhal's agar and incubated overnight. A heavy inoculum of bacteria from the subculture was inoculated into 40 ml of heart infusion broth containing 0.002 M $CaCl_2$ in a 125-ml culture tube and incubated at 37 C with active aeration for 6 to 8 hr. Phage was added and incubation continued until clearing of the culture was obtained, usually within 4 to 6 hr. Lysates with 10^{10} to 10^{11} particles per ml were regularly obtained. After filtration through sterile, washed, Seitz filter pads, the lysates were stored for several months in screw-cap bottles in the refrigerator without loss in titer. Occasionally phage was prepared in liter quantities: D29 was propagated in 2-liter bottles with active aeration, whereas D28 and D32 were prepared in Florence flasks on a rotary-type shaker. Phage was assayed either by the soft agar overlay method or by a modification of this technique. Overlay of the following composition was employed (Froman et al., 1954): heart

infusion broth (Difco), 25 g; Bordet-Gengou agar base (Difco), 12 g; proteose peptone (Difco), 10 g; glycerol, 10 ml; and distilled water, 1,000 ml. Bottom agar was prepared by the addition of 1.5 per cent agar (Difco) either to heart infusion or to nutrient broth (Difco).

Antisera were prepared by inoculating guinea pigs intraperitoneally with 3 ml of sterile lysate. Injections were given three times a week for 5 weeks, with a test bleeding taken 1 week after the last injection. Two or three courses of injections were required for the production of high-titered antisera. Guinea pigs proved to be superior to rabbits for the production of sufficiently high-titered mycobacteriophage antisera. The sera could be stored at −20 C for a period of 6 to 12 months before a loss in titer occurred.

RESULTS AND DISCUSSION

Assay procedures. At the beginning of the study, phage assays were carried out by the semisolid agar overlay method. Either heart infusion agar or nutrient agar was used for the base layer. The desirability of a simpler assay led to a comparative study of the conventional method with overlay over an agar base and a modification using a large volume of overlay alone. As can be seen (experiment 1, Table 1) semisolid agar alone or over nutrient agar base was greatly superior to semisolid agar over heart infusion agar. Further study (experiments 2 and 3, Table 1) revealed that some lots of heart infusion media were inhibitory whereas others were satisfactory. Therefore, the effect on plaque formation by each new lot of medium incorporated in the overlay was carefully evaluated before it was used. No attempt was made to determine what toxic factor(s) might be responsible for the deleterious effect observed.

Phage, then, was routinely assayed by adding the proper dilution of virus to 0.5 ml of a dense culture of bacteria suspended in 6 ml of 0.7 per cent agar overlay mixture described under Materials and Methods. The agar was evenly distributed over the bottom of a sterile petri dish and incubated for 24 to 48 hr. The plaques were distinct and easily counted, and the results compared very favorably with assay procedures employing an agar base. Thus, the necessity of preparing numerous petri dishes containing an agar base was eliminated.

Antigenic analysis. Separation of bacteriophages into groups on the basis of inactivation rates in homologous and heterologous antisera is a very reliable method of establishing relationships among them (Adams, 1950). To investigate possible serological relationships among the mycobacteriophages each virus, at a concentration of 10^7 particles per ml, was tested against a 1:1,000 dilution of the homologous and heterologous antisera at 37 C.

The results, expressed in terms of a velocity

TABLE 1

Comparison of phage counts obtained by different planting procedures using different lots of heart infusion broth in the media

Expt No.	Phage	SSA*	SSA† / HIA	SSA‡ / NA
1	D28	115	32	116
	D29	80	12	77
	D32	124	64	120
	A new lot of HIB in agar base			
2	D28	111	120	110
	D29	91	91	82
	D32	117	121	130
	A new lot of HIB in agar overlay			
3	D32	55	168	130

* Semisolid agar (6 ml) overlay mixture only, no base agar.

† Semisolid agar (2.5 ml) overlay mixture over heart infusion agar base.

‡ Semisolid agar (2.5 ml) overlay mixture over nutrient agar base.

Phage stocks were diluted and plated in triplicate employing the three different plating procedures simultaneously.

TABLE 2

Cross-neutralization tests of mycobacteriophages

Phage	*k* Value*		
	D28 Antiserum	D29 Antiserum	D32 Antiserum
D28	380	0	0
D29	0	480	0
D32	0	0	330

* Phages neutralized per minute at 95 to 99 per cent neutralization.

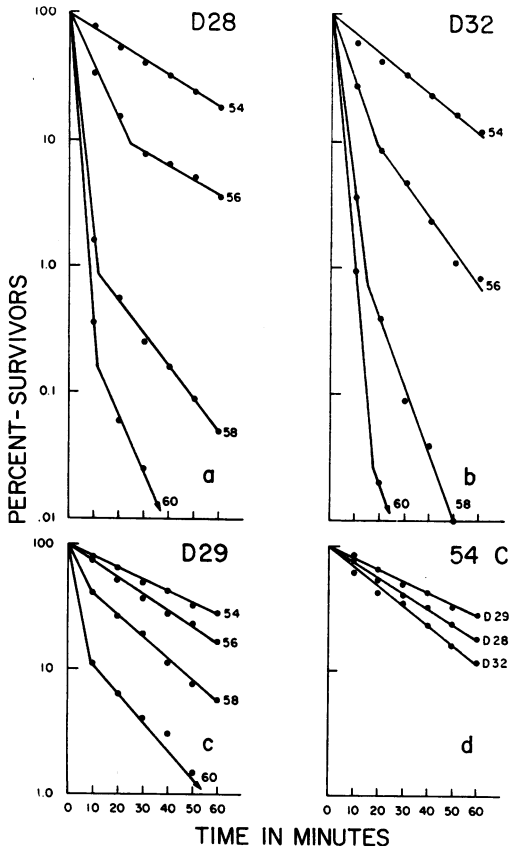

Figure 1a–d. Thermal inactivation of mycobacteriophages D28, D29, D32 at 54, 56, 58, and 60 C. Phage at a concentration of 10^7 particles per ml was incubated in a water bath for 1 hr; at 10-min intervals, samples were assayed for viable phage and the log per cent survivors plotted against time. To conserve space, the following values at 60 min are not shown: 0.8 per cent survival of D29, 0.001 per cent survival of D28, and 0.005 per cent survival of D32.

constant k, representing the fractional rate of phage neutralization per minute, are recorded in Table 2. Each phage was neutralized by the homologous antiserum only. There was no cross-neutralization by heterologous antisera which indicated each of these phages is antigenically distinct.

Thermal inactivation. In an effort to further characterize the mycobacteriophages, the effect of temperature on phage survival was studied. Phage was diluted to a concentration of 10^7 particles per ml in nutrient broth at pH 7 and incubated in a closed water bath at 50, 56, 58, and 60 C. At 10-min intervals, samples were withdrawn and assayed for the number of surviving phage particles. The per cent of viable phage was determined by comparison of the results with those for an unheated control sample.

When log per cent survivors is plotted against time (Fig. 1*a–d*) a linear relationship is obtained on exposure of all three phages to 54 C; a similar relationship is obtained for D29 at 56 C. At 56, 58, and 60 C, D28 and D32, as well as D29 at 58 and 60 C, exhibited two-component inactivation curves: a rapid-inactivation component, followed by a slower-inactivation component. Generally, two-component inactivation curves are explained on the basis of inherent population heterogeneity. However, as has been discussed by Woese (1960), this explanation cannot account for all the observed results; it is more reasonable to postulate two or more heat-mediated reactions involving different components of phage. One reaction is evident during the first part of the response and accounts for up to 90 per cent of the inactivation; the second reaction is a more thermal-resistant one and accounts for the death of 10 per cent or less of the population at higher temperatures. Woese further points out that the linear or one-component curve obtained at lower temperatures could result from the fact that at lower temperatures the reaction rate of the second component is much greater than that of the first component. Therefore the measured inactivation rate reflects predominantly the reaction responsible for the second-component of the two-component curve obtained at higher temperatures. The observed features of the thermal inactivation of mycobacteriophages appear to be similar to the characteristics of the inactivation of most, if not all, animal viruses, other bacteriophages and tobacco mosaic virus (Woese, 1960).

Of the three phages studied, D29 (Fig. 1*c* and *d*) is the most heat stable: it has single-component curves at both 54 and 56 C, and 6 and 0.08 per cent survival for 60 min at 58 and 60 C, respectively. D32 is the most heat-labile, with less than 1 per cent survival after 10 min at 60 C and after 20 min at 58 C (Fig. 1*b* and *d*). D28 is intermediate between D29 and D32 (Fig. 1*a* and *d*).

pH sensitivity. Bacteriophages vary in their sensitivity to the pH of the surrounding medium. They are stable in the vicinity of pH 6 to 8, but

TABLE 3

Effect of pH on mycobacteriophage survival

Phage	Per Cent Survival at 37 C for 1 Hr													
	Nutrient broth at pH:							Heart infusion broth at pH:						
	4	5	6	7	8	9	10	4	5	6	7	8	9	10
D28	0	16	99	100	98	100	97	0	1	94	100	100	97	65
D29	0	6	78	100	100	98	92	0	2	77	100	97	94	72
D32	0	1	67	93	91	89	65	0	7	76	94	93	90	60

Phage at a concentration of 10^7 particles per ml was suspended in the medium which had been adjusted with KOH to the specified pH. After incubation for 1 hr at 37 C, assays were made and the per cent survival of virus determined by comparison with controls simultaneously incubated at pH 7.

within each phage system individual viruses may vary, some being stable in suspensions as acid as pH 5 or as alkaline as pH 10 (Friedman and Cowles, 1953; Alexander and McCoy, 1956; Kerby et al., 1949).

For the determination of the effect of pH on the viability of mycobacteriophages, heart infusion broth and nutrient broth were adjusted to the desired pH with KOH; allowance was made for changes in pH produced by autoclaving. The pH was again checked after autoclaving. Stock phage was diluted in broth of the desired pH to a concentration of 10^7 particles per ml, incubated in a water bath at 37 C for 1 hr, and then assayed for viable phage. The per cent phage survival was determined by comparisons with controls simultaneously incubated at pH 7.

The results recorded in Table 3 show that, in nutrient broth, the pH range of greatest stability is pH 6 to 10 for D28, pH 7 to 10 for D29, and pH 7 to 9 for D32. The results in heart infusion broth are similar except for D28 and D29 at pH 10: these two phages are 33 and 22 per cent, respectively, less stable in infusion broth at this pH than in nutrient broth at the same pH. These results might be due to some component present in heart infusion broth but not in nutrient broth. One such component is sodium chloride, which is added (5 g per liter) to the infusion of beef heart used in heart infusion broth, but not to the beef extract of nutrient broth.

To determine whether the sodium chloride present in heart infusion broth could account for the diminished survival of D28 and D29 in this medium at pH 10, a comparison was made between phage survival at this pH in ordinary nutrient broth and in broth plus 0.5 per cent sodium chloride. It was found that addition of salt caused a diminished survival of both phages.

The influence of other metallic ions on phage survival was investigated: Li^+ and K^+, in addition to Na^+, were added as neutral salts to broth, adjusted to pH 10 with NH_4OH, and tested for activity. Pertinent results from these experiments are summarized in Table 4. All the monovalent cations were active; the addition of Mg^{++} or Ca^{++} did not reduce the survival of any of the phages to a significant degree. In fact, the deleterious effects produced by the monovalent cations could be reversed by the addition of the divalent ions to the medium. In only one instance was the survival of phage diminished to any significant degree by the monovalent ions in the presence of a divalent cation: D32 was 30 per cent inactivated by 0.2 M KCl, notwithstanding the presence of magnesium. However, 96 per cent of D32 was inactivated in broth containing the KCl only. Both 0.1 and 0.01 M concentrations of calcium and magnesium were used to reverse the monovalent cation effects; the lower concentration had to be employed because of the precipitate which formed when the higher salt concentration was used. D28 displayed the greatest susceptibility to monovalent cation inactivation whereas D29 was the least affected; the effects on the survival of D32 are less significant since pH 10 is not within the range of greatest pH stability for this virus. The order of monovalent cation activity against D28 was K > Li > Na and, against D29 and D32, K > Na > Li.

The exact mechanism of action of these cations is not apparent. The most reasonable possibilities

TABLE 4

Influence of cations on mycobacteriophage survival in nutrient broth at pH 10

Supplement Added	Survival of Phage		
	D28	D29	D32
	%	%	%
0.1 M LiCl	22	79	50
0.1 M LiCl + 0.01 M $CaCl_2$	91	100	67
0.1 M NaCl	30	53	25
0.1 M NaCl + 0.01 M $CaCl_2$	100	100	74
0.1 M KCl	5	25	6
0.1 M KCl + 0.01 M $CaCl_2$	89	90	76
0.2 M KCl	3	12	4
0.2 M KCl + 0.01 M $CaCl_2$	99	90	76
0.2 M KCl + 0.01 M $MgCl_2$	96	85	54
None	90	95	74

Phage at a concentration of 10^7 particles per ml was added to nutrient broth supplemented with a given cation and adjusted to pH 10 with ammonium hydroxide. After incubation for 1 hr in a water bath at 37 C, samples were assayed for viable phage.

appear to be a swelling of the molecules or an unfolding of the polypeptide chains in the phage protein coat resulting from electrostatic repulsions between the negative charges introduced by raising the pH. Since the increase in net negative charge on the protein would tend to repulse the negatively charged deoxyribonucleic acid, the addition of relatively high concentrations of cations might promote the release of the polyanion. However, an examination of the samples in which inactivation of phage had occurred failed to show an increase in adsorption at λ 260 mμ. This finding indicates that deoxyribonucleic acid is not being released into the surrounding medium to any great extent.

An alternative hypothesis is to assume that monovalent cations combine with vital negatively charged groups on the proteins which have become accessible at pH 10. If this occurs in the area at the tip of the tail where attachment occurs (Anderson, 1953; Williams and Frazer, 1956) the molecular configuration might be sufficiently altered to prevent attachment or penetration of phage into the host bacterium (Puck, 1954). When phage is held in ordinary broth at pH 10 and subsequently diluted into a neutral medium before plating, the protein molecule is able to resume its original specific configuration and, thus, attachment and penetration can proceed. The different phages interact in specific ways with the cations, although the pH dependence reflects similarities in the underlying mechanisms. The number and chemical nature of groups which become available at pH 10 apparently vary from phage protein to phage protein.

Divalent cations may be necessary for the functioning of some enzyme involved in the penetration of phage nucleic acid into the host cell (Lark and Adams, 1953). Such an enzyme might readily form complexes with various metal cations but become activated only when complexed with specific divalent ions. The enzyme would preferentially bind divalent cations, and bind them more strongly than univalent cations (Gurd, 1954). This would account for the fact that calcium and magnesium not only do not inactivate the phage but are able to reverse the monovalent cation inactivation.

The inactivation of phage by monovalent cations does not occur at neutral pH; experiments employing up to 1 M concentrations of the salts revealed no adverse effects.

SUMMARY

Three phages, D28, D29, and D32, active against both saprophytic and virulent mycobacteria, have been characterized with respect to thermal inactivation rates, pH stabilities, and serological relationships. A study of the effect of temperature on phage survival shows that when log per cent survivors is plotted against time, the curves obtained are characteristic of viruses in general, i.e., they are composed of one component at lower temperatures, but with increasing temperature a two-component relationship obtains. No serological cross-neutralization was found, indicating that the three phages are antigenically distinct. The range of greatest pH stability is pH 6 to 10 for D28, pH 7 to 10 for D29, and pH 7 to 9 for D32. The addition of alkali-metallic ions to phage suspended in nutrient broth at pH 10 results in a loss in viability of varying degrees; this loss can be prevented by the addition of calcium or magnesium ions to the medium. It is proposed that the influence of monovalent metallic ions on phage viability at pH 10 is related to interactions of cations with specific viral protein, the result of which is an

alteration in molecular specificity required for successful attachment or penetration of phage to host bacterium.

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