FURTHER STUDIES OF INFECTIOUS DNA EXTRACTED FROM MYCOBACTERIOPHAGES*

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In the previous publication (1) it was reported that deoxyribonucleic acid (DNA) extracted from mycobacteriophage D29 could infect its host, *Mycobacterium smegmatis* 607. No alteration in the structural integrity of the bacteria such as the production of protoplasts was required; cultures susceptible to intact phage could also be infected with phage DNA.

This investigation has been extended to include three additional mycobacteriophages. In the experiments reported here, DNA extracted from D4 and D32 but not that from D28, was found to be infectious. Attempts to extend the host range of intact phage with phage DNA were not successful.

Materials and Methods

The source and preparation of reagents, enzymes, buffers, and chromatography procedures were the same as those described in the preceding publication (1). One additional buffer used in this study, Versonate-potassium trichloroacetate (V-KTCA), Nutrional Biochemicals Company, Cleveland, Ohio, was prepared according to Weil (2). Versonate buffer was composed of 0.2 g disodium Versonate, NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.02 g, distilled water to 1000 ml. Potassium trichloroacetate: 0.15 M, pH 7.6. The final V-KTCA buffer was made up of 1.5 parts Versonate and 2.5 parts potassium trichloroacetate. Compounds not previously described (1), will be noted in the text.

Phages.—D28, D29, D32, and D4 were obtained originally from Dr. Seymour Froman. They were isolated by soil-enrichment techniques and except for D4, are active against certain strains of both virulent and saprophytic acid-fast bacilli; D4 is active against *Mycobacterium smegmatis* 607 (3, 4). Methods for the production of high-titered lysates and for phage assay have been described (5).

Bacteria.—*Mycobacterium smegmatis* ATCC 607 (propagating host for D4 and for D29), *Mycobacterium* sp. ATCC 9033 (propagating host for D32), and *Mycobacterium* sp. ATCC 9626 (propagating host for D28) were employed. These bacteria will be referred to in the text by their ATCC numbers. Two additional species of *Mycobacterium* were employed in host-range experiments. They were obtained from Dr. Froman and, according to him, one is *M. phlei* (F 89) and the other *M. smegmatis* (F 87). The latter can be distinguished from 607 by its phage susceptibility pattern. These bacteria will be referred to in the text as F 89 and as F 87. Stock cultures were maintained, and log-phase cultures prepared as described (5).

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Phage DNA.—High-titered phage lysates were cleared of bacterial debris either by passage through a Sharples centrifuge (Sharples Chemical Division, Pennsylvania Salt Manufacturing Co., Philadelphia) at 45,000 RPM or by centrifugation in an International centrifuge (International Centrifuge, Boston) at 5000 RPM for 90 min. Some lots of lysate were cleared by Seitz filtration. Phage was concentrated by differential centrifugation in a Spinco Model L, (Beckman Instruments, Inc., Spinco Division, Palo Alto, California) centrifuge, first at 20,000 *g* for 75 min to sediment the phage, resuspended in heart infusion broth containing 0.002 M $CaCl_2$ (this prevented a substantial loss in plaque-forming activity which resulted when buffers were used at this step), and then centrifuged at 3500 *g* for 30 min to remove clumped debris. The phage suspension was digested with deoxyribonuclease (DNAase) and ribonuclease (RNAase), each at a final concentration of 50 μg/ml, for two hr at room temperature. Phage was again subjected to a second cycle of high and low speed centrifugation. The second phage pellet was resuspended in V-KTCA buffer at pH 7.6. Plaque titrations were carried out and the phage extracted with cold phenol (6). DNA was precipitated out of the aqueous phase phenol extract by the addition of an equal volume of 2-ethoxyethanol (7). The resultant DNA fibers could be stored conveniently for long periods of time in the ethoxyethanol at −20°C. The final DNA solution was prepared by dissolving the fibrous precipitate in V-KTCA buffer and then dialyzing against the same buffer to remove any residual phenol or ethoxyethanol. The final solution was stored at 4°C until used. DNA concentration was determined by the method of Keck (8) using calf thymus DNA as standard (Worthington Biochemical Corporation, Freehold, New Jersey). DNA was analyzed by the methods of Fink et al. (9, 10) and its buoyant density in CsCl determined according to Meselson et al. (11), as described in the previous publication (1). Although D29 DNA was characterized previously, it was included in the experiments reported here for comparative purposes; the values obtained agree, within the limits of experimental error, with those previously reported (1).

Bacterial Nucleic Acids.—Bacteria were inoculated into heart infusion broth and actively aerated at 37°C for 18 to 24 hr. Wet, packed cells obtained by Sharples centrifugation were immediately frozen and then disrupted by passage through a modified Hughes type press (12). The cell paste was diluted with V-KTCA buffer and the nucleic acids were extracted with cold phenol, then precipitated out of the aqueous phase phenol extract with 2-ethoxyethanol. The resultant fibrous precipitate composed of both DNA and RNA, was dissolved in buffer and digested with RNAase; DNA was precipitated out of the solution with 2-ethoxyethanol and analyzed as indicated above for phage DNA. When an analysis of bacterial RNA was to be made, the RNAase digest was not removed as indicated above but was left in the solution and the analyses of both RNA and DNA carried out simultaneously on the one sample. The final digest, containing both deoxyribo- and ribonucleotides or their nucleosides, was separated into its 8 component parts by paper chromatography employing the same solvents as described for DNA only (9, 10, 1). Recovery and base analyses of RNA as well as of the DNA were carried out using the methods described (9, 10, 1).

Determination of Thermal Denaturation Temperature (Tm).—DNA was diluted in SSC buffer (0.15 M NaCl plus 0.015 M sodium citrate adjusted to pH 7) at a concentration of approximately 20 μg/ml and exhaustively dialyzed in the cold, against the same buffer. Glass-stoppered quartz cuvettes containing 3 ml of DNA solution were placed in a Beckman DU Spectrophotometer (Beckman Instruments Inc., Fullerton, California) equipped with a Gilford model 2000 multiple absorbence recording device (Gilford Instrument Co., Oberlin, Ohio) and a Gilford model 207 thermosensor. The temperature was adjusted to give a linear rise of approximately 1.5°C/min in the chamber by circulating a hot ethylene glycol-water mixture through two thermal spacers essentially as described by Szybalski and Mennigmann (13). The optical density of the DNA solution at 260 mμ and the temperature of the chamber were recorded sequentially every 5 sec; a sharp increase in absorbence occurs at the temperature at which DNA denatures. The Tm values were determined graphically according to

Marmur and Doty (14), Tm being the temperature at the midpoint of the transition of the absorbence temperature curve.

RESULTS

Data on several preparations of infectious DNA are recorded in Table I. The extraction process results in a profound loss in plaque-forming activity. For example, a final yield of only 5.28×10^3 infectious centers is obtained from 7.4×10^{12} PFU (plaque-forming units) of D4 phage (sample 1). A major contributing factor is undoubtedly the hydrodynamic shear which occurs as phage is being shaken with phenol in the DNA extraction process (15).

TABLE I

Infectivity of Different Preparations of Mycobacteriophage DNA

Sample No.	Phage	Total PFU extracted	Buffer	Final volume DNA extracted	DNA/ml in final extract	DNA extracted per PFU	Infectivity of DNA	Total PFU in DNA
				ml	*μg*	*g*	*PFU/μg*	
1	D4	7.4×10^{12}	V-KTCA	10	440	5.9×10^{-16}	1.2	5.28×10^3
2	D28	7.5×10^{12}	V-KTCA	5	800	5.3×10^{-16}	0	0
3*a*	D29	2.8×10^{13}	Tris	5	908	1.6×10^{-16}	0.95	4.31×10^3
3*b*	D29	2.8×10^{13}	V-KTCA	5	898	1.6×10^{-16}	2.4	1.08×10^4
4	D29	3.6×10^{14}	V-KTCA	33	1420	1.3×10^{-16}	3.2	1.50×10^5
5*a*	D32	2.9×10^{13}	Tris	10	770	2.7×10^{-16}	0.31	2.39×10^3
5*b*	D32	2.4×10^{13}	V-KTCA	10	784	3.3×10^{-16}	1.0	8.62×10^3
6	D32	7.7×10^{13}	V-KTCA	15	500	1.0×10^{-16}	0.93	6.96×10^3

Of interest was the finding that phenol extraction of DNA in V-KTCA buffer increased the number of infectious units in the DNA. This is illustrated by samples 3*a* and 3*b*: D29 was divided into two equal samples after the first high and low speed centrifugation; sample 3*a* was processed in Tris and 3*b* in K-TCA buffer. Although the total quantity of DNA extracted did not differ significantly, the total yield of PFU was 1×10^4 in K-TCA compared with 4.3×10^3 in Tris. Weil (2) demonstrated that the efficiency of plating (EOP) of polyoma virus DNA could be increased from approximately 5×10^{-6} to approximately 10^{-4} by employing K-TCA buffer in the extraction procedure.

D4, D28, and D29 are all 65 mμ in diameter; D32 is larger with a head diameter of 80 mμ (16). The amount of DNA extracted per phage particle was not directly proportional to its size: D4 and D28 yielded 5.9 and 5.3×10^{-16} respectively, D29 yielded an average of 1.5×10^{-16} g, and D32 between 3.3 and 1.0×10^{-16} g. By comparison, T1 coliphage, which has a head diameter of 50 mμ contains 0.7×10^{-16} g of DNA; T5, with a head diameter of 65 mμ contains 1.8×10^{-16} g and T2 with head dimensions of 65×95 mμ, contains 2.3×10^{-16} g of DNA (17).

Infectivity of the DNA was quickly abolished by incubation with DNAase (5 μg/ml) whereas phage specific antiserum was without effect.

Phage and Bacterial Nucleic Acids.—The buoyant densities, the thermal denaturation or melting temperatures (Tm) and the base ratios of DNA from each of the 4 phages examined and from each of the 3 propagating bacteria are recorded in Table II. The bases are paired, adenine (A) with thymine (T) and guanine (G) with cytosine (C). The over-all base compositions are quite similar with a proportionately higher per cent of G and C present than of A and T. DNA from two of the bacteria, 607 and 9033, has a GC content of 68% which is the same as that found in D32 phage DNA. The moles per cent of GC in the bacterium 9626 DNA is 66. D4 and D29 phage DNA is 64% GC and

TABLE II

DNA Composition of Host Bacteria and Phage

DNA	Base proportions				$\frac{A+T}{G+C}$	Density CsCl	Tm
	A	T	G	C			
	mole %	*mole %*	*mole %*	*mole %*		*g/cm⁻³*	°C
9626	17	17	33	33	0.51	1.7247	94.70
D28	20	20	30	30	0.66	1.7192	92.45
607	16	16	34	34	0.47	1.7264	94.20
D4	18	18	32	32	0.56	1.7222	92.70
D29	18	18	32	32	0.56	1.7232	92.40
9033	16	16	34	34	0.47	1.7263	94.75
D32	16	16	34	34	0.47	1.7274	94.75

that of D28 is 60%. Bases other than those present in calf thymus DNA were not found in any of the DNA extracted from either mycobacteria or mycobacteriophage.

The buoyant density in CsCl and the thermal denaturation temperature of DNA are linearly related to its GC content (18, 14). The buoyant densities of the phage and bacterial DNA recorded in Table I show this linear relationship. The densities in CsCl range from 1.7192 g/cm^{-3} for D28 to 1.7274 g/cm^{-3} for D32 DNA. When these values are substituted in the formula of Schildkraut et al. (19), using *E. coli* K12 DNA with a density of 1.710 g/cm^{-3} as reference DNA to compute the mole proportions of GC, the results are within 1% of the moles per cent of GC found by direct measurement (Table I). On the other hand, a linear relationship between thermal denaturation temperature and GC content was not found except for D28 DNA. The moles per cent GC of D28 DNA, computed from either its Tm (14) or its density in CsCl (19) was within 1% of the value obtained by direct measurement. The thermal transi-

tion profiles were characteristic of double-stranded DNA. However, the melting out or change from a double-stranded structure to a denatured state occurred at a lower temperature than the GC content of the DNA predicted with the exception noted above. Since the moles per cent GC of the 7 different species of DNA examined, found by direct measurement and correlated with their densities in CsCl, ranged from 60 to 68%, the midpoint of their denaturation temperatures, according to the formula of Marmur and Doty (14), should have ranged between 92° and 98°C. The actual values found ranged from 92.4° to 94.75°C. Stated in another way, using the Tm found by experimentation to calculate the GC pairing of each DNA studied, the resultant values would fall between 58.5 and 60.4% GC. We have no satisfactory explanation for this apparent discrepancy. Several experiments were carried out using different DNA preparations in normal and in dilute SSC buffer (20) and employing *E. coli* K12 DNA (Tm, 90.5°C) as a control.

TABLE III

RNA Composition of Host Bacteria

RNA	Base proportions			
	A	U	G	C
	mole %	*mole %*	*mole %*	*mole %*
9626	34	14	33	19
607	36	11	36	17
9033	34	12	34	20

After centrifugation of one preparation of D29 DNA in CsCl to determine its buoyant density, a small band of UV-absorbing material heavier than the main component was observed. In a second preparation the heavy band was not seen but in addition to the main component, a small amount of UV-absorbing material appeared as a lighter, rather diffuse band. In view of the inconsistency in the appearance of these minor components, it is possible that they represent preparation artifacts. Nevertheless, their appearance was peculiar to D29 DNA, not having been observed in any of the preparations of the other species of DNA, examined.

The RNA composition of the propagating strains of mycobacteria is found in Table III. Again, the over-all base proportions in each RNA are similar; the proportion of A and G in each of the three bacteria is approximately two times that of uracil (U) and C. These results differ significantly from those previously reported on the composition of RNA from other species of mycobacteria (21). An average of 23 mole % of U and 21 mole % of A have been reported as compared with our analysis in which an average of 12 mole % of U and 35 mole % of A, was found. Perhaps the difference in extraction and digestion procedures

account for the different results. On the other hand, the composition of DNA from the mycobacteria employed in our experiments is very similar to the base ratios of other mycobacteria, reported previously (21).

Comparison between the Host Range of Infectious DNA and Intact Phage.—The data in Table IV show the host range of phage and of phage DNA, employing five different mycobacteria as hosts. Intact D4 is active against only one of these bacteria, 607, as is D4 DNA. Infectivity of D28 DNA was not demonstrated, even for its propagating host, 9626. Although D29 phage was found to be active against 3 of the 5 bacteria employed, D29 DNA was infectious only for the propagating host, 607. However, the same phage preparation which has a titer of 2.9×10^{10} on 607, has a titer of only 6.6×10^{7} on F 87 and on F 89.

TABLE IV

Plating Efficiency of Intact Phage and of Phage DNA on Different Hosts

Host	D4		D28		D29		D32	
	Phage	DNA	Phage	DNA	Phage	DNA	Phage	DNA
9626	$<10^2$	0	2.6×10^{10}	0	$<10^2$	0	$<10^2$	0
607	3.5×10^{10}	299	5×10^{6}	0	2.9×10^{10}	331	1.4×10^{10}	365
9033	$<10^2$	0	$<10^2$	0	$<10^2$	0	2.9×10^{10}	138
F 87	$<10^2$	0	$<10^2$	0	6.6×10^{7}	0	3.3×10^{9}	26
F 89	$<10^2$	0	$<10^2$	0	6.6×10^{7}	0	1.0×10^{3}	0

Replicate samples of a given phage and of a given DNA preparation were added to soft agar seeded with the specified host bacteria and assayed. Results were recorded as plaques formed per ml of phage or of DNA.

Although 607 and F 87 are both *smegmatis* species, the EOP of D29 is almost 1000 times greater on the former than on the latter. The efficiency of plating of the DNA is very much lower than that of intact phage; infection of a given host was not accomplished by DNA when titration for plaque formation by intact phage was less than 10^9 PFU. The EOP of D32 phage on both 607 and 9033 is high; however, the titer is always 2 to 3 times higher on 9033, the strain used to propagate D32 phage, than on 607. Results are reversed with DNA, that is, the EOP of D32 DNA is 2 to 3 times greater on 607 than on 9033. D32 DNA is also infectious for F 87 but at a lower efficiency; this is also true for the intact phage. Species barriers were not crossed by DNA; bacteria not susceptible to phage were not infected with DNA.

These experiments were extended to include several species of mycobacteria in liquid medium.

DNA was added to log-phase cultures in heart infusion broth containing 0.002 M $CaCl_2$ and shaken on a wrist action shaker at 37°C overnight. Samples of the culture were then plated with the propagating host of the phage from which the DNA being employed in the experiment had been extracted.

As a matter of fact, many attempts were made to infect various microorganisms, including staphylococci, streptococci, pneumococci, and species of bacilli and *Nocardia*. Efforts were also made to propagate phage in HeLa cells by exposing them to phage DNA. In none of these experiments was intact phage recovered. Plaques were formed when samples of some cultures were plated back on the indicated hosts; however, pretreatment of these samples with DNAase completely abolished plaque formation, indicating that the activity was due to the survival of input DNA.

TABLE V

Inhibition of D29 DNA Plaque Formation as a Function of Time at which RNAase is added to the Culture

Time	Culture, only	Culture plus RNAase added at			
hr		10 min	30 min	60 min	120 min
0	340				
1	320	44	58		
2	200	26	38	40	
3	310	42	50	60	80
5	5000	810	900	1360	1800

DNA was added to a log-phase culture of 607. The culture was then divided into 5 equal samples and incubated at 37°C on a wrist action shaker; at the times specified, 50 μg of RNAse was added to a given sample as indicated. At hourly intervals beginning with time 0, samples were withdrawn and assayed; the data are recorded as PFU/ml of culture.

Interactions of D29 DNA with Basic Proteins.—In the preceding paper it was reported that RNAase inactivated D29 DNA by some mechanism other than enzymatic (1). In further efforts to characterize infectious D29 DNA, the effects of additional basic proteins as well as RNAase on infectivity, were investigated.

The inhibition of plaque formation by infectious D29 DNA, as a function of the time at which RNAase is added to the culture, was investigated.

DNA (350 PFU/ml) was added to a log-phase culture of 607 in heart infusion broth containing 0.002 M $CaCl_2$, the culture divided into 5 equal samples, and incubation carried out at 37°C on a wrist action shaker. At 10, 30, 60, and 120 min after addition of DNA to the bacteria, 50 μg of RNAase was added to a given sample. At hourly intervals, samples were withdrawn and assayed for plaques.

Table V shows the results. Addition of RNAase to the culture results in a marked reduction of plaque formation even when added as late as 120 min after the addition of DNA. However, there is an increase with time in plaque survival although the rate of this increase is very slow. These results indicate that after infectious DNA is added to sensitive bacteria, it remains susceptible to inactivation by RNAase for relatively long periods of time. Apparently, the uptake of DNA is a slow process as indicated previously in a time course analysis

of the production of infective centers by the DNA (1). In the culture only, there is a slow decrease in PFU which is most prominent at 2 hr, postinfection. This phenomenon is also observed in samples containing RNAase. It is probably due to the eclipse of input DNA and emphasizes again the relative slowness of the infectious cycle initiated by DNA as compared with the infectious cycle following infection by intact D29 phage (1). Okubo et al. (22) have reported similar observations in cultures of *Bacillus subtilis* infected with phage DNA. These authors propose that time is required for the cells to take up several molecules of DNA and recombine them to produce an undamaged phage genome, a proposal consistent with the recombination frequencies observed in their experiments.

TABLE VI

Comparative Effects of Different Basic Proteins on D29 DNA Infectivity

Trypsin		Histone		RNAase	
Conc.	Plaque survival	Conc.	Plaque survival	Conc.	Plaque survival
μg/ml	*No./ml*	*μg/ml*	*No./ml*	*μg/ml*	*No./ml*
0	495	0	210	0	208
10	442	5	214	5	201
50	480	10	195	10	131
500	163	50	46	50	39

Increasing concentrations (Conc.) of a given basic protein were added to replicate samples of DNA in Tris buffer at pH 7.4. After incubation at 37°C min, the samples were assayed for plaque-forming activity. Pertinent data only were recorded here.

In parallel experiments in which intact phage was substituted for phage DNA, RNAase had no influence on plaque formation.

The effects of additional basic proteins on the infectivity of D29 DNA were investigated.

Replicate samples of DNA in Tris buffer at pH 7.4 were incubated with increasing concentrations of trypsin, of calf thymus histone (Nutritional Biochemicals Corporation, Cleveland), dissolved in water, and of RNAase. After incubation at 37°C for 30 min, the different samples were assayed for plaques.

The data in Table VI reveal that incubation of D29 DNA with 10 μg of RNAase per ml for 30 min resulted in a 37 % drop in plaque formation whereas in the same concentration of histone, the drop in plaque formation was only 7 %. At concentrations of 50 μg per ml, these compounds were equally effective and increasing the concentrations beyond this level was not accompanied by a significant decrease in DNA infectivity. Trypsin was weakly reactive; only at a concentration of 500 μg per ml was a significant loss in DNA activity demon-

TABLE VII

Effects of Calf Thymus DNA on the Inactivation of D29 DNA by Histone and RNAase

Experiment I

Tube No.	1	2	3	4	5
Compound					
RNAase	−	+	+	−	−
Histone	−	−	−	+	+
Calf thymus DNA	−	−	+	−	+
D29 DNA	+	+	+	+	+
Incubated for 30 min at 37°C					
Plaques/ml	253	55	45	119	114
Per cent survival	100	22	17	47	45

Experiment II

Tube No.	1	2	3	4	5
Compound					
RNAase	−	+	+	−	−
Histone	−	−	−	+	+
Calf thymus DNA	−	−	+	−	+
Incubated for 30 min at 37°C					
D29 DNA	+	+	+	+	+
Incubated for 30 min at 37°C					
Plaques/ml	222	45	63	80	208
Per cent survival	100	20	28	36	94

The test compounds at a concentration of 50 μg were added, in the order indicated, to Tris buffer at pH 7.4. Replicate samples of D29 DNA were added to each of the tubes and after the specified periods of incubation, samples were assayed for plaque formation.

strated. Incubation periods could be terminated either at 10, at 30, or at 45 min without significantly altering the final results. A fourth compound, cadaverine, was tested but had no effect on D29 DNA activity. When a high concentration of histone was added to a concentrated DNA solution, a somewhat gelatinous, fibrous precipitate formed which could easily be lifted out of the surrounding solution with a glass rod. When this material was added to soft agar seeded with 607 cells and assayed for plaques, no infectivity was found indicating that strong

binding between D29 DNA and histone had occurred. However, the remaining solution always yielded between 10 and 20% of the input DNA infectivity.

In order to determine something about the binding between D29 DNA and RNAase and histone, attempts were made to reverse the interactions with calf thymus DNA.

Five replicate samples were prepared: 1 sample contained RNAase only and 1 sample RNAase plus calf thymus DNA; 1 sample contained calf thymus histone and 1 sample, histone plus calf thymus DNA. The fifth sample served as the control. The compounds were employed at a final concentration of 50 μg/ml and Tris buffer at pH 7.4 was used to equalize the volume in each tube. DNA (253 PFU/ml) was added to each tube and, after incubation at 37°C for 30 min, the samples were assayed for plaques. A second experiment was carried out as described above except that before being assayed for plaques, the tubes were incubated before and again after the addition of DNA, for 30 min at 37°C.

The results recorded in Table VII show (*a*) that when either RNAase or calf thymus histone are reacted with D29 DNA, simultaneously with calf thymus DNA, the binding between D29 DNA and RNAase or histone is unimpaired; (*b*) when histone is incubated for 30 min with calf thymus DNA prior to contact with D29 DNA, the deleterious effect of histone is completely nullified whereas, (*c*) preincubation with calf thymus DNA does not reverse the effect of RNAase. Thus, the sites on calf thymus histone but not those on RNAase, which bind to D29 DNA, were displaced by calf thymus DNA.

DISCUSSION

The presence of internal basic polyamines in the phage head has been explained by assuming that they serve to neutralize, along with divalent cations, the very high density of negative charges accumulated by the phosphate residues on the DNA molecule. Otherwise, tremendous repulsive forces would exist between different parts of the viral DNA, tightly packed into the phage head (17).

Stoichiometric binding of most basic proteins to the highly charged, acidic phosphate groups on DNA readily occurs in solution to form DNA-protein complexes. Strong binding occurs between RNAase or calf thymus histone and D29 DNA, resulting in the loss of biological activity. Trypsin is only weakly reactive; very high concentrations of this protein are required to bring about a significant decrease in D29 DNA infectivity. It is probable that these interactions involve a measure of specificity since the configurational groups involved may differ from one DNA to another and from one basic protein to another. Guthrie and Sinsheimer (23) found that trypsin formed a complex with the single-stranded infectious DNA from ØX174 phage, which resulted in the loss of biological activity. Their results indicated that strong binding occurred between these two molecules.

The interactions involved in the fixation of phage to host-cell surface which

initiate infection are of a highly specific nature. Much evidence is available concerning these interactions and their role in determining the host range of a given phage system (24). It has been possible to bypass "requirements" involving specific cell wall receptor sites with infectious DNA. Guthrie and Sinsheimer (23) were able to infect protoplasts of strains of *E. coli*, usually resistant to the intact phage, with ØX174 DNA. Okubo et al. (22), obtained mutants of a competent strain of *B. subtilis* with resistance to SPO-1 phage, but which could be infected with the phage DNA. It would be interesting to know the reasons for the apparent specificity of mycobacteriophage DNA. It is possible that the DNA penetrates by a highly specific, host controlled mechanism. Alternatively, it may be that DNA readily passes through the cell wall but its replication is prevented in nonreceptive hosts. Results of some of our experiments suggest that, in some hosts, the latter may occur (26). Answers to these questions can be more readily found when we develop more efficient and reproducible methods for preparing infectious DNA and learn something more specific about "competence" in the host bacteria.

Dussoix and Arber (25) have demonstrated that λ-phage DNA of a given host specificity is accepted upon injection by a certain number of host strains (nonrestricting strains), and is degraded soon after its injection into other hosts (restricting strains). This host specificity of λ-phage is under the control of the bacteria on which it is grown; the host range of a given phage can be changed by propagating it on a different host. Restriction, in some cultures, is not an all-or-none phenomenon; the final outcome of phage multiplication may be influenced by (*a*) the rate at which the injected DNA becomes integrated; (*b*) degraded pieces of DNA which may be genetically rescued by superinfecting, nonrestricted phage; and (*c*) the particular physiological condition of a bacterium at the moment of infection. In the light of these findings, the EOP of D32 phage and D32 DNA on the two *smegmatis* species, 607 and F 87, are of interest (Table IV). Replicate samples which produced 1.4×10^{10} plaques on 607, produced only 3.3×10^{9} plaques on F 87 and the same concentration of D32 DNA that produced 26 plaques on F 87, produced 365 plaques on 607. D32 rapidly adsorbs to F 87, even at a faster rate than to 607 (26). Within 10 min after mixing D32 and F 87, almost 90% of the phage had been adsorbed. A characteristic feature of the ensuing cycle of infection is the high rate of abortive or nonproductive infection which follows adsorption. Within a matter of minutes, over half of the adsorbed phage loses the capacity to produce an infectious center as indicated by failure to form plaques when assayed on indicator bacteria in soft agar. In terms of Dussoix and Arber's results (25) we may describe F 87 as a partially restricting strain for D32 phage and 607 as a nonrestricting strain. That the mechanism(s) restricting infection by intact D32 and by D32 DNA are the same, is indicated by the parallelism found between the EOP of intact phage and of phage DNA, on the two hosts. The only

definitive information concerning D32 phage restriction is that it occurs subsequent to adsorption. Whether the DNA is injected and then degraded by the bacterium, a set of events analogous to the restriction found by Dussoix and Arber for λ-phage in restricting strains of *E. coli*, remains to be determined. It is possible that although D32 adsorbs to F 87, the mechanism for DNA injection is missing or incomplete on a proportion of the bacteria; thus, restriction of phage replication would occur at this step in the multiplication cycle. Whether host specificity of D32 is under the control of the host on which it is propagated has not been established; experiments in progress show that host-range mutants can be derived from wild type phage with relative ease, thus providing appropriate phages with which to test the hypotheses (26).

SUMMARY

Results of the previous investigation in which it was found that DNA extracted from D29 mycobacteriophage was infectious for *Mycobacterium smegmatis* 607, have been extended. DNA extracted from mycobacteriophage D4 and D32 produced plaques when plated on their respective hosts; D28 DNA, extracted in the same manner and tested under similar conditions, failed to show infectivity. Species barriers were not crossed by mycobacteriophage DNA; bacteria resistant to intact phage were not infected with the phage DNA. The efficiency of plating of the DNA is very much lower than that of intact phage; infection of a given host was not accomplished by DNA when titration for plaque formation by the intact phage was less than 10^9 PFU.

The base composition of DNA extracted from the four mycobacteriophages and the three propagating hosts was very similar. The bases were paired, adenine with thymine and guanine with cytosine. A relatively higher per cent of guanine-cytosine than of adenine-thymine, was found. The buoyant density of each DNA in CsCl was linearly related to its guanine-cytosine content whereas with the exception of D28 DNA, thermal denaturation temperatures failed to show this relationship. However, the thermal transition profiles were characteristic of double stranded DNA.

Additional evidence that D29 DNA forms complexes with basic proteins was obtained. Binding between calf thymus histone and between RNAase and D29 DNA readily occurs with a resultant loss in DNA infectivity. Trypsin and D29 DNA are only weakly reactive.

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BIBLIOGRAPHY

1. Tokunaga, T., and Sellers, M. I., Infection of *Mycobacterium smegmatis* with D29 phage DNA, *J. Exp. Med*., 1964, **119**, 139.

2. Weil, R., A quantitative assay for a subviral infective agent related to polyoma virus, *Virology*, 1961, **14,** 46.
3. Froman, S., Will, D. W., and Bogen, E., Bacteriophage active against virulent *Mycobacterium tuberculosis*. I. Isolation and activity, *Am. J. Public Health*, 1954, **44,** 1326.
4. Froman, S., personal communication.
5. Sellers, M. I., and Runnals, H. R., Mycobacteriophage. I. Physiochemical characterization, *J. Bact.*, 1961, **81,** 442.
6. Gierer, A., and Schramm, G., Infectivity of ribonucleic acid from tobacco mosaic virus, *Nature*, 1956, **177,** 702.
7. Kit, S., Fractionation of deoxyribonucleic acid preparations on substantial cellulose anion exchangers, *Arch. Biochem. and Biophysics*, 1960, **87,** 318.
8. Keck, K., An ultramicro technique for the determination of deoxypentose nucleic acid, *Arch. Biochem. and Biophysics*, 1956, **63,** 446.
9. Fink, K., Cline, R. E., and Fink, R. M., Paper chromatography of several classes of compounds: Correlated R_f values in a variety of solvent systems, *Anal. Chem.*, 1963, **35,** 389.
10. Fink, R. M., and Fink K., Biosynthesis of radioactive RNA and DNA pyrimidines from thymidine-2-C^{14}, *Biochem. and Biophysic. Research Commun.*, 1961, **6,** 7.
11. Meselson, M., Stahl, F. W., and Vinograd, J., Equilibrium sedimentation of macromolecules in density gradients, *Proc. Nat. Acad. Sc.*, 1957, **46,** 581.
12. Sagers, R. D., An efficient, easily constructed cell homogenizing press, *Appl. Microbiol.*, 1962, **10,** 37.
13. Szybalski, W., and Menningmann, H. D., The recording thermospectrophotometer, an automatic device for determining the thermal stability of nucleic acids, *Anal. Biochem.*, 1962, **3,** 267.
14. Marmur, J., and Doty, P., Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature, *J. Mol. Biol.*, 1962, **5,** 109.
15. Mandel, J. D., and Hershey, A. D., A fractionating column for analysis of nucleic acids, *Anal. Biochem.*, 1960, **1,** 66.
16. Sellers, M. I., Tokuyasu, K., Price, Z., and Froman, S., Electron microscopic studies of mycobacteriophages, *Am. Rev. Tuberc. and Pulmonary Dis.*, 1957, **76,** 964.
17. Stent, G., The infective unit, *in* Molecular Biology of Bacterial Viruses, Springfield, Illinois, Charles C. Thomas, 1961.
18. Sueoka, N., Marmur, J., and Doty, P., Heterogeneity in deoxyribonucleic acids II. Dependence of the density of deoxyribonucleic acids on guanine-cytosine content, *Nature*, 1959, **183,** 1429.
19. Schildkraut, C. L., Marmur, J., and Doty, P., Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl., *J. Mol. Biol.*, 1962, **4,** 430.
20. Marmur, J., Schildkraut, C. L., and Doty, P., Biological and physical chemical aspects of reversible denaturation of deoxyribonucleic acids, *in* The Molecular Basis of Neoplasia, Austin, Texas, University of Texas Press, 1961.

21. Belozersky, A. N., and Spirin, A. S., Chemistry of the nucleic acids of microorganisms, *in* The Nucleic Acids, Volume III, (E. Chargaff and J. N. Davidson, editors) New York, Academic Press, Inc., 1960.
22. Okubo, S., Strauss, B., and Stodolsky, M., The possible role of recombination in the infection of competent *Bacillus subtilis* by bacteriophage deoxyribonucleic acid, *Virology*, 1964, **24,** 552.
23. Guthrie, G. D., and Sinsheimer, R. L., Infection of protoplasts of *Escherichia coli* by sub-viral particles of bacteriophage ϕX174, *J. Mol. Biol.*, 1960, **2,** 297.
24. Stent, G., Infection, *in* Molecular Biology of Bacterial Viruses, Springfield, Illinois, Charles C. Thomas, 1961.
25. Dussoix, D., and Arber, W., Host specificity of DNA produced by *Escherichia coli*. II. Control over acceptance of DNA from infecting phage λ, *J. Mol. Biol.*, 1962, **5,** 37.
26. Mizuguchi, Y., and Sellers, M. I., unpublished experiments.