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The Role of Polyamines in the Neutralization of Bacteriophage Deoxyribonucleic Acid

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It was previously reported that bacteriophage T4 contains the polyamines putrescine, $NH_2(CH_2)_4NH_2$, and spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, in amounts sufficient to neutralize about half of the viral deoxyribonucleic acid (1). The putrescine and spermidine in the phage were found to be derived from the large amount of these polyamines normally present in the host bacterium, *Escherichia coli* B. It was also shown that these cations are the unidentified compounds in phage T2 reported by Hershey to be injected into the bacteria along with the viral DNA (2).

In the present communication we have attempted to answer certain questions raised by these findings:

1. Is the role of the polyamines in phage that of specific or nonspecific cations for neutralizing the negatively charged phosphate groups in the DNA?
2. Are the amounts and kinds of polyamines in the phage determined by the phage or by the bacterial pool of cations?
3. Can stoichiometry between cations in the phage and the phosphate anions of the DNA be demonstrated?
4. What is the distribution of polyamines in viruses?

The cations of T4 phage have been examined and a balance has been obtained between total cations and total DNA anions. The normal cation content of T4 (putrescine^{++}, spermidine^{+++}, and Mg^{++}) was changed markedly under certain conditions. When the host bacterium *E. coli* B was grown on minimal medium containing spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, a polyamine present in animal tissues (3, 4) but not generally present in bacteria (5), the putrescine and spermidine normally present in the *E. coli* were replaced by spermine and acetylated spermine (6). These abnormal polyamines were found as the main polyamines in the T4 phage grown on these bacteria.

The replacement of the normal polyamines suggested that the polyamines may be acting as nonspecific cations. Two types of evidence support this hypothesis. The lack of polyamines in various bacteriophages (T3, T5, P22) has been correlated with the permeability of these phages to cations; it seems as if the polyamines were displaced by other cations during the purification of the phage.

When Brenner's (7) permeable (osmotic-shock resistant) mutant of T4 was washed with Mg^{++}, a preparation of phage was obtained containing essentially no polyamines; when the mutant phage was washed with spermidine and then with water, a balance was obtained between the DNA anions and the spermidine cations. The properties of preparations of T4 phage containing various cations have been examined.

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EXPERIMENTAL

Materials and Methods

Determination of Phage and Bacterial Titers—The techniques used for assaying the various phages were those described by Adams (8). The bacterial titer was assayed by determining the absorbancy of the culture at 650 mμ in a Beckman DU spectrophotometer. A standard curve of viable bacteria (as determined by plating) *versus* absorbancy was used in converting from absorbancy to bacterial titer.

Growth of Bacteria—Bacteria were grown in 1 liter of minimal medium in a 2-liter flask shaken at 37° on a rotary shaker (New Brunswick Company). The minimal medium used was the medium E of Vogel and Bonner (9) which was supplemented with 1 ml per liter of trace element solution (10). After the medium was autoclaved sufficient sterile glucose to make a 0.5% solution was added.

Preparation of T4 Phage—E. coli B was allowed to grow to a titer of about 8×10^8 bacteria per ml, at which time from 3 to 5 T4 phages[1] per bacterium and 5 μg per ml of L-tryptophan were added. After about 7 hours of further aeration, several milliliters of chloroform were added and the flask was stored in the cold room overnight. The final titer was usually about 5×10^{10} phages per ml. The phage was purified as follows: A low speed centrifugation of 15 minutes at 4,000 × *g* removed most of the bacteria from the crude lysate. The supernatant solution was cleared of bacterial debris by passage through a No. 02 Selas filter candle (Selas Corporation of America) with 850 mμ diameter pores, followed by passage through either a PH Millipore filter (Millipore Filter Corporation) with 300 mμ diameter pores, or an HA Millipore filter with 450 mμ diameter pores. The resulting filtrate was centrifuged at 20,000 × *g* for 2 hours in a Lourdes SL centrifuge. The phage pellet was taken up in "storage buffer" which consisted of 0.01 M $MgSO_4$ and 0.02 M potassium 3,3-dimethylglutarate (Aldrich Chemical Company) buffer at pH 7.0. The phage was further purified by successive low speed (4,000 × *g* for 15 minutes) and high speed (25,000 × *g* for 1 hour) centrifugations. The final pellet was completely transparent and was kept as a suspension in storage buffer. About 50% of the phage, as measured by plaque count, was lost during the purification.

Preparation of P-22 Phage—Salmonella typhimurium strain LT-2 was grown to a titer of 5×10^8 at which time 4 P-22 phages[2]

[1] The initial stock of T4 phage was obtained from Dr. S. Benzer.
[2] The initial P-22 phage stock was obtained from Dr. P. E. Hartman.

TABLE I
Cation content of T4 phage

Cation*	Phage† incubated in	
	$MgCl_2$	$CaCl_2$
	meq/eq P	
$Putrescine^{++}$	250	290
$Spermidine^{+++}$	75	87
Ca^{++}	<2	96
Mg^{++}	340	360
Na^{+}	90	30
K^{+}	60	<30
Total meq per eq P...	815	863

* An analysis of NH_4^+ was not done on these samples, but from the 2,4-dinitrofluorobenzene analysis on several other samples it can be stated that the NH_4^+ content of the phage is <100 mmoles per mole of P. The analyses for Mg^{++} were done by a thiazole-yellow colorimetric procedure (13) in the National Institutes of Health clinical laboratories and we are indebted to Dr. Arthur Ness for these determinations. The Ca^{++}, Na^+, and K^+ analyses were done using a flame photometer and we are indebted to Dr. E. Frame of the Clinical Laboratories for these determinations.

† This particular sample of phage was obtained from somewhat older cells than usual; 1 liter of bacteria (10^9 per ml) was infected with 4×10^{12} phages and after 10 hours the culture, containing 1.7×10^{11} phages per ml, was harvested.

were added per bacterium. The culture was shaken for an additional 5 hours and then several milliliters of chloroform were added and the flask was stored at 4° overnight. The phage was harvested and purified in a manner similar to that used for T4 phage. Since phage P-22 is about one-third the size of T4 it was necessary to spin at 35,000 × *g* for 2 to 3 hours in order to sediment the phage. The final pellet was kept as a suspension in storage buffer.

Preparation of T2 Phage—The preparation and purification of the T2 phage[3] was similar to that described for phage T4. The bacteria were usually grown to a titer of about 1×10^9 per ml before phage (3 to 5 per bacterium) was added; the final yield was about 1.7×10^{11} T2 per ml. Tryptophan, which is necessary for T4 adsorption, is not necessary for T2 and was omitted.

Preparation of T3 Phage—The preparation of the T3 phage[3] was similar to T4 except that the growth medium was made 1 mM with respect to $CaCl_2$. The purification of the phage was the same as that for P-22.

Polyamine Determination—The purified phage preparations were treated with 0.3 N trichloracetic acid at 25° for 10 minutes, and the precipitate of protein and nucleic acid was sedimented by centrifugation and discarded. If the volume of the precipitate was appreciable as compared with that of the supernatant solution, the precipitate was treated with a second portion of acid before being discarded. The total acid supernatant solution was shaken with ether to remove the trichloroacetic acid and then analyzed for polyamines by means of Dowex 50 and paper chromatography, and by the 2,4-dinitrofluorobenzene and ninhydrin assays described elsewhere (6, 11). The polyamine determinations in bacteria were done in a similar way (6, 11).

Phosphate Determination—The phage sample (0.01 to 0.05 ml) in a 13 × 100 mm Pyrex test tube was mixed with 0.05 ml of 10% $Mg(NO_3)_2 \cdot 6H_2O$ in ethanol; the mixture was evaporated to dryness over a strong flame with rapid shaking and further heated in the flame until the brown fumes had disappeared.[4] After the tube had cooled, 0.3 ml of 1 N HCl was added. The tube was capped with a marble and heated in a boiling water bath for 15 minutes to hydrolyze to inorganic phosphate any pyrophosphate formed in the ashing procedure. The inorganic phosphate was then determined by a slight modification of the very sensitive procedure of Chen *et al.* (12); 0.7 ml of ascorbic-molybdate mixture was added to the tube and after 20 minutes at 45° the solution (1 ml) was read at 820 mμ against a blank containing water instead of phage. The ascorbic-molybdate mixture, which was made up daily, contained 1 part of 10% ascorbic acid to 6 parts of 0.42% ammonium molybdate·4 H_2O in 1 N H_2SO_4. An absorbancy of 0.240 was obtained from 0.01 μmole of phosphate (about 10^{10} T4 phages). An inorganic phosphate value was obtained for each phage preparation, as well as a total phosphate, and the former (which was usually negligible) was subtracted from the total to give the organic phosphate content of the phage. The inorganic phosphate determination was done on the supernatant solution from a cold trichloroacetic acid treatment of the phage; the acid treatment precipitates the phage protein and nucleic acid.

RESULTS

Phage T4 of E. coli

Cation Analysis—In order to determine what cations besides putrescine and spermidine could neutralize the anion phosphate in the phage DNA, a sample of purified T4 phage was analyzed for Mg^{++}, Ca^{++}, Na^+, and K^+ as well as for putrescine and spermidine. The major cations in the growth medium were K^+ (110 mM), Na^+ (10 mM), NH_4^+ (10 mM), and Mg^{++} (0.8 mM). $Putrescine^{++}$ and $spermidine^{+++}$ were the polyamines present in the host bacterium, *E. coli* B. Half (3×10^{13}) of the purified T4 phage to be analyzed was incubated 15 hours at 4° in 0.02 M $MgCl_2$ (1 ml) and half in 0.02 M $CaCl_2$ (1 ml). Each sample was then dialyzed at 4° against three 2-liter portions of distilled water for a total of 7 hours. The samples were then assayed for viable phage, total phosphate and cations, and the optical density at 260 mμ was determined. The cation analysis is given in Table I. About 85% of the negatively charged phosphate groups of the DNA could be neutralized by the cations present in the virus. Table I shows that Mg^{++}, putrescine, and spermidine account for over 80% of the cations present in the phage. The phage sample incubated in Ca^{++} did not have a diminished Mg^{++} content and contained only a relatively small amount of Ca^{++}, indicating that the phage is not freely permeable to divalent cations. External putrescine-C^{14} had previously been shown not to exchange with putrescine in the virus (1).

Polyamine Content of Phage DNA Isolated by Phenol Method—In order to determine if the polyamines in phage stayed with the DNA during isolation, phage DNA was separated from phage protein by means of the phenol procedure. This method was developed for the isolation of RNA from tobacco mosaic virus by Schuster *et al.* (14), and consists of shaking the virus with the two-phase system phenol-water. It has been used on polio virus by Koch *et al.* (15). They found that all the viral protein dis-

[3] The T2 and T3 phage stocks were obtained from Dr. G. Streisinger.

[4] This method of ashing was suggested by Dr. R. Kielley.

solves in the phenol layer and the RNA remains in the aqueous layer.

We have found that the phenol procedure is also effective in isolating from T2 bacteriophage a protein-free, highly polmerized DNA.[5] The effectiveness of this phenol procedure on phage contrasts with its ineffectiveness in extracting mammalian DNA (16).

Reagent grade phenol was washed several times with water and distilled under reduced pressure. The phenol was stored under water at 4°. Diethyl ether was washed several times with water and stored over water at 4°. The T2 phage[6] used (1.5×10^{14} T2 in 4 ml) was dialyzed against 0.02 M potassium dimethylglutarate buffer at pH 7 and diluted to 16 ml with distilled water. The phage was then shaken with an equal volume of water-saturated phenol in a polyethylene centrifuge tube for 30 seconds in the cold room. The tube was then centrifuged briefly to separate the layers. The DNA-containing aqueous layer was quite viscous at this point due to the presence of DNA. The phenol layer (lower) was sucked off with a syringe and the aqueous layer was extracted twice more with 16-ml portions of phenol. The viscous layer containing the DNA was extracted 10 times with 25-ml portions of ether to remove traces of phenol. Helium was then bubbled through the DNA solution at 37° for 15 minutes to remove the ether. The polyamines in the phage suspension used for DNA isolation contained 22.5 μmoles of N (as determined by the dinitrofluorobenzene reaction). The DNA solution contained 20.8 μmoles or 93% of the total polyamine content of the phage. Since the DNA interfered in the assay for polyamines, it was precipitated with 0.3 N trichloroacetic acid and the assay was performed on the supernatant solution.

A control experiment was done by a similar partitioning of polyamines (in amounts equivalent to that in the added phage) between the potassium dimethylglutarate buffer and phenol. In the control, only 39% of the polyamine N was in the aqueous layer.

Effect of Spermine on Polyamine Content of T4 and E. coli B—When *E. coli* B is grown in spermine-containing medium, the normal putrescine and spermidine in the bacteria are replaced by spermine, monoacetylspermine, and diacetylspermine (6). The phage was grown on these spermine-containing bacteria to see whether the polyamine content of the phage paralleled that of the bacteria. The lysis of the spermine-containing bacteria by the phage was similar to that of normal bacteria (Fig. 1). An analysis of this phage as well as of the host bacteria is given in Table II. It can be seen that qualitatively the phage contains the polyamines present in the bacteria at the time of infection. The ratio of the total basic polyamine nitrogen to DNA phosphate, however, remained fairly constant at about 50% of the phosphate neutralized.

Transfer of Polyamines from Host Bacteria to Phage—The experiments presented in Table II were examined from the point of view of efficiency of transfer from host to phage. Table III shows a comparison of the polyamine content of the bacteria at the time of infection with that of the phage in the total lysate. This comparison does not take into account any change in the polyamine content of the cells during the period from infection

[5] We are indebted to Dr. G. Koch for suggesting this method. Phage DNA prepared by this method was found to be of high molecular weight by ultracentrifuge studies (J. Johnson and D. Bradley, personal communication).

[6] We are indebted to Dr. W. Dreyer for one of the samples of purified T2 used in this experiment.

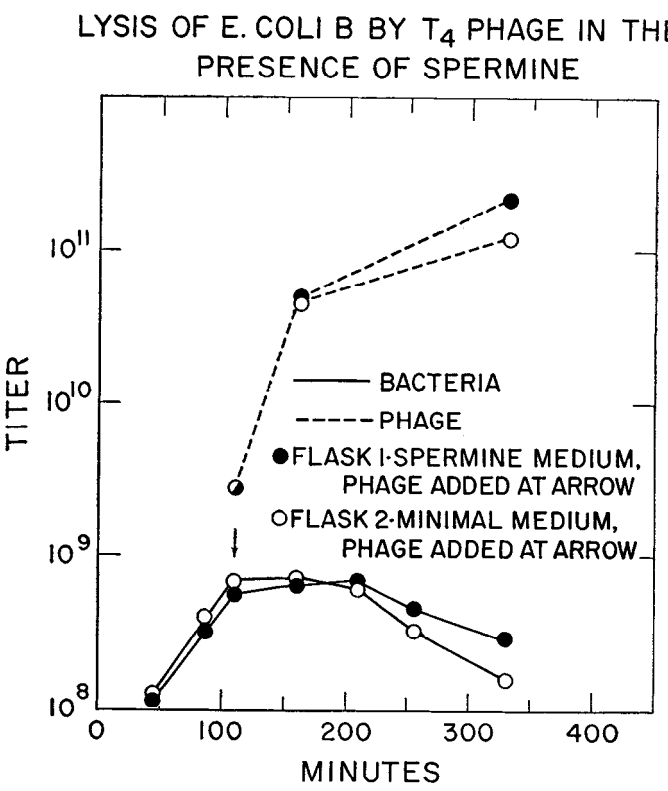

FIG. 1. Lysis of *Escherichia coli* by T4 phage in the presence of spermine. Two flasks of minimal medium, one supplemented with spermine (1 mM), were inoculated with *E. coli* B and grown as described under "Methods." The absorbancy at 650 mμ was converted to titer of bacteria as described. At 110 minutes, 5 T4 phages were added per bacterium along with an L-tryptophan supplement (5 μg per ml). Both the phage titer and 650 mμ absorbancy were determined at intervals. As the phage kills the bacteria on adsorption, the bacterial titers after phage infection are a measure of turbidity rather than of viable cells.

to lysis. From the data on two successive bacterial aliquots in Table II it can be seen that any change would probably be in the direction of decreasing the putrescine and spermidine content of the cells and increasing the spermine and acetylated spermine. Table III shows that the transfer seems to be most efficient for spermine, spermidine, and putrescine and least efficient for diacetylspermine, and that the bulk of the spermine, spermidine, and putrescine in the bacteria is transferred to phage.

Properties of T4 Phage Containing Spermine—Various properties of normal T4 phage and T4 phage that contains spermine have been compared. The absorbancy at 260 mμ and the phosphate content per plaque-forming unit was the same for both types of phage (*cf.* Table II). The efficiency of plating of the two batches of phage was comparable on all strains of *E. coli* tested: *E. coli* B, BB, W 3104, K 12 (λ) and K/6. The ultraviolet inactivation curves were also the same for both (Fig. 2).

T2 Bacteriophage of E. coli

A preparation of purified T2 phage was obtained as described under "Methods." It contained 107 mmoles of putrescine and 25 mmoles of spermidine per mole of P, *i.e.* 29% of the DNA phosphate could be neutralized by polyamines.

T3 Bacteriophage of E. coli

A preparation of purified T3 phage obtained similarly contained <12 mmoles of putrescine and <7 mmoles of spermidine per

TABLE II

Effect of spermine on polyamine content of T4 and Escherichia coli B

	Polyamines*					
	Putrescine	Spermidine	Spermine	Monoacetylspermine	Diacetylspermine	Meq amino N/eq. P
Minimal medium†						
E. coli B	11.7	1.6				
T4 phage	170	47				481
Spermine medium‡						
E. coli B (a)	1.0	<0.2	5.9	3.8	12.5	
(b)	0.2	<0.05	9.3	5.9	22.0	
T4 phage (I)	26	1	72	27	21	466
(II)	10	1.7	87	30	35	533

* Bacterial polyamines are expressed as μmoles per g of wet weight and phage polyamines as mmoles per mole P.

† The sample of bacteria was of exponentially growing cells. The purified T4 preparation had a 280 mμ to 260 mμ ratio of 0.76, a 260 mμ absorbancy of 8.7 per cm for a suspension of 10^{12} T4 per ml, and contained 1.0 μmole organic phosphorus per 10^{12} phages.[7]

‡ Two parallel bacterial cultures were grown in minimal medium which was 1 mM in spermine. Aliquots of bacteria were taken from one culture at titers of 2×10^8 (*a*) and at 2×10^9 (*b*), washed in 0.9% sodium chloride solution and analyzed as described by Dubin and Rosenthal (6). (These are the same as cultures 2*A* and 2*B* of Table V of their paper.) Phage was added to the other culture at a bacterial titer of 2×10^8 and the phage (Analysis I) prepared as described under "Methods." Phage analysis II is from a similar experiment. The phage (II) had a 280 mμ to 260 mμ ratio of 0.73, a 260 mμ absorbancy of 8.8 per cm for a suspension of 10^{12} phages per ml and contained 1.0 μmole organic phosphorus per 10^{12} phages.[7]

mole of phosphate. No other substances which react with 2,4-dinitrofluorobenzene were detected.

Phage P-22 of S. typhimurium

The temperate phage, P-22, grown on *Salmonella typhimurium* LT-2 in minimal medium and prepared as described under "Methods," was found to be free of polyamines (less than 1.5 mmoles of polyamine N per mole of DNA phosphate). The purified phage had a 280 to 260 absorbancy ratio of 0.65, had an absorbancy of 2.40 per cm at 260 mμ for a suspension of 10^{12} phages per ml, and contained 0.51 μmole of phosphate per 10^{12} phages. P-22 obtained from the host *Salmonella* grown in nutrient broth (which contains spermine) contained a trace of spermine (7 mmoles per mole of P) and of monoacetylspermine (9 mmoles per mole of P).

When *S. typhimurium* was grown in spermine medium (1 mM), there was little or no inhibition of growth or loss in viability. The amine content of the bacteria was about the same as that of *E. coli* grown in spermine medium. No phage could be obtained from these bacteria, however, as long as spermine was present in the medium even though the P-22 adsorbed normally.

[7] The absorbancy of the phage suspension at 260 mμ per μmole P is in agreement with the value of 8.4 reported for T4 (8). The values for the μmoles of P/10^{12} phages reported here are somewhat higher than the value of 0.75 reported for T4 (8). This discrepancy may represent some inactive phage in our preparations which are not being scored in the plaque assay method. Our values, however, have been constant for each strain of phage used and are not influenced by the polyamine content of the phage.

TABLE III

Transfer of polyamines from bacteria to phage

	Putrescine	Spermidine	Spermine	Monoacetylspermine	Diacetylspermine
Coli host*	0.50	<0.1	3.0	1.9	6.3
Phage yield†	0.41	0.07	3.5	1.2	1.4
Percentage of transfer	80	>70	120	60	20

* The polyamine content in μmoles of a liter of spermine-grown bacteria of 2×10^8 titer has been calculated from the data in Table II.

† The polyamine content in μmoles of the phage from the spermine grown bacteria (Table II, phage analysis II) has been calculated for the total number of phages present in the crude lysate and the polyamine content per phage in the purified preparation.

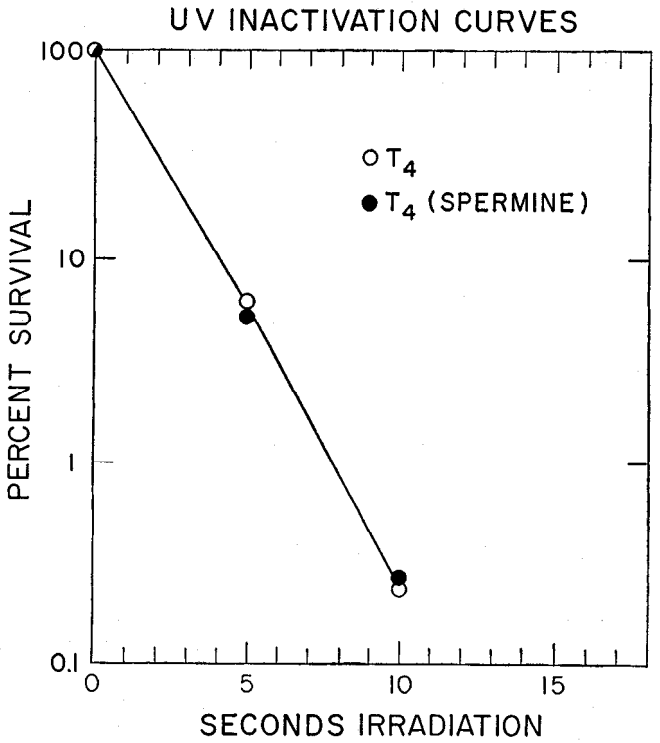

FIG. 2. Ultraviolet inactivation of T4 phage and T4 phage containing spermine. Samples of T4 phage from the experiment presented in Table II were suspended in minimal medium and irradiated (8). Aliquots were taken at various times and assayed for viable phage. The assay manipulations were done in subdued light under conditions where no photoreactivation could take place.

When the bacteria grown in the presence of spermine were infected with phage and then centrifuged and suspended in fresh medium without spermine, they lysed, producing a burst of P-22. These P-22 have been found to contain traces of spermine (5 mmoles per mole of P) and of monoacetylspermine (5 mmoles per mole of P) similar to that found in the P-22 grown in nutrient broth.

O_1 Mutant of T4

After various phages were analyzed for polyamines, it became obvious that a correlation exists between the permeability of the phage to cations and the lack of polyamines. In order to test

whether the polyamines in some phages were displaced from the phage DNA by the Mg^{++} in the washing buffer, the O_1 mutant of T4 isolated by Brenner[8] (7), was examined for polyamines. This phage was isolated as an osmotic shock resistant mutant of T4, and shown to be permeable to various ions as contrasted with wild-type T4 (7). The O_1 mutant was grown and purified as described for T4.[9] One aliquot of the purified phage was allowed to stand in storage buffer for 12 hours at 28°. This sample was then sedimented in a preparative ultracentrifuge and resuspended in storage buffer. The phage was washed by two more sedimentations and resuspensions and finally suspended in distilled water. This phage contained 4.7 moles of polyamine N[10] per 100 moles of DNA phosphate and contained 1.4 μmoles of phosphate per 10^{12} phages. The absorbancy of the phage suspension at 260 mμ was 9.5 per cm per 1.0 μmole of P.[7]

A second aliquot of the O_1 mutant was incubated in 0.01 M spermidine·3 HCl (neutralized to pH 6.5 with NaOH) for 12 hours at 28° and then freed of unbound spermidine by three successive sedimentations and resuspensions in distilled water. This phage contained 89 moles of spermidine N[10] per 100 moles of DNA phosphate and contained 1.4 μmoles of P per 10^{12} phages. The absorbancy of the phage suspension at 260 mμ was 9.5 per cm per 1.0 μmole of P.[7]

Tobacco Mosaic Virus

A sample of 10 mg of tobacco mosaic virus[11] was analyzed for polyamines. The virus contained <4 mmoles of polyamine N per mole of phosphate, *i.e.* less than 0.4% of the RNA phosphate could be neutralized by polyamines.

Cucumber Virus

A sample of 10 mg of cucumber virus[11] was analyzed for polyamines. The virus contained <10 mmoles of polyamine N per mole of phosphate, *i.e.* less than 1% of the RNA phosphate could be neutralized by polyamines.

Tomato Bushy Stunt Virus

A sample of 87 mg of bushy stunt virus[11] was analyzed for polyamines. The virus contained 3.4 mmoles putrescine N, 13.2 mmoles spermidine N, and 2.2 mmoles spermine N per mole of phosphate. Traces of amino acids totaled about 8 mmoles of N per mole of phosphate. The putrescine and spermidine were identified by column and paper chromatography (4, 6, 11) while the spermine was identified only by a single R_F value on paper and is therefore not firmly established. The total polyamines, however, account for less than 2% of the RNA phosphate neutralization and may represent a small impurity in the virus preparation. Spermidine appears to be the main polyamine in tomato leaves.[12]

Polio Virus

A sample of 1.2 mg of pure polio virus (17)[13] was analyzed for polyamines. Less than 20 mmoles of polyamine N were found per mole of RNA phosphate assuming the virus was 22% RNA. This amount of polyamine is only sufficient to neutralize 2% of the phosphate and is probably not significant.

DISCUSSION

There are several pieces of evidence supporting the idea that the polyamines in T2 and T4 phages function as cations in the neutralization of the negative charges of the phosphate groups of phage DNA.

1. The polyamines appear to be surrounded by the protein of the phage; *i.e.* they are inside the phage head with the DNA. It has been established that the putrescine of the phage will not exchange with putrescine-C^{14} added externally, and that the polyamines of the phage are not displaced when the phage are incubated in Mg^{++} or Ca^{++}. Hershey (2) has shown that when the phage head was broken open by osmotic shock the "A" compounds (the polyamines) were not associated with the protein coat (*i.e.* they were not sedimentable). The "A" compounds were dialyzable when osmotically shocked phage were dialyzed against buffer, as contrasted to their nondialyzability in unshocked phage (2). The polyamines were also easily extractable in cold acid. It thus appears that nonexchangeability and nondialyzability of the polyamines of the phage is due to their location inside the protein coat rather than due to some sort of covalent linkage of the polyamine with the phage.

2. The polyamines appear to be associated with the DNA rather than the protein of the phage. Hershey (2) has shown that, during phage infection, the "A" substances were injected into the bacterium with the DNA, while over 95% of the protein remained outside. A small amount of basic internal protein does seem to be associated with the DNA (2, 18), but this protein can account for the neutralization of less than 5% of the DNA. The remainder of the protein of the phage seems to be essentially neutral (8). The protein, polyamines, and nucleic acid account for all the carbon of the phage (2, 8). In the phenol extraction the polyamines remained with the DNA despite a large excess of K^+ ions, indicating that their affinity for DNA is considerably greater than that of K^+.

3. The polyamines have a high affinity for nucleic acid as compared to protein. Various workers have made qualitative observations on the affinity of the polyamines for nucleic acid[12] (19, 20). Quantitative observations on polyamine-nucleic acid binding have recently been made by Razin and Rozansky (21) and by Felsenfeld and Huang (22). The latter workers, in a detailed study, showed that putrescine interacts strongly with nucleic acid as contrasted to the weak interaction of monovalent ions. Spermine is even more tightly bound than putrescine and resembles polylysine in its behavior; spermine was found to displace Ba^{++} stoichiometrically from polyuridylic acid, precipitating a spermine-polyuridylic acid complex. Felsenfeld and Huang come to the conclusion from their binding studies that "in a physiological situation . . . which involves high polyamine concentrations it is likely that nucleic acids will carry significant amounts of polyamines."

4. The data presented show a balance in the phage between the negative charges of the DNA and the positive charges of the

[8] We wish to thank Dr. S. Brenner for a stock of this mutant, and Dr. G. Streisinger for suggesting its use in this experiment.

[9] In this experiment the minimal medium used for the growth of *E. coli* B contained a supplement of 1.5% Casamino acids and 2.5% glycerol as a source of carbon.

[10] The polyamine N was all spermidine as determined by 350 mμ to 390 mμ ratio (11), and by paper chromatography (6).

[11] We are indebted to Dr. C. A. Knight for a generous sample of this virus.

[12] S. M. Rosenthal and C. W. Tabor, unpublished observations.

[13] We are indebted to Drs. L. Levintow and J. E. Darnell for this preparation.

cations. Titration curves of the polyamines indicate that all the amino groups are protonated at pH 7.[14]

The experiments with spermine-containing bacteria show that qualitatively the polyamine content of the phage is not determined by the phage but by the polyamine content of the bacteria. The precise amounts of each cation in the phage may be determined, however, by the affinity of the particular cation for the phage DNA and the amount of the cation in the bacterial pool at the time of phage assembly. The results of the transfer experiments and Mg^{++} assays are consistent with this hypothesis. It would be expected (22) that the relative binding affinities of cations for phage DNA would be: spermine^{++++} > spermidine^{+++} > putrescine^{++}, $Mg^{++} \gg NH_4^+$, K^+, Na^+. The acetyl spermines which are divalent or trivalent presumably would have an affinity similar to putrescine or spermidine, although the presence of secondary rather than primary amino groups might change this somewhat. The main cations in the bacteria at the time of phage assembly are presumably spermidine^{+++}, putrescine^{++}, Mg^{++}, NH_4^+, Na^+, and K^+.

During the course of this work, it seemed puzzling that phages T3 and P-22 which are assembled in essentially the same polyamine environment as T2 and T4 (based on the bacterial polyamine content) should not contain polyamines. One explanation for this is that these phages are more permeable than T2 or T4 and that there is a displacement of the polyamines in the phage by Mg^{++} during the purification and washing of the phage. Support for this hypothesis is found in the observations that T3 and P-22 are quite permeable while T2 and T4 are not; T3 and P-22, unlike T2 and T4, are both resistant to osmotic shock with $MgCl_2$ or NaCl[15] (23). Furthermore, dye-binding studies also show that T3 is readily permeable to dyes while T2 and T4 are not (24). T4-O_1, a permeable mutant of T4, was found to have only a trace of polyamine left after washing it in the Mg^{++} buffer used in the purification.

Though T4 phage contains polyamines which serve to neutralize about one-third to one-half of the DNA phosphate, it was not clear at first whether these substances were acting in a role other than as cations. The evidence that T4 phage containing spermine, and acetylated spermines and T4 phage containing putrescine and spermidine are biologically indistinguishable seemed to indicate that polyamines act only as nonspecific polyvalent cations. Further support of this hypothesis was obtained by displacing all the DNA neutralizing cations in T4-O_1 with Mg^{++} on the one hand or with spermidine on the other with no loss in infectivity.

The cations in phage may have to be polyvalent, however, in order to stabilize the nucleic acid if only a stoichiometric amount is present (*cf.* Felsenfeld and Huang, (22)). Various stability studies on T5 phage, which is also quite permeable to metal ions (23), and which lacks polyamines[16] when purified by standard techniques, indicated that small amounts of a divalent cation (Mg^{++} or Ca^{++}), or large amounts of a monovalent cation are necessary for the stability of stored phages (25). Studies on the transforming ability of urea-shocked T2 phage (26) indicated that any of several polyamines can stabilize activity.

The cation differences between permeable and nonpermeable phages may explain some recent observations on phage density. On centrifuging in a CsCl density gradient (27, 28), phages T2[17] and T4[17] give diffuse peaks indicating phage populations heterogeneous with respect to density, while phages λ (28), T3[17], ε15[18], and φX174[19] give much sharper peaks indicating more nearly homogeneous populations. The sharpness of the peak in a density gradient seems to be correlated with permeability; the heterogeneous phages are shockable with salt and the homogeneous phages are not. If the cations in the bacteria change during the period of phage assembly, then this might explain the heterogeneous density of the nonpermeable phages. The polyamines presumably are taken up preferentially by the phage assembled early (1) (Table III) whereas the later phage probably contains more Mg^{++}, which is in excess in the medium. The permeable phages on the other hand, would equilibrate whatever cations they contained initially with the 6 M CsCl and thereby give homogeneous peaks. This explanation of the CsCl banding experiments was in part suggested by the experiments of Pratt and Stent[20] who have reached a similar conclusion as to the cause of the density heterogeneity in T4. They have found that incubating T4 in 6 M CsCl at 45° (a temperature which would increase permeability) (23), results in a population of phage with reduced density heterogeneity.[20] Their results lend further support to the idea that a variety of cations can serve as neutralizing agents for the phage DNA.

SUMMARY

The cations putrescine^{++}, spermidine^{+++}, and Mg^{++} neutralize the deoxyribonucleic acid of the T4 bacteriophage obtained from *Escherichia coli* grown in minimal medium. Their relative amounts are a function of both the composition of the pool of cations in the host bacterium at the time of phage synthesis, and the affinity of each species of cation for the phage nucleic acid. Viable T4 phage have been obtained with various cations as the deoxyribonucleic acid-neutralizing agent; the role of the polyamines in phage seems to be that of a nonspecific cation for deoxyribonucleic acid neutralization and stabilization.

The absence of polyamines in certain *E. coli* and *Salmonella typhimurium* phages is correlated with their permeability to cations; it seems that the polyamines are displaced by other cations during purification of the phage. Polyamines are not present in tobacco mosaic virus, cucumber virus, tomato bushy stunt virus, or polio virus.

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