# EFFECT OF OSMOTIC SHOCK AND LOW SALT CONCENTRATION ON SURVIVAL AND DENSITY OF BACTERIOPHAGES T4B AND T4Bo$_1$

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ABSTRACT Measurements of survival and buoyant densities of bacteriophages T4B, T4Bo$_1$, and T4D have demonstrated the following: (*a*) After suspension in a concentrated salt solution, T4B and T4D are sensitive both to osmotic shock and to subsequent exposure to low monovalent salt concentrations. (*b*) Sensitivity of T4B to dilution from a concentrated salt solution is dependent on dilution rate, that of T4D is less dependent, and that of T4Bo$_1$ is independent. (*c*) Sensitivity of all three phages to low salt concentrations depends on initial salt concentrations to a variable extent. (*d*) Density gradient profiles indicate that nearly half of osmotically shocked T4B retain their DNA. Similar analysis demonstrates that few, if any, T4Bo$_1$ lose DNA when subjected to a treatment causing 90% loss of infectivity. (*e*) The effective buoyant densities of T4B and T4Bo$_1$ depend significantly on the dilution treatments to which the phages are subjected prior to centrifugation in CsCl gradients. These data are explicable in terms of the different relative permeabilities of the phages to water and solutes, and of alterations in the counterion distribution surrounding the DNA within the phage heads.

## INTRODUCTION

The precise packing of DNA within the head of T-even bacteriophages and the forces responsible for the specific packing are obscure. Since there is increasing evidence that water may play a role in the conformation of macromolecules, water may well play a role in the structural organization of phage. One way to study the role of water is to perturb it, and two ways to perturb it are to subject it to osmotic flow and to the structural influence of electrolytes. The effects of these two parameters on bacteriophages were the subject of this investigation.

Anderson (1949, 1953) first described the inactivation of T-even phages that occurs when they are rapidly diluted from a concentrated solution, calling this effect "osmotic shock." Other workers have used osmotic shock as an investigative

tool to study the biology and chemistry of bacteriophages (Hershey and Chase, 1952; Herriott and Barlow, 1957; Kleinschmidt et al., 1962). In a study of the genetics of phage protein, Brenner and Barnett (1959) decribed the isolation of phage T4 mutants that are resistant to osmotic shock.

Investigation of the effects of subzero temperatures on several phage T4 strains suggested that some of the inactivation observed results from osmotic shock (Leibo and Mazur, 1965). But upon comparing the osmotic sensitivity of the shock-sensitive strains, T4B and T4D, with a shock-resistant strain, $T4Bo_1$, we found that phage infectivity also depends on the minimum salt concentration to which the particles have been exposed. This additional effect of osmotic shock treatment had not previously been recognized, since Anderson, Rappaport, and Muscatine (1953) and Herriott and Barlow (1957) used only 100-fold and 20-fold dilutions, respectively, rather than the 1000-fold or more dilutions described here. Therefore, they did not achieve low enough salt concentrations to observe the effect.

The purpose of the present study, then, was to investigate this effect, determine its relationship to osmotic shock, and to seek an explanation of it.

## MATERIAL AND METHODS

*Bacteria.* *Escherichia coli* B, the host organism, was grown in 1.5% nutrient broth plus 0.8% NaCl at 37°C either in 50-ml volumes on a rotary shaker or in 800-ml volumes with aeration and stirring in an incubator. Stocks were maintained on 1.5% nutrient agar slants.

*Bacteriophages.* Osmotic shock–sensitive bacteriophages T4B and T4D and a shock-resistant phage, $T4Bo_1$, were obtained from Doctors R. B. Setlow, R. C. Curtiss, 3rd, and D. J. Cummings, respectively. Phages for survival experiments were prepared from 50-ml lysates, and those for density analyses were prepared from 800-ml lysates. The phages were purified by differential centrifugation, filtration through 0.45-$\mu$ filters (Millipore Filter Corporation, Bedford, Massachusetts), and incubation at 37°C for 30 min with pancreatic deoxyribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey). Purified stocks were stored in nutrient broth at 5°C.

The phage titer was determined by standard techniques (Adams, 1959). Phages were mixed with bacteria in soft agar at 45°C, and the mixture was poured onto the bottom agar layer. The titer was the same on plates dried from 1 to 9 days at 25°C and 65% relative humidity. (If, however, the phage suspension was added directly to the agar surface, and bacteria in soft agar were poured onto the suspension, the titer decreased as much as 50% with increasing dryness of the bottom agar layer.)

*Osmotic Shock Procedures.* The osmotic shock treatments were modifications of those of Anderson et al. (1953) and Herriott and Barlow (1957). Since we found that minor differences in procedure had marked effects on survival, the procedures will be described in detail.

Experimental material was prepared by diluting 0.1 ml of purified phage with 100 ml of the suspending medium to be tested. The suspension was equilibrated for 15 min at 25°C. (Equilibration times from 1 to 180 min did not affect survival except for one case in which glycerol was the dilution medium.) It was then subjected to osmotic shock treatment consisting of a second 1000-fold dilution of 0.1 ml of diluted phage with a

given dilution medium. The second dilution was performed either rapidly (by pouring 100 ml of dilution medium within 2 sec onto 0.1 ml of phage suspended in the solution to be tested) or slowly (by adding volumes of the dilution medium stepwise to 0.1 ml of phage suspension so that the osmotic pressure of the solution, calculated from the data of Robinson and Stokes (1959, p. 483), decreased at about 15 atm/min). The media used for the second dilution were deionized glass–triple-distilled water, nutrient broth, glycerol, or ionic solutions of various concentrations. Although it could not be controlled because of the low salt concentrations required, the pH of all solutions used was between 6 and 8.

Some experiments required a third 10- to 100-fold dilution with distilled water. The solute concentrations of these very dilute solutions were calculated by the dilution factor, and were checked occasionally by measurement of their electrical conductivity. Subsequent dilutions for assay purposes, if required, were made with nutrient broth.

One set of samples suspended in and diluted with nutrient broth served as a control in every experiment. When appropriate, additional sets of samples of phages suspended in and diluted with either water or 0.065 molal NaCl (isosmolal to nutrient broth) were also used as controls. Each control and experimental set consisted of triplicate samples. All data shown, with the exception of those in Fig. 3, represent two or more replicate experiments, in which freshly prepared phage stocks were used. The mean ± standard error of the percentage survival was calculated on the basis of six or more samples, each sample representing the average of duplicate plates.

*CsCl Density Gradient Analysis.* Cesium chloride (American Potash and Chemical Corporation, Los Angeles, California) was incinerated, purified with Norite, and filtered. The solution was adjusted to pH 7.0 and a refractive index of 1.400. The absorbance at 260 $m\mu$ was no greater than 0.025. Refractive indices of CsCl solutions were read at 25°C, and were converted to density using the equation of Ifft, Voet, and Vinograd (1961). Previously purified phage stocks were washed twice by centrifugation in either 0.1 M sodium phosphate buffer, pH 7.0, or 3.0 molal NaCl. One-tenth-ml volumes of the phage suspensions, at a concentration of approximately $2 \times 10^{13}$ phage/ml, were subjected to various osmotic shock treatments by 1000-fold dilution with appropriate dilution media. Depending on the gradient desired, 1.2- to 1.8-ml volumes of the diluted phage were carefully layered over 3.8 to 3.2 ml of CsCl ($\rho = 1.707$) in 5-ml cellulose nitrate tubes. The tubes were centrifuged in a Spinco SW-39 rotor at 32,500 RPM (average force = 90,000 *g*) at 25°C for 18 hr. Varying the centrifugation time from 12 to 24 hr resulted in no discernible differences in the measured densities. Following centrifugation, 0.3-ml fractions were collected and monitored by a modification of the technique of Fisher, Cline, and Anderson (1964), and the refractive indices of the fractions were measured. After converting refractive index to density, density gradient plots were constructed in which T4-DNA was used as a marker and was assumed to have a density at 25°C of 1.690 g/ml (Vinograd and Greenwald, 1965). Each density centrifugation was repeated four times, using a freshly prepared phage stock each time. Assays of infectivity of the diluted samples were also performed before centrifugation in CsCl.

## RESULTS

### *Effects of Osmotic Pressure Changes and of Low Salt Concentration*

The results in Fig. 1 show that the survival of T4B decreased sharply when the

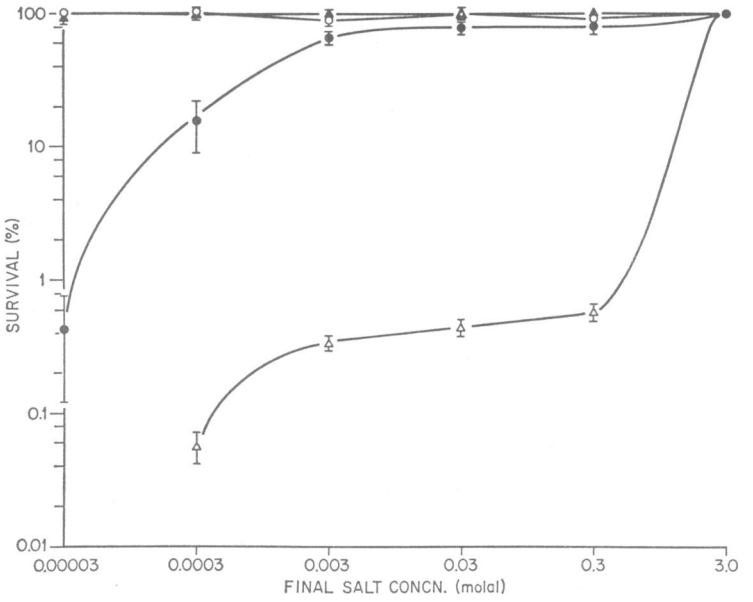


FIGURE 1 The effect of final salt concentrations on survival of phages T4B and T4Bo$_1$. The phages were suspended in 3.0 molal NaCl or $MgSO_4$, and were rapidly diluted $10^3$-, $10^4$-, or $10^5$-fold to various final salt concentrations. Standard errors were omitted from the succeeding figures for clarity, but were similar to those shown in this figure. Phage T4B in 3 molal NaCl (△) and $MgSO_4$ (▲). Phage T4Bo$_1$ in 3 molal NaCl (●) and $MgSO_4$ (○).

phages were rapidly diluted from suspension in 3.0 molal NaCl (153 atm) to 0.3 molal NaCl (13 atm), but the survival of T4Bo$_1$ decreased only slightly after the same treatment. Reducing the final concentration from 0.3 to 0.003 molal NaCl had little additional effect, but reducing it below 0.003 molal produced a marked drop in survival of T4Bo$_1$ and a second drop in survival of T4B. These data demonstrate:

1. The survival of T4B is markedly reduced, but that of T4Bo$_1$ is hardly affected by a large, rapid decrease in NaCl concentration, and, therefore, in osmotic pressure. This justifies their designation as "osmotic shock sensitive" and "osmotic shock resistant," respectively.

2. The infectivity of both T4B and T4Bo$_1$ is reduced when the final salt concentration is lowered below 0.003 molal, even though the osmotic pressure difference between 0.003 and 0.00003 molal salt is much less than 1 atm.

Therefore, these findings suggest that when T4 phages are rapidly diluted from 3 molal NaCl, they are subject to two separate effects: those due to a large, rapid change in osmotic pressure of the suspending solution, and those due to exposure to low salt concentrations.

*Effects of Osmotic Pressure Changes*

*Phage T4B.* When phage T4B was rapidly transferred from a concentrated to a dilute solution (0.065 molal NaCl or less), survival depended critically on the initial concentration in which the phages were suspended. The results in Fig. 2, like those reported by Anderson et al. (1953) for T6rS, show that the infectivity of T4B was unaffected when the initial concentration was less than 1.5 molal NaCl (70 atm), but decreased logarithmically above that initial concentration. (The effect of final salt concentration noted in Fig. 1 is also evident in Fig. 2).

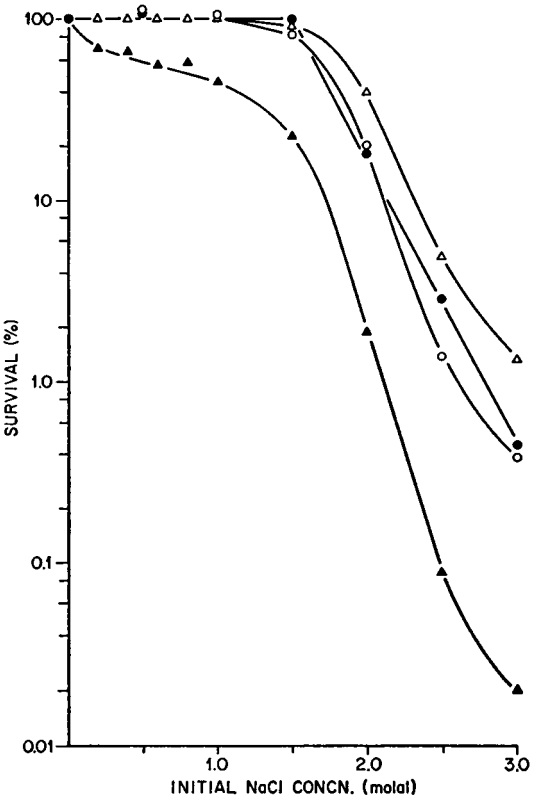


FIGURE 2 The effect of initial salt concentrations on survival of phage T4B. The phages were initially suspended in various concentrations of NaCl, and then rapidly diluted $10^3$-, $10^4$-, or $10^5$-fold with water or dilute NaCl solutions to each of the following final salt concentrations: △, 0.065 molal NaCl; ●, $10^{-3}$ × initial concentration; ○, 0.0005 molal NaCl; ▲, $10^{-5}$ × initial concentration.

However, phage inactivation only occurred with rapid dilution (Table I, Fig. 3): the survival of T4B decreased 100-fold with rapid dilution, but showed no decrease with slow dilution, even when the final concentration was $3 \times 10^{-5}$ molal NaCl. The dilution volume by itself did not affect the survival of T4B provided that the dilution rate and final concentration were held constant. For example, when T4B was rapidly diluted 100- or 1000-fold from 3.0 to 0.3 molal NaCl, survivals were 0.5 and 0.8%, respectively.

The results in Table I show that osmotic shock of T4B occurred equally well in

TABLE I

SURVIVAL OF PHAGES T4B, T4Bo$_1$, AND T4D AFTER RAPID OR SLOW DILUTION IN VARIOUS SOLUTIONS

The phages were suspended in the initial medium for 15 min and then diluted either rapidly or slowly $10^3$-, $10^4$-, or $10^5$-fold with water or a dilute salt solution to the indicated final concentration.

| Dilution medium | | Dilution rate | Per cent survival | | |
|---|---|---|---|---|---|
| Initial | Final | | T4B | T4Bo$_1$ | T4D |
| | 0.003 molal NaCl | Slow | 111.5 | 73.2 | — |
| | 0.003 molal NaCl | Fast | 1.1 | 68.5 | — |
| 3.0 molal NaCl | 0.00003 molal NaCl | Slow | 100.0 | 0.8 | 23.9 |
| | 0.00003 molal NaCl | Fast | 0.1 | 0.1 | 0.1 |
| | 0.003 molal CsCl | Slow | 76.6 | — | — |
| | 0.003 molal CsCl | Fast | 0.3 | — | — |
| 3.0 molal CsCl | 0.3 molal CsCl | Fast | — | 78.8 | — |
| | 0.0003 molal CsCl | Fast | — | 0.02 | — |
| | 0.003 molal LiCl | Slow | 72.6 | — | — |
| | 0.003 molal LiCl | Fast | 1.3 | — | — |
| 3.0 molal LiCl | 0.3 molal LiCl | Fast | — | 89.0 | — |
| | 0.0003 molal LiCl | Fast | — | 0.05 | — |
| 6.0 molal glycerol | 0.006 molal glycerol | Fast | 0.7 | 99.2 | 75.9 |

NaCl, CsCl, and LiCl, and that the effect of dilution rate was observed with all three solutions.

Another characteristic that distinguished osmotic shock from the effect of the final salt concentration is that the former could occur in a nonelectrolyte solution, namely, glycerol (Table I). Anderson et al. (1953) were also able to shock T6rS using glycerol, but Cummings (1964) found that 60 to 70% of T4B survived a similar treatment. This difference between his data and ours may be due to differences in the dilution procedures. Cummings diluted T4B only 20-fold from a very viscous solution of 8.5 M glycerol, whereas we diluted T4B 1000-fold from a less viscous 4.2 M solution. The dilution rate in the former case was probably appreciably lower.

The behavior of T4B in $MgSO_4$ solutions differed from its behavior in $MgCl_2$ solutions, and its behavior in both these solutions differed from that in solutions of monovalent salts. Approximately 100% of T4B in 0.5 to 3.0 molal $MgSO_4$ survived a 1000-fold rapid dilution (Fig. 1). Phage T2r in $MgSO_4$ also appears to be insensitive to osmotic shock (Herriott and Barlow, 1957), whereas T6rS in $MgSO_4$ is sensitive (Anderson et al., 1953). On the other hand, virtually none of T4B in 3.0 molal $MgCl_2$ survived after either rapid or slow dilution. Anderson et al. (1953) found that T6rS in $MgCl_2$ are destroyed by rapid dilution, but survive slow dilution.

*Phage T4Bo$_1$.* Phage T4Bo$_1$ behaved quite differently. When it was rapidly

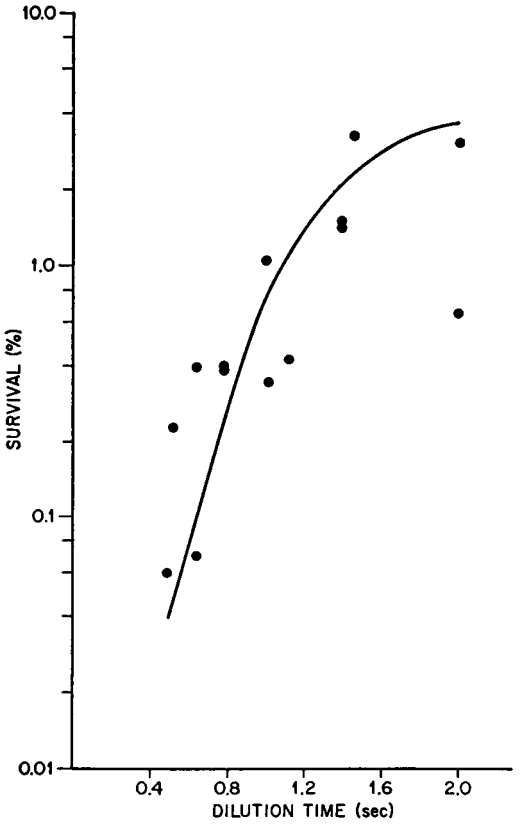


FIGURE 3 The effect of dilution rate on survival of phage T4B. The phages were suspended in 3.0 molal NaCl, and 0.025-ml samples were then diluted 1000-fold to a final concentration of 0.003 molal NaCl. The various dilution rates were achieved by adjusting the delivery rate of diluent with a Brewer Automatic Pipetting Machine, Model 40 (Baltimore Biological Laboratory, Inc.).

transferred from suspension in concentrated solutions to more dilute ones, survival was nearly independent of the initial concentration as long as the final solute concentration was constant (Fig. 4). For example, 80% of T4Bo$_1$ survived rapid dilution to 0.065 molal NaCl, regardless of whether the initial concentration was 0.5 or 3.0 molal NaCl, even though the total changes in osmotic pressure were 20 and 150 atm, respectively. (The decreasing survivals in Fig. 4 of the phage after 1000-fold dilutions from initial concentrations below 1.5 molal were due to the progressively lower values of the final concentration.) Unlike T4B, the survival of T4Bo$_1$ after dilution to a given final concentration was similar with both slow and rapid dilution (Table I).

Phage T4Bo$_1$ also withstood large osmotic pressure changes in solutions of CsCl, LiCl, and glycerol (Table I). This last result differs from that of Cummings (1964), who found that 20 to 30% of T4Bo$_1$ was inactivated after rapid dilution from 67% glycerol; we found that no more than a few per cent of T4Bo$_1$ was inactivated after rapid dilution from 36% glycerol. This difference may result from the fact that we exposed T4Bo$_1$ to a change of 150 atm, whereas Cummings exposed it to an

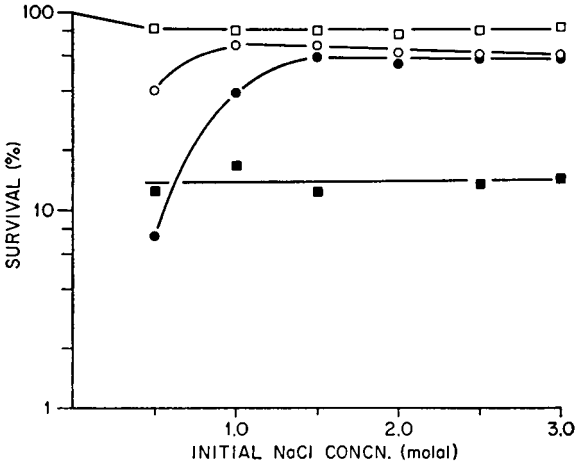


FIGURE 4 The effect of initial salt concentrations on survival of phage T4Bo$_1$. The phages were initially suspended in various concentrations of NaCl, and were then rapidly diluted $10^3$- or $10^4$-fold with water or dilute NaCl solutions to each of the following final salt concentrations: □, 0.065 molal NaCl; ○, 0.003 molal NaCl; ●, $10^{-3}$ × initial concentration; ■, 0.0005 molal NaCl.

osmotic pressure change of at least 550 atm. Alternatively, it might be explained by an inactivation of the phage resulting simply from exposure to 67% glycerol. T4Bo$_1$ was also resistant to a large, rapid change in osmotic pressure of a divalent salt solution, $MgSO_4$ (Fig. 1). However, as noted for T4B, exposure of T4Bo$_1$ to 3.0 molal $MgCl_2$ resulted in complete loss of infectivity, regardless of subsequent dilution treatment.

*Phage T4D.* The response of phage T4D to rapid dilution from concentrated salt solutions was intermediate to that of T4B and T4Bo$_1$ (Fig. 5). Like T4B and unlike T4Bo$_1$, the infectivity of T4D was unaffected by being rapidly diluted from NaCl solutions below 1.5 molal to a final concentration of 0.065 molal NaCl, but was adversely affected by being rapidly diluted from initial concentrations above 1.5 molal. But unlike T4B (Fig. 2) and like T4Bo$_1$ (Fig. 4), rapid dilution to a final concentration of $3 \times 10^{-5}$ molal or below produced substantial inactivation even with initial concentrations of NaCl as low as 0.2 molal. The response of T4D in solutions of KCl, $NaNO_3$, and $KNO_3$ was similar but not identical to that in NaCl. Phage T4D was also more sensitive to osmotic shock with glycerol and to differences in dilution rate than was T4Bo$_1$, but was less sensitive than T4B (Table I).

### *Effect of Low Salt Concentration*

*Phage T4B.* The results in Fig. 6 show that when phage T4B was diluted rapidly from suspension in a concentrated salt solution to various final salt con-

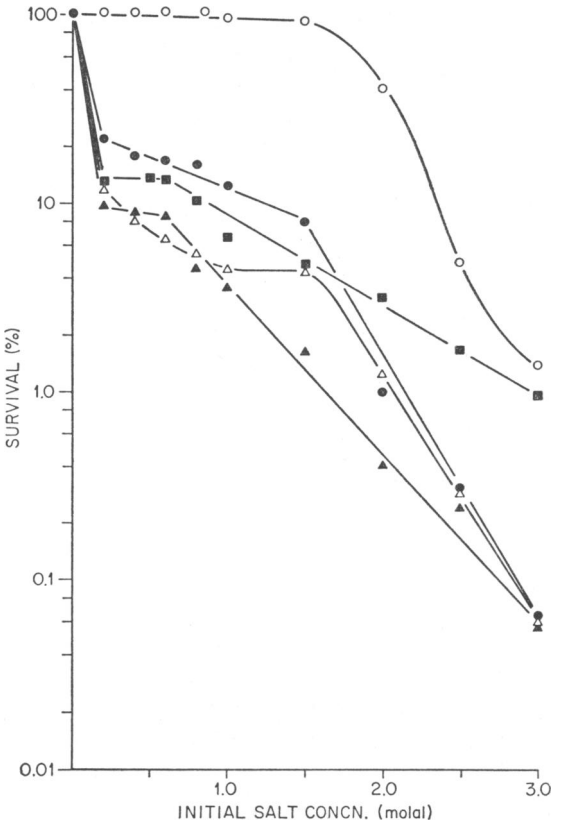


FIGURE 5 The effect of initial concentrations of various salts on survival of phage T4D. The phages were suspended in various initial concentrations of NaCl, KCl, $NaNO_3$, or $KNO_3$, and were then rapidly diluted to the following final concentrations of these salts: ○, 0.065 molal NaCl; ●, $10^{-5}$ × initial NaCl concentration; ■, $10^{-5}$ × initial $KNO_3$ concentration; △, $10^{-5}$ × initial KCl concentration; ▲, $10^{-5}$ × initial $NaNO_3$ concentration.

centrations, survival first decreased precipitously, then plateaued, and finally dropped again at final concentrations below $10^{-3}$ molal salt. The first drop is associated with a large change in osmotic pressure of the suspending solution, and is interpreted as osmotic shock. But the second decrease occurred with an osmotic pressure change of only a fraction of 1 atm, and is therefore believed due to the low ionic strength itself. Neither drop occurred when the dilution was carried out slowly (Table I). That is, 100% of T4B survived slow dilution from 3 molal to $3 \times 10^{-5}$ molal NaCl, whereas only 0.1% survived rapid dilution of the same magnitude. Slow dilution, in other words, eliminated both the osmotic shock and the deleterious effect of low salt concentrations.

The extent of inactivation after rapid dilution to low salt concentrations de-

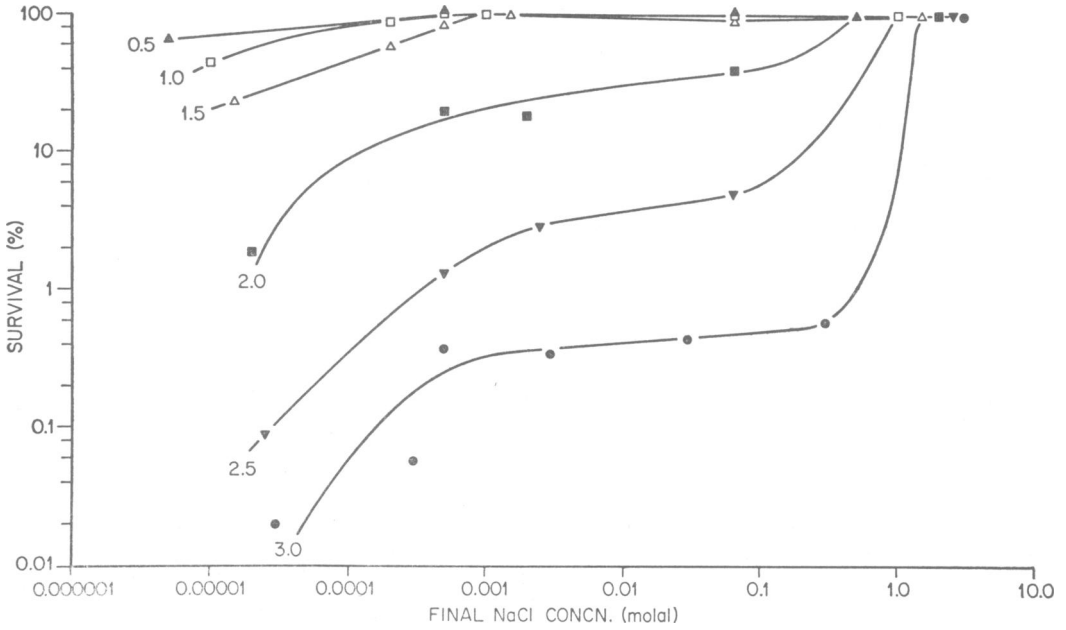


FIGURE 6 The effect of final NaCl concentrations on survival of phage T4B. The phages were initially suspended in NaCl solutions of the indicated molalities, and were then rapidly diluted $10^3$-, $10^4$-, or $10^5$-fold with water or dilute NaCl solutions to achieve various final salt concentrations.

pended greatly on the initial concentration to which the phages were exposed (Fig. 6). But deleterious effects of low salt concentration were observed with all initial concentrations used. The magnitude of the second drop in survival was much larger when the initial concentration was sufficient for osmotic shock, i.e., 2 molal NaCl or above. Nevertheless, a slight loss in survival below a final concentration of $10^{-3}$ molal NaCl occurred even when the initial concentration was too low to effect osmotic shock, i.e., 1.5 to 0.5 molal NaCl.

When suspended in concentrated solutions of $MgSO_4$ rather than NaCl, T4B was completely resistant to both large, rapid changes in osmotic pressure and to dilution to very low final salt concentrations, such as $3 \times 10^{-5}$ molal (Fig. 1).

Experiments with glycerol as the suspending medium lend further support to the view that damage from osmotic shock and low salt concentrations are separable phenomena in T4B (Table II). Thus, diluting T4B $10^5$-fold from 6.0 molal glycerol (treatment 3) produced the same survival as a $10^3$-fold dilution from 3.0 molal NaCl (treatment 7), but it produced a 30-fold higher survival than a $10^5$-fold dilution from 3.0 molal NaCl (treatment 8). In other words, in glycerol, the only effect observable appears to be the osmotic shock associated with a large, abrupt change in osmotic pressure.

TABLE II

THE EFFECT OF VARIOUS DILUTION MEDIA ON SURVIVAL OF PHAGES T4B, T4Bo$_1$, AND T4D

The phages were diluted 1000-fold from storage in 1.5% nutrient broth into the initial medium. One-tenth-ml samples of diluted phage were then diluted rapidly with 100 ml of the intermediate medium, and finally, these dilutions were rapidly diluted 100-fold with the final medium.

| Treatment | Dilution medium | | | Per cent survival | | |
|---|---|---|---|---|---|---|
| | Initial | Intermediate | Final | T4B | T4Bo$_1$ | T4D |
| 1 | Broth | Broth | Broth | 100 | 100 | 100 |
| 2 | $H_2O$ | $H_2O$ | $H_2O$ | 17.0 | 100.0 | 76.0 |
| 3 | 6.0 molal glycerol | $H_2O$ | $H_2O$ | 0.7 | 99.2 | 75.9 |
| 4 | 3.0 molal NaCl | 6.0 molal glycerol | 6.0 molal glycerol | 35.4 | 70.7 | — |
| 5 | 6.0 molal glycerol | 6.0 molal glycerol | 6.0 molal glycerol | 39.4 | 89.4 | — |
| 6 | 3.0 molal NaCl | 6.0 molal glycerol | $H_2O$ | 0.8 | 69.5 to 0.3 | — |
| 7 | 3.0 molal NaCl | $H_2O$ | Broth | 0.7 | — | — |
| 8 | 3.0 molal NaCl | $H_2O$ | $H_2O$ | 0.02 | — | — |

The data in Table II also indicate (with one exception) that exposure to NaCl solution is required to observe subsequent deleterious effects from low ionic strength. Thus, no adverse effects were noted after $10^5$-fold dilution from broth (treatment 1), and relatively high survivals were obtained after dilution from glycerol with glycerol (treatment 5). (The drop in survival from 100 to 35% is believed to have been the result of osmotic shock during the assay procedure when the phage were diluted from 6 molal glycerol (154 atm) to nutrient broth (6 atm).) Glycerol is also apparently able to prevent or reverse the effects of a prior exposure to NaCl (treatments 3 and 6 vs. 8). The observation that about 80% of T4B were inactivated by a $10^8$-fold dilution with distilled water seems inconsistent with the other data, but Adams (1948, 1949) reported analogous results.

*Phage T4Bo$_1$.* The results in Fig. 4 suggested that the survival of the osmotic shock–resistant mutant, T4Bo$_1$, after rapid dilution of the NaCl suspending solution depended only upon the final salt concentration in the medium. This suggestion is borne out by the results in Fig. 7, which shows the survivals of T4Bo$_1$ after suspensions in several initial concentrations had been rapidly diluted to various final salt concentrations. There was no inactivation over the concentration range of 3 to 0.3 molal salt that was most deleterious to T4B, and over which the largest absolute change in osmotic pressures occurred. But there was marked inactivation below about $10^{-3}$ molal salt. The exact final concentration at which substantial inactivation began to occur appeared to depend upon the initial concentration. That is, inactivation started below about $3 \times 10^{-3}$ molal NaCl when the initial concentrations were high (0.5 to 3 molal), but below about $1 \times 10^{-3}$ molal when they were

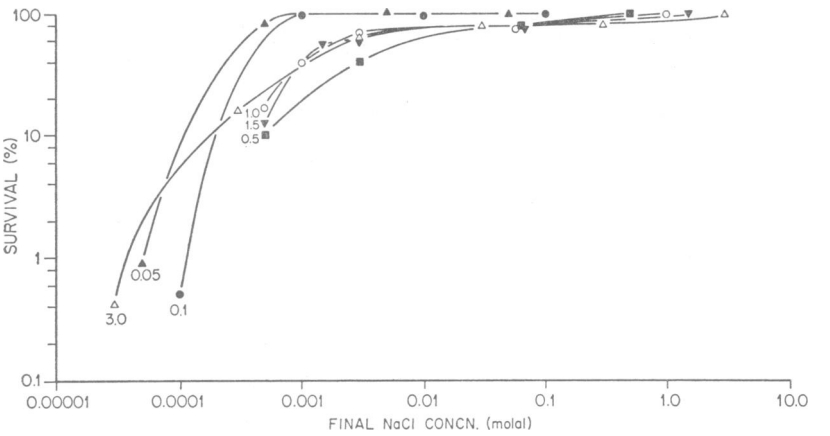


FIGURE 7 The effect of final NaCl concentrations on survival of phage T4Bo$_1$ initially suspended in NaCl solutions of the indicated molalities. Other details were the same as in Fig. 6.

low (0.1 and 0.05 molal). However, the total extent of inactivation at the lowest concentrations achieved (3 to 10 $\times$ $10^{-5}$ molal) appeared to be independent of initial concentration (cf. curves for initial concentrations of 0.05, 0.1, and 3.0 molal). This last finding differs from that with T4B (Fig. 6). As already mentioned, the extent of inactivation of T4B from lowering the salt concentration below 0.001 molal is less when the initial concentration is below 1.5 molal than when it is above.

The effects of final salt concentration on survival of T4Bo$_1$ were independent of dilution rate. For example, about 70% of T4Bo$_1$ in 3 molal NaCl survival slow or rapid dilution to 0.003 molal NaCl, and about 0.5% survived slow or rapid dilution to 0.00003 molal NaCl (Table I). This is in sharp contrast to T4B, in which survivals after rapid or slow dilution were 0.1 to 1% and 100%, respectively.

Phage T4Bo$_1$ suspended in LiCl and CsCl behaved like phages in NaCl in showing the detrimental effect of low salt concentrations (Table I). But those suspended in $MgSO_4$ showed no detrimental effects, since 100% of T4Bo$_1$ survived even after rapid dilution from 3.0 to 3 $\times$ $10^{-5}$ molal $MgSO_4$ (Fig. 1).

Although the curves in Fig. 7 demonstrate the deleterious effects on T4Bo$_1$ from lowering the salt concentration below 0.001 molal even when the initial concentration was as low as 0.05 molal, the effects were not observed if the phages were not initially exposed to some NaCl; that is, little or no inactivation of T4Bo$_1$ occurred when phages in broth, water, or glycerol were diluted $10^5$-fold with broth or water (Table II, treatments 1, 2, 3) or when phages in glycerol were diluted with glycerol (treatment 5). Furthermore, even when the phages were initially suspended in concentrated NaCl, subsequent dilution with glycerol eliminated any

deleterious effects, even though the dilution was sufficient to reduce the initial NaCl concentration to $10^{-5}$ of its original value (treatment 4). When phages in concentrated NaCl were first diluted in glycerol and then further diluted with water, survivals were variable (treatment 6). The source of variation was found to be the length of exposure to concentrated glycerol prior to the final dilution in water; i.e., over the time range of 1 to 55 min, survival after further dilution with water was found to vary exponentially according to the relationship, survival (%) = $0.067e^{t/12.9}$ when the time of exposure to glycerol is expressed in minutes. This time effect was present even though in all cases the NaCl was reduced to $10^{-5}$ of its original value.

In summary, it appears that glycerol is able to block or reverse the consequences of an initial exposure to NaCl, but that its ability to do so requires that it be in contact with the phage for some length of time. We believe that the inactivation of phage at low salt concentration is due to the reduction of electrolyte concentration in the head, and that the glycerol may protect because its high viscosity reduces the rate at which ions can leave the head.

*Phage T4D and Others.* Phage T4D was intermediate to T4B and $T4Bo_1$ not only with respect to osmotic sensitivity, but also with respect to the effects of low salt concentrations. For example, 100% T4B, 24% T4D, and less than 1% $T4Bo_1$ survived slow dilution from 3 to $3 \times 10^{-5}$ molal NaCl (Table I).

Preliminary experiments with two other osmotic shock–resistant mutants, $T4Bo_5$ (obtained from Dr. R. B. Setlow) and $T4Dos_{41}$ (obtained from Dr. R. Edgar), suggested that these phages are similar to $T4Bo_1$ in that they are inactivated by exposure to very low monovalent salt concentrations.

### *Loss of DNA from Bacteriophage Heads*

It has been demonstrated both microscopically (Kleinschmidt et al., 1962) and chemically (e.g. Hershey and Chase, 1952) that DNA is lost from the heads of osmotically shocked T-even phage. It was of interest to determine if DNA was also lost into solution from the heads of $T4Bo_1$ inactivated by exposure to low salt concentrations. In order to do this, phages T4B and $T4Bo_1$ were subjected to dilution treatments causing various degrees of inactivation, and then centrifuged to equilibrium in CsCl gradients. The results are shown in Figs. 8 and 9 and Table III.

The absorbancy curve in Fig. 8*a* demonstrates that the control phage T4B stock banded as a single peak. Three peaks appeared in the osmotically shocked T4B sample (Fig. 8*c*). On the basis of their densities and comparison with control samples, we identified these three peaks (from left to right) as DNA, intact phage, and phage ghosts. Comparison of the density profile in Fig. 8*b* of T4B slowly diluted from 3 molal NaCl (no osmotic shock, 100% survival) with that in Fig. 8*c* of T4B rapidly diluted from 3 molal NaCl (osmotic shock, 1% survival) clearly

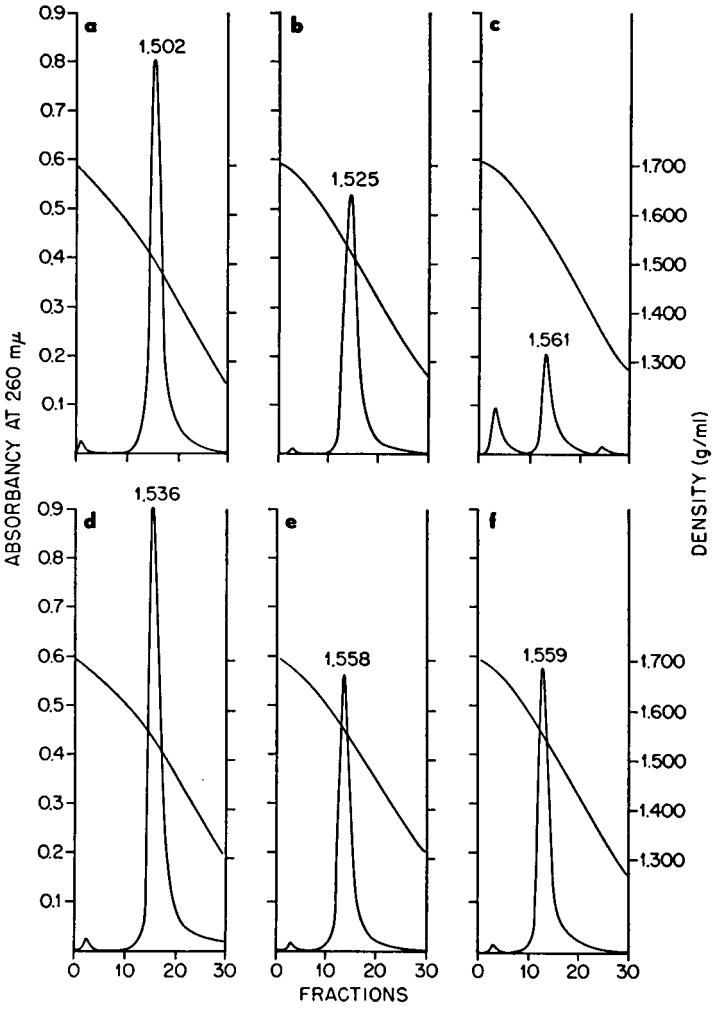


FIGURE 8 Density gradient profiles of variously treated phages T4B and T4Bo$_1$. The phages were subjected to the following dilution treatments, and were then centrifuged in CsCl density gradients. (*a*) T4B in 0.1 M phosphate buffer rapidly diluted to 0.1 molal NaCl; (*b*) T4B in molal NaCl slowly diluted to 0.003 molal NaCl; (*c*) T4B in molal NaCl rapidly diluted to 0.003 molal NaCl; (*d*) T4Bo$_1$ in 0.1 M phosphate buffer rapidly diluted to 0.1 molal NaCl; (*e*) T4Bo$_1$ in 3 molal NaCl rapidly diluted to 0.3 molal NaCl; (*f*) T4Bo$_1$ in 3 molal NaCl rapidly diluted to 0.0003 molal NaCl. Absorbancy at 260 mμ was read continuously as the fractions were collected. The upper curve is the CsCl density gradient constructed from the refractive indices of the collected fractions. The concentration in (*f*) was only 1/10 of that in the other cases, so the observed absorbancy of the centrifuged samples has been multiplied by 10 in the figure.

shows that DNA was lost from the heads of osmotically shocked T4B. But the area of the central, phage peak in Fig. 8*c* is about 40% of the phage peak in Fig. 8*b*, suggesting that nearly half of the osmotically shocked phage retained their DNA. That the total absorbancy in Fig. 8*c* is less than that in Fig. 8*b* may be partially due to the anomalous absorption resulting from light scattering by the intact phage particles (McLaren and Shugar, 1964, p. 83), or to the adsorption of about 10% of the free DNA on the surface of the centrifuge tubes (Billen and Hewitt, 1966).

The density profile in Fig. 8*d* demonstrates that the untreated phage $T4Bo_1$ stock banded as a single peak. Comparison of the results in Fig. 8*e* of $T4Bo_1$ diluted from 3 to 0.3 molal NaCl (82% survival) with those in Fig. 8*f* of $T4Bo_1$ diluted from 3 to 0.0003 molal NaCl (10% survival) indicates that little or no DNA was lost from $T4Bo_1$ after a dilution treatment that caused a 70% loss of survival. Moreover, these results also prove that the loss of infectivity of $T4Bo_1$ from exposure to low salt concentrations was not due to an absolute loss of phage particles, e.g. by adhesion of the particles to the glass of the dilution vessel, since approximately all the UV-absorbing material was recovered, regardless of survival. The widths at half-height of the phage peaks in Fig. 8*e* and *f* are about the same, suggesting that the observed 70% loss of infectivity was not due to clumping of infective phage particles.

### *Changes in Bacteriophage Density*

The densities of T4B and $T4Bo_1$ depended on the dilution treatment to which the phages were exposed prior to centrifugation in CsCl density gradients (Table III).

TABLE III

DENSITY OF PHAGES T4B AND $T4Bo_1$ AS A FUNCTION OF DILUTION TREATMENT

The phages were suspended in the initial medium. One-tenth-ml samples of these suspensions were then diluted rapidly or slowly with 100 ml of a dilute NaCl solution or with water to yield the indicated final salt concentration. In one case, $T4Bo_1$ was diluted an additional 10-fold to give a final salt concentration of 0.0003 molal NaCl. The diluted phage were centrifuged in CsCl density gradients, fractions collected, and their densities determined refractometrically. Survival was measured before centrifugation.

| Phage | Suspending medium | | Dilution rate | Per cent survival | Density |
|---|---|---|---|---|---|
| | Initial | Final | | | |
| | | | | | g/ml |
| T4B | $PO_4$ buffer | 0.1 molal NaCl | Fast | 100 | 1.502 ± 0.003 |
| $T4Bo_1$ | $PO_4$ buffer | 0.1 molal NaCl | Fast | 100 | 1.536 ± 0.005 |
| T4B | 3.0 molal NaCl | 0.003 molal NaCl | Slow | 100 | 1.525 ± 0.003 |
| T4B | 3.0 molal NaCl | 0.003 molal NaCl | Fast | 0.5 | 1.561 ± 0.006 |
| $T4Bo_1$ | 3.0 molal NaCl | 0.3 molal NaCl | Fast | 82 | 1.558 ± 0.003 |
| $T4Bo_1$ | 3.0 molal NaCl | 0.03 molal NaCl | Fast | 80 | 1.554 ± 0.003 |
| $T4Bo_1$ | 3.0 molal NaCl | 0.003 molal NaCl | Fast | 65 | 1.557 ± 0.003 |
| $T4Bo_1$ | 3.0 molal NaCl | 0.0003 molal NaCl | Fast | 10 | 1.559 ± 0.002 |

The approximate survival value found with each treatment is also presented in that table for reference. The density of 1.502 g/ml for T4B diluted from 0.1 M phosphate buffer into 0.1 molal NaCl agrees with the value of 1.505 g/ml for T2L found by Cummings (1963). These results also show:

1. The density of control $T4Bo_1$ was higher than that of control T4B.
2. The density of T4B previously exposed to concentrated NaCl was greater than that of T4B not previously exposed.
3. The density of osmotically shocked T4B was greater than that of unshocked phage.
4. The density of $T4Bo_1$ previously exposed to concentrated NaCl was greater than that of $T4Bo_1$ not previously exposed, and was the same regardless of subsequent dilution and survival.

The reality of these density differences was confirmed by centrifuging mixtures of variously treated phages in CsCl gradients (Fig. 9).

## DISCUSSION

### *Summary of Significant Findings*

Measurements of survival and buoyant densities of bacteriophages T4B, $T4Bo_1$, and T4D have demonstrated the following significant points:

1. After suspension in a concentrated salt solution, phages T4B and T4D are inactivated both by osmotic shock and by subsequent exposure to low concentrations of monovalent salt. After similar treatment, $T4Bo_1$ is resistant to osmotic shock, but is also inactivated by exposure to low salt concentrations.
2. Sensitivity of T4B to dilution from a concentrated salt solution depends on dilution rate. Sensitivity of $T4Bo_1$, however, is independent of rate, and that of T4D is less dependent.
3. Sensitivity of T4B and T4D to osmotic shock depends critically on the initial salt concentration in which the phages were suspended.
4. Sensitivity of all three phages to low salt concentrations depends on initial salt concentrations to a variable extent.
5. Since no more than 60% of osmotically shocked T4B seem to lose DNA when subjected to a treatment that causes 99% loss of infectivity, loss of DNA from the phage head may not be the primary cause of loss of infectivity from osmotic shock.
6. No DNA is lost into solution from $T4Bo_1$ exposed to low salt concentrations that produce a 90% loss of infectivity.
7. The effective buoyant densities of T4B and $T4Bo_1$ depend significantly on the dilution treatments to which the phages are subjected prior to centrifugation in CsCl gradients.

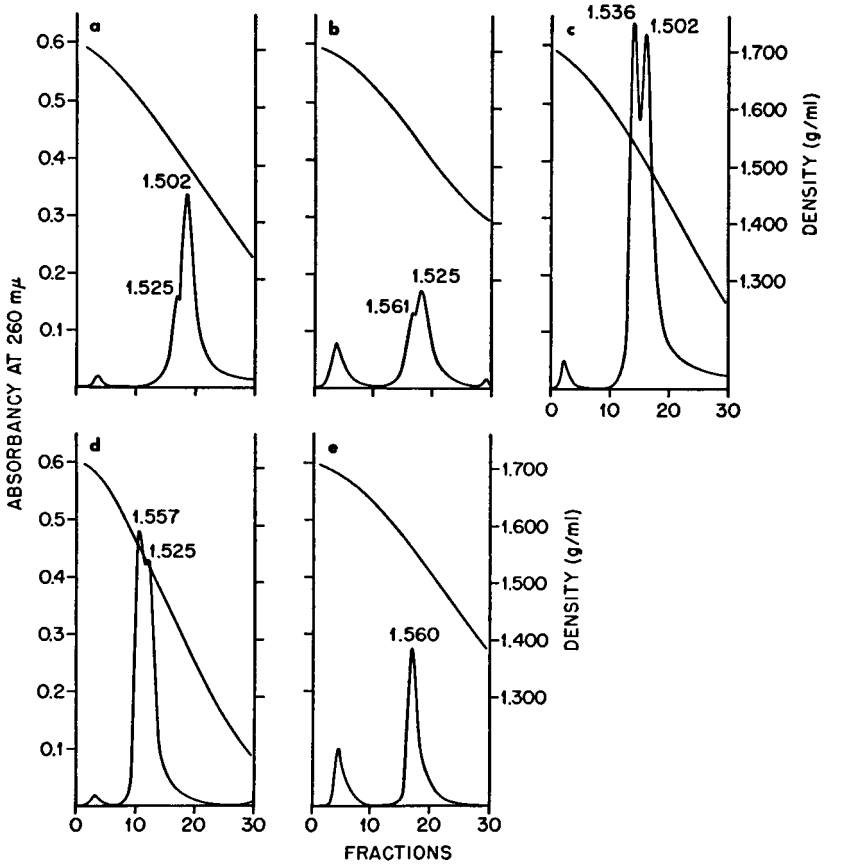


FIGURE 9 Density gradient profiles of mixtures of phages T4B and T4Bo$_1$. After dilution, equal volumes of the samples were mixed in the following combinations, and centrifuged in CsCl density gradients. Other details are given in Fig. 8. (*a*) T4B in 0.1 M phosphate buffer rapidly diluted to 0.1 molal NaCl plus T4B in 3 molal NaCl slowly diluted to 0.003 molal NaCl; (*b*) T4B in 3 molal NaCl *slowly* diluted to 0.003 molal NaCl plus T4B in 3 molal NaCl *rapidly* diluted to 0.003 molal NaCl; (*c*) *T4B* in 0.1 M phosphate buffer rapidly diluted to 0.1 molal NaCl plus *T4Bo$_1$* in 0.1 M phosphate buffer rapidly diluted to 0.1 molal NaCl; (*d*) *T4B* in 3 molal NaCl *slowly* diluted to 0.003 molal NaCl plus *T4Bo$_1$* in 3 molal NaCl *rapidly* diluted to 0.003 molal NaCl; (*e*) *T4B* in 3 molal NaCl rapidly diluted to 0.003 molal NaCl plus *T4Bo$_1$* in 3 molal NaCl rapidly diluted to 0.003 molal NaCl.

We propose that these data can be explained in terms of the different relative permeabilities of the phage particles and the polyelectrolyte behavior of the DNA contained within the phage heads.

### *Osmotic Shock*

The explanation of osmotic shock, as originally proposed by Anderson et al. (1953),

assumes that the following conditions exist: (*a*) Low molecular weight solutes can diffuse in and out of the phage head to maintain equilibrium between the internal and external environments of the phage. (*b*) Therefore, there must be solvent water present in the phage head. (*c*) The phage possess a differentially permeable barrier, so that water diffuses in and out faster than solutes. (*d*) Osmotic shock occurs when the osmotic pressure drop across the barrier exceeds a critical value. Phage $T4Bo_1$, being more permeable to solutes than T4B, loses the solutes almost as fast as it takes up water when rapidly transferred into dilute media. Therefore, an injurious pressure differential can not be established and the phage survives.

The evidence on these points is as follows:

A variety of data indicates that solutes can diffuse through the phage membrane. The sensitivity to osmotic shock (Table I) and the increased density (Table III) of phage suspended in CsCl is consistent with cesium having permeated the phage head. Second, Ames and Dubin (1960) showed that the cation content of T4 phage varies as a function of the solutes present in the suspending medium. Third, studies of photodynamic inactivation of phages incubated in various dyes further substantiate the supposition of solute flux (Helprin and Hiatt, 1959; Cummings and Kozloff, 1962; Ritchie, 1965).

These facts and the X-ray diffraction patterns of phage as a function of relative humidity (North and Rich, 1961) indicate the presence of solvent water in the head of T-even phage.

The fact that dilution rate is critical in osmotic shock (Table I) is consistent with the hypothesis that water diffuses in and out of the phage head faster than solutes. Furthermore, there is evidence that T4B and $T4Bo_1$ differ with respect to permeability to solutes. That is, the rate of dye uptake and that of dye removal by washing is faster in shock-resistant (permeable) phages than in shock-sensitive (less permeable) ones (Helprin and Hiatt, 1959; Cummings and Kozloff, 1962; Ritchie, 1965). In addition, Ames and Dubin (1960) noted that those phages whose polyamine content could be removed by washing are resistant to osmotic shock.

It has usually been assumed that the differentially permeable barrier of phage is the protein coat (Anderson, 1953; Anderson et al., 1953). This is especially likely in view of the fact that the *os* mutation, which controls osmotic sensitivity in phage T4, is located on gene 24 (A. S. Sarabhai and S. Brenner, 1966, personal communication), amber and temperature-sensitive mutants of which do not produce normal phage coats when grown under nonpermissive conditions (Epstein et al., 1963).

However, the nature of the alteration of the protein coat which determines osmotic sensitivity remains uncertain. So also does the exact factor responsible for loss of infectivity. This phage inactivation has usually been ascribed to loss of DNA, but the data in Fig. 8 indicate that the former can occur without the latter. There-

fore, damage may be more subtle than gross rupture of the phage head. The actual site of damage is also unknown. Since osmotically shocked phage retain cofactor requirements, and can adsorb and kill sensitive bacteria (Herriott, 1951; Duckworth and Bessman, 1965), the properties of the tail appear not to have been destroyed. However, the DNA itself, the protein coat, the injection mechanism, or any combination of these may be the the primary factor inactivated by osmotic shock.

*Effects of Low Salt Concentration*

We have demonstrated that phages T4B and $T4Bo_1$ are inactivated when rapidly diluted from a concentrated univalent salt solution to a very dilute one, and propose the following sequence of events to explain this phenomenon.

(*a*) When phage are transferred into concentrated ionic solutions, these exogenous ions permeate the phage head. (*b*) The exogenous cations compete with those normally present in the head (polyamines, $Mg^{+2}$, $Ca^{+2}$, $Na^+$, $K^+$, internal protein )that serve to neutralize the negative charges of the DNA phosphate groups. The exogenous cations replace the naturally occurring counterions to an extent dependent upon their relative concentrations and binding affinities for phosphate groups. (*c*) While the phage is still suspended in the concentrated solution, those endogenous counterions that have been replaced tend to diffuse out of the phage head in response to the resultant concentration gradient. The extent to which this diffusion occurs depends on the permeability of the phage membrane to the particular ion. For example, since $T4Bo_1$ is permeable to its endogenous cations, after these counterions have been displaced by the exogenous ion, they diffuse out of the phage. But since T4B is impermeable to polyamines and internal protein, it retains these organic cations within the confines of its head, but loses its normal inorganic counterions. (*d*) When the external medium is diluted, the free exogenous cations in the head also diffuse out into the medium. As a result of the lowered ionic strength, shielding of $PO_4^-$ groups by the counterions becomes less effective so that the repulsive forces between $PO_4^-$ groups on the DNA increase. This repulsion alters the DNA, rendering the phage inactive. In $T4Bo_1$ the exogenous cation, e.g. $Na^+$, is the sole counterion so that the concentration at which $PO_4^-$ repulsion becomes critical is dictated solely by the binding affinity of the cation, e.g. $Na^+$, to DNA. But in T4B, when sufficient $Na^+$ has diffused out of the head, the polyamines, which are still present within its confines, again act as DNA counterions. Because of their higher binding affinity to the $PO_4^-$ groups, the polyamines are able to prevent damaging $PO_4^-$ repulsion at lower ionic strengths than $Na^+$. However, since there are insufficient polyamines to neutralize all the $PO_4^-$ groups, the remainder are neutralized by the more weakly bound $Na^+$, and some inactivation does occur at extreme dilutions. Osmotically shocked T4B, having become permeable to polyamines as a result of the shock, behaves similarly to $T4Bo_1$.

Support for these suppositions is the following:

(*a*) *Cation Permeability.* The evidence indicating that cations can permeate the heads of phages T4B and $T4Bo_1$ was discussed in the previous section on osmotic shock.

(*b*) *Replacement of Endogenous Counterions.* Ames and Dubin (1960) demonstrated that several cations (polyamines, $Mg^{+2}$, $Ca^{+2}$, $Na^+$, $K^+$) serve as counterions of DNA in T-even phage. Extensive work on the interaction of cations with DNA in solution indicates that the binding affinity increases in the order: $Cs^+$, $K^+$, $Na^+$, $Li^+$, $Mg^{+2}$, putrescine$^{+2}$, spermidine$^{+3}$ (Ross and Scruggs, 1964; reviews by Jordan, 1960; Steiner and Beers, 1961; Felsenfeld, 1962; Michelson, 1963; Tabor and Tabor, 1964). In addition, a number of investigators have shown that high enough concentrations of cations of relatively low binding affinity can reversibly replace those with higher binding affinities in solutions of DNA (Jordan, 1960, p. 231; Michelson, 1963, p. 505; Scruggs and Ross, 1964).

Ames and Dubin (1960) observed that polyamines remain attached to T2-DNA in solution in the presence of 0.02 M $K^+$, suggesting that this concentration of potassium is too low to displace the polyamines. But they found that there are no polyamines present in intact $T4Bo_1$ washed in the presence of 0.01 M $Mg^{+2}$, indicating that this concentration of magnesium is high enough to displace the polyamines from the phosphate groups. These data support the contention that the naturally occurring counterions are displaced from DNA phosphate groups when T4B and $T4Bo_1$ are suspended in various concentrated electrolyte solutions.

(*c*) *Loss of Endogenous Cations.* The data of Ames and Dubin (1960) demonstrate that endogenous counterions, e.g. polyamines, can be removed from $T4Bo_1$, but not T4B, by washing it in 0.01 M $Mg^{+2}$, or can be replaced by washing the phage in 0.01 M spermidine.

The density differences of variously treated T4B and $T4Bo_1$ (Table III) also suggest the loss and replacement of endogenous cations. Calculated phage densities based on this assumption (in Table IV, to be discussed below) are similar to the observed densities.

(*d*) *Alteration of Counterion Charge Density Distribution.* If the behavior of DNA in the phage head is similar to that of DNA in solution, and if ions do diffuse into and out of the head, then, reducing the salt concentration of the suspending medium should produce the same effect on DNA in the phage head as on DNA in solution, namely, an alteration of the three-dimensional structure.

Thomas (1954) and others have demonstrated that the stability of nucleic acids is decreased by decreasing the salt concentration of the solution, whether the assay involves shifts in the absorbancy-temperature profile (Colvill and Jordan, 1963; Gordon, 1965; Schildkraut and Lifson, 1965), viscosity (Scruggs and Ross, 1964), or transforming activity (Cavalieri, Rosoff, and Rosenberg, 1956). Electron micrographs of DNA exposed to 0.002 M NaCl indicate that portions of the molecule separate into occasional lengths of single strands, giving rise to small loops (Das

Gupta, Sarkar, and Misra, 1966). The calculations of Schildkraut and Lifson (1965) suggest that the energy for such alterations of DNA results from the potential due to interchain phosphate repulsion as the salt concentration is lowered. We suggest that effects such as these on the DNA in the phage head could result in an irreversible loss of infectivity, regardless of whether such alterations to DNA are reversible or irreversible.

The data of Thomas (1954) illustrate one similarity between the inactivation of phage and the alteration of DNA in solution. He observed that DNA in solution first exhibits hyperchromicity when the sodium concentration is reduced to $10^{-3}$ M. We observed that the survival of both T4B and T4Bo$_1$ first decreased sharply, as a function of final concentration, at about $10^{-3}$ molal $Na^+$ (Figs. 6 and 7).

An additional similarity between phage and DNA in solution is that polyamines stabilize both (Tabor and Tabor, 1964). The stabilizing influence of polyamines on DNA can explain the different susceptibilities of T4B and T4Bo$_1$ to low salt concentrations (Figs. 6 and 7); namely, in T4Bo$_1$ the extent of inactivation from low salt concentrations was independent of initial concentration, but in T4B it was dependent. Since T4Bo$_1$ is permeable and T4B is impermeable to polyamines (section on Osmotic Shock above), and since DNA is stabilized by much lower concentrations of polyamines than inorganic cations (Kaiser, Tabor, and Tabor, 1963), we propose that only those phages that have lost all or the portion of their normal polyamine content are inactivated by exposure to low salt concentrations.

For T4Bo$_1$, loss of infectivity, and therefore the presumed loss of polyamines, occurs only when the phages are exposed to NaCl solutions of 0.05 molal or above prior to dilution. This is consistent with the finding of Kaiser et al. that 0.1 M NaCl completely eliminates the protective effect of spermine on DNA. In other words, if the phage were not previously exposed to a salt solution to bring about the replacement and loss of polyamines, then the polyamines "protected" the phage against inactivation from low salt concentrations. Thus, T4Bo$_1$ completely survived $10^5$-fold dilution from water or glycerol into distilled water (Table II).

But T4B is normally impermeable to polyamines. Therefore, we propose that those T4B phages that survive rapid dilution from 0.5 to 3 molal NaCl to $10^{-3}$ molal NaCl but are inactivated by dilution below $10^{-3}$ molal have undergone a nonlethal alteration in permeability as a result of stresses from the rapid change in osmotic pressure. This alteration is sufficient to allow the escape of polyamines. The findings in Fig. 6 are consistent with this view; most of the phages diluted rapidly from initial concentrations above 1.5 molal NaCl were inactivated by osmotic shock after dilution to 0.3 molal, but those that survived underwent a further 10-fold drop in infectivity upon dilution below $10^{-3}$ molal. Those phages that were diluted rapidly from initial concentrations below 1.5 molal survived the large, rapid change in osmotic pressure upon dilution to final concentrations of 0.003 to 0.3 molal; but their survival still dropped by 35 to 50% upon dilution below $10^{-3}$

molal. In contrast, when the stresses from the changes in osmotic pressure were reduced by slow dilution, the T4B phages survived (Table I), and presumably did not undergo any membrane alterations leading to loss of polyamines.

The differences in density of the variously treated phages (Table III) are also consistent with our proposed mechanism of injury. As shown in Table IV, the observed density changes can be accounted for if we assume the following:

TABLE IV

COMPARISON OF OBSERVED AND CALCULATED DENSITIES OF PHAGES T4B AND T4Bo$_1$ AS A FUNCTION OF DILUTION TREATMENT

See text and appendix for details of the calculation. Statements I to IV represent four assumptions made as to the binding equivalence of cations to DNA phosphate groups.

I All cations bind according to their charge.
II Each cation binds to one phosphate group only.
III Each $Mg^{+2}$ binds to two phosphate groups; each of the other cations binds to one phosphate group.
IV Polyamines bind according to their charge; each other cation binds to one phosphate group only.
Statements (*a*) to (*c*) refer to assumptions made as to volume changes of the head and changes in water content.
(*a*) Head volume and water content remain constant.
(*b*) Phage head volume remains constant, but water enters or leaves head to compensate for volume differences between counterions and cesium.
(*c*) Water content remains constant, but phage head volume changes to compensate for volume differences between counterions and cesium.

| Phage | Treatment* | Observed density | Cations assumed to be replaced by cesium | | Calculated density (g/ml) I | II | III | IV |
|---|---|---|---|---|---|---|---|---|
| | | g/ml | | | | | | |
| T4B | $PO_4$ buffer to 0.1 molal NaCl; Fast dilution | 1.502 | Na, K | (*a*) | 1.50 | 1.50 | 1.50 | 1.50 |
| | | | | (*b*) | 1.49 | 1.49 | 1.49 | 1.49 |
| | | | | (*c*) | 1.49 | 1.49 | 1.49 | 1.49 |
| T4Bo$_1$ | $PO_4$ buffer to 0.1 molal NaCl; Fast dilution | 1.536 | Na, K, Mg | (*a*) | 1.56 | 1.53 | 1.56 | 1.53 |
| | | | | (*b*) | 1.54 | 1.52 | 1.54 | 1.52 |
| | | | | (*c*) | 1.53 | 1.51 | 1.53 | 1.51 |
| T4B | 3 molal NaCl to 0.003 molal NaCl; Slow dilution | 1.525 | Na, K, Mg | (*a*) | 1.56 | 1.53 | 1.56 | 1.53 |
| | | | | (*b*) | 1.54 | 1.52 | 1.54 | 1.52 |
| | | | | (*c*) | 1.53 | 1.51 | 1.53 | 1.51 |
| T4B | 3 molal NaCl to 0.003 molal NaCl; Fast dilution | 1.561 | Na, K, Mg, polyamines, internal protein | (*a*) | 1.59 | 1.52 | 1.55 | 1.56 |
| | | | | (*b*) | 1.61 | 1.56 | 1.59 | 1.59 |
| | | | | (*c*) | 1.63 | 1.58 | 1.61 | 1.64 |
| T4Bo$_1$ | 3 molal NaCl to 0.3–0.0003 molal NaCl; Fast dilution | 1.557 | Na, K, Mg, polyamines, internal protein | (*a*) | 1.59 | 1.52 | 1.55 | 1.56 |
| | | | | (*b*) | 1.61 | 1.56 | 1.59 | 1.59 |
| | | | | (*c*) | 1.63 | 1.58 | 1.61 | 1.64 |

* Bracketed items refer to the initial and final dilution media into which the phage were transferred at the indicated rate.

1. When phage are transferred into CsCl without having been exposed to concentrated NaCl, the weakly binding cesium replaces all the endogenous inorganic counterions; but only $Na^+$ and $K^+$ are able to diffuse out of the relatively impermeable T4B, whereas $Na^+$, $K^+$, and $Mg^{+2}$ can leave the more permeable $T4Bo_1$. The cesium, however, is not able to replace the polyamines and internal protein.

2. When the two phages are first suspended in 3 molal NaCl, the $Na^+$ replaces *all* the endogenous counterions, since it binds more strongly to DNA than does $Cs^+$. However, only $Na^+$ and $K^+$ are able to diffuse out of T4B, whereas all endogenous cations leave $T4Bo_1$. When T4B is then diluted slowly to avoid osmotic shock, the permeability barrier is slightly altered so that $Mg^{+2}$ is lost. Transfer of the phage to CsCl then results in the $Cs^+$ replacing $Na^+$, $K^+$, and $Mg^{+2}$ in the head. But when T4B is diluted rapidly, the permeability barrier is disrupted enough by shock to allow all endogenous cations to leave. It then behaves like $T4Bo_1$, and when shocked T4B or $T4Bo_1$ are transferred to CsCl, $Cs^+$ becomes the sole cation in their heads.

The evidence for these selective cation replacements and for the differences in the permeability of the two phages has already been discussed.

Calculating the numerical densities to be expected from these cation alterations required knowledge, or estimates, of the concentration of endogenous cations in a phage, of the equivalence of the endogeneous cations and cesium with respect to the DNA phosphate, and of the total mass of a single phage particle. These estimates are given in the appendix, together with the method of calculation. Assumptions also had to be made as to whether net volume changes associated with ion replacement would be compensated by changes in head volume or by changes in water volume.

It can be seen from the figures in Table IV that the calculated densities agree rather well with the observed; however, in view of the uncertainties and assumptions inherent in the calculations, the agreement must be viewed with caution.

## CONCLUSIONS AND IMPLICATIONS

We conclude that phages T4B, $T4Bo_1$, and T4D possess various degrees of differential permeability to organic and inorganic solutes. The infectivity and density of these phages, after having been exposed to several solutions, reflect their permeability properties and the binding affinities of the cations that serve to neutralize the phosphate groups of the phage DNA. Changes in charge neutralization, we believe, may result in slight, reversible, or irreversible alterations of the DNA in the phage head leading to irreversible inactivation of these bacteriophages. The fact that solute and solvent flux seem to occur through the phage head strongly suggests that there is solvent water present within the head of T-even phage.

A number of questions remain unanswered: (*a*) If the differentially permeable barrier in phage is the protein coat, what is the difference between coats of T4B and $T4Bo_1$? (*b*) What causes osmotically shocked phage to be inactive? Is the loss

of DNA a cause or consequence of inactivation? (*c*) Do low salt concentrations produce alterations in the DNA as hypothesized? If so, what are these alterations, what is their relation to phage inactivation, and what information do they give about the packing arrangement of DNA in the head? (*d*) What is the state of water in the phage head that almost certainly has low activity, yet possesses solvent properties? Fortunately, most of these questions are experimentally answerable.

## APPENDIX

The density of phage as a function of its cationic content was calculated on the basis of the following assumptions:

1. The normal cation content of T4B is that found by Ames and Dubin (1960, their Table I). (Their analysis was performed on phage previously suspended in 0.01 M $MgSO_4$ and 0.02 M $MgCl_2$. It is possible that the cation composition of phage in nutrient broth might be somewhat different.)

2. The normal cation content of phage $T4Bo_1$ is the same as that of phage T4B (Ames and Dubin, 1960).

3. The mass of both phages T4B and $T4Bo_1$ is the same as that of T2r, $5.70 \times 10^{-16}$ g (Cummings and Kozloff, 1960); their density with normal counterions is the same as that of T2L measured by sedimentation in NaCl, 1.47 g/ml (Cummings, 1963); and their volume, $3.88 \times 10^{-16}$ ml, is that calculated using electron microscope data available for T-even phages (Bradley, 1963; Brenner et al., 1959; Cummings and Kozloff, 1960).

4. The number of binding sites on DNA is equal to the molecular weight of DNA ($1.30 \times 10^8$) divided by the average nucleotide molecular weight (331) and is $3.9 \times 10^5$.

5. The moles of DNA phosphate is equivalent to the number of binding sites on DNA/$6.02 \times 10^{23}$, and is $6.48 \times 10^{-19}$ moles.

6. If the cesium replaces any counterion on DNA, the mass of cesium replacing the exogenous cations is equal to (moles of cation replaced) $\times$ (molecular weight of cesium) $\times$ (valence of cation replaced). Because of uncertainty as to whether the polyamines and magnesium bind according to their charge to DNA phosphate, we calculated phage densities with four assumptions regarding the equivalence of the cations.

7. The volume of cesium replacing the cations is equal to (mass of cesium replacing the cations)/(density of cesium). We calculated phage densities with three assumptions regarding changes in phage head volume.

On the basis of these assumptions, we calculated phage densities resulting from the replacement of any one or all of the exogenous cations of T4 phage with cesium, using the equation:

$$\rho_2 = \rho_1 v_1 m_2 / m_1 v_2$$

in which $\rho$, $m$, and $v$ are phage density, mass, and volume, and the subscripts *1* and *2* refer to conditions before and after cesium replacement, respectively.

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