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Inactivation of bacteriophage T4 by organic and inorganic tin compounds

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SUMMARY

Butyltins and inorganic tins inactivated bacteriophage T4. The effect was on the phage and not on its host, *Escherichia coli*. The order of effectiveness was $SnCl_4 \geq$ monobutyltin > dibutyltin > tributyltin $\geq SnCl_2$. For the butyltins and $SnCl_4$ this was the reverse of the order of effectiveness usually observed for plants, animals, and microorganisms. This pattern suggests that degradation of tributyltin does not always detoxify it. Monobutyltin (MBT), the most effective organotin, was more effective at pH 4 than at higher pH values and it was more effective at low ionic strength. Inactivation proceeded more rapidly at 37 °C than at 18 °C. The results of experiments in which the ratio of phage to MBT was varied suggests that tin compounds may act by flocculating phage particles. Zinc, which is bound by phage short tail fibers (P12), inhibited phage inactivation by MBT, suggesting that MBT may act upon these tail fibers.

INTRODUCTION

Tin compounds can be toxic to a wide variety of macro- and microorganisms. Organotins are more toxic than inorganic tins. Among organotins, tri-substituted alkyl and aryl compounds are considered most toxic, followed by di- and then mono-substituted compounds. Tetra-substituted organotins are not regarded as toxic. Among tri-substituted organotins, propyl-, butyl-, pentyl-, phenyl- and cyclohexyltins are generally most toxic to microorganisms [14,36,37]. Tributyltin (TBT) is of concern in aquatic ecosystems because it is used in antifouling paints that are applied to boat hulls, to cages used in fish farming, and to other surfaces that may be immersed in water [7,16,37].

Because of its toxicity to nontarget organisms, the use of paints containing TBT has been regulated in several European countries and in the United States [9,38]. Even in these countries, its use has not been banned, however, and it should continue to enter aquatic and terrestrial ecosystems from a variety of sources.

Degradation of TBT can occur both chemically and biologically [12,28], although the relative contributions of each mode are not clear. Degradation is thought to involve sequential debutylation to yield dibutyltin (DBT), monobutyltin (MBT) and inorganic tin, and microorganisms can participate in the process [12]. Sn^{4+} can be reduced to Sn^{2+}, but Sn^{4+} predominates under aerobic conditions in the environment. Thus, aquatic systems which receive TBT usually contain DBT, MBT and inorganic tin [10,17,19,27,29,32,33,35,39].

TBT, DBT, MBT and inorganic tin can be toxic to algae, bacteria and fungi [14], but their activity against viruses has not been determined. Therefore, we examined the ability of several forms of tin to inhibit virus replication using a bacteriophage system.

MATERIALS AND METHODS

Organisms

Bacteriophage $T4D^+$ and its host bacterium, *Escherichia coli B* were obtained from Todd Gary of Vanderbilt University, Nashville, TN. Phage lysates were made according to standard procedures [1,11] by infecting 30 ml of log phase cultures of *E. coli B* grown in Luria Bertani (LB) broth with phage from a plaque (soft agar overlay) and shaking the suspension at 250 rpm for 10 to 12 h at 37 °C. The suspension was cooled to 4 °C and cell debris was removed by centrifugation at $5000 \times g$ for 10 min at 4 °C. The supernatant fluid was filtered through a 0.45-μm pore size filter. Phages were pelleted from the filtrate by high speed centrifugation at $45\,000 \times g$ for 70 min at 4 °C, resuspended in 0.5 ml of storage medium (SM)

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[30], and used directly or further purified through CsCl step gradients [30] to remove ghosts (defective particles).

Stock cultures of *E. coli B* were maintained on LB agar plates. Bacteria used in phage plating assays were grown by transferring a single colony from the surface of the LB agar plate to LB broth and incubating it overnight at 37 °C; 0.1 ml of this stationary phase culture was used as inoculum for 50 ml of LB broth which was shaken at 37 °C for 2.5 h [1,11]. The flask of plating bacteria was put on ice during use, and could be stored at 4 °C for up to 1 week without significant loss of efficiency of plating.

Chemicals

TBTCl (> 97% pure liquid) and $MBTCl_3$ (> 96% pure liquid) were purchased from Pfaltz & Bauer, Waterbury, CT. $DBTCl_2$ (> 96.5% pure solid) was purchased from Alpha Products, Danvers, MA. $SnCl_2$ (solid, anhydrous flake) and $SnCl_4$ (99.999% pure liquid) were purchased from Fisher Scientific Co., Pittsburgh, PA, and Aldrich Chemical Co., Milwaukee, WI, respectively. $ZnCl_2$ (≥ 95% pure hygroscopic solid) was purchased from Sigma Chemical Co., St. Louis, MO. Concentrated stock solutions of butyltins (8.4 mM), inorganic tins (8.4 mM), and zinc chloride (15 mM) were made by dissolving or diluting the compounds in HPLC-grade methanol. Stocks were stored in the dark at 4 °C in acid-cleaned tubes with Teflon-lined caps and used for up to 6 months. Dilutions of all stock solutions were made in methanol, to maintain solubility and sterility, before adding them to aqueous suspensions of phage. No precipitates were observed when tin compounds were added to aqueous solutions lacking phage. All glassware was soaked in hot, concentrated nitric acid (HNO_3) before use, to oxidize organics and remove contaminating metals.

Assay conditions

The standard 10-ml saline solutions used in experimental reactions contained 0.03% NaCl and 1 mM $MgCl_2$, unless stated to be different. The salinity of aqueous solutions to which phage was added was altered by the addition of appropriate volumes of 10% NaCl. The pH was changed by adding HCl or NaOH. $ZnCl_2$ was added to certain reactions at time zero and after different intervals of incubation. Phage T4 from concentrated stocks was added to the saline solutions, which were then mixed before the addition of TBT, DBT, MBT, $SnCl_2$, $SnCl_4$, or methanol (control, no tin). Reaction volumes were incubated in glass test tubes with caps at 18 or 37 °C. At time zero and at appropriate intervals, 100-μl samples were serially diluted in dilution fluid containing 1 g peptone, 0.25 g $MgSO_4 \cdot 7H_2O$, and 3 g NaCl per liter to reduce phage titers to platable numbers. Aliquots of phage dilutions were added to molten LB soft agar along with approx. 10^7 bacteria and then spread on LB agar plates. Soft agar overlay plates were incubated 10 to 12 h at 37 °C and then plaques were counted. Bacteriophage plaque-forming units (PFUs) are the means of values from duplicate plates. Data in the figures represent the means of two or three replicates of the same experiment, in most cases, or the best representative experimental replicate from two, three, or more experiments.

RESULTS AND DISCUSSION

Comparison of tin compounds

Among the five tin compounds examined, the order of toxicity to bacteriophage T4 was $Sn^{4+} \geq MBT > DBT > TBT \geq Sn^{2+}$ (Fig. 1). This is the reverse of the order generally observed for toxicity toward plants, animals and microorganisms, which is $TBT > DBT > MBT > > Sn^{4+}$ [14]. $SnCl_2$ is not regarded as a toxic compound. The effect(s) of the tin compounds was on the phage and not on the host bacterium, as *E. coli B* cells did not lose their viability or plating efficiency at the concentrations of tin compounds to which they were exposed. The peptone present in the dilution fluid and the yeast extract in the LB soft agar probably bound the tin, as they do other metals [20,31], thereby preventing further phage inactivation or loss of viability of the host bacterium. The tin-mediated loss of phage infectivity was not correlated with their exposure to soft agar at 55 to 60 °C as tin-inactivated phage were unable to infect and replicate in *E. coli B* cells grown at 37 °C in LB broth (data not shown).

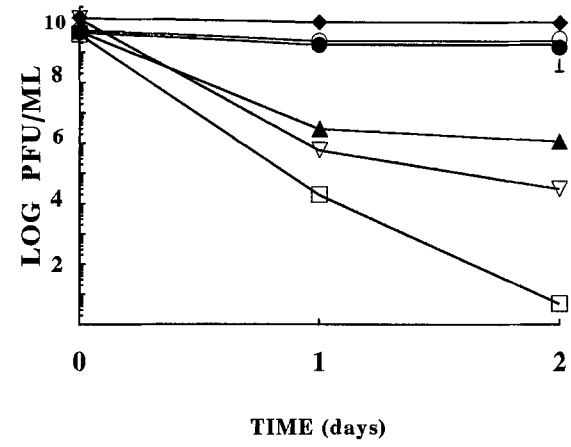


Fig. 1. Effect of tin compounds on infectivity of T4 phage. Phage were incubated with 84 μM of the indicated tin compound at pH 6 and at 18 °C for the times indicated. Error bars indicate the standard error of the mean (SEM). Where no bar is shown, the SEM was smaller than the symbol. ◆, no tin added; ●, Sn^{2+}; ○, TBT; ▲, DBT; ▽, MBT; □, Sn^{4+}. In this figure and in later figures, the no-tin controls received methanol.

TBT is not always more toxic to microorganisms than its degradation products: MBT is more toxic than TBT to methanogens and sulfate reducers [5,8], and MBT caused as much leakage of K^+ as TBT from some marine yeasts [13].

These findings suggest that it is not sufficient to relate environmental toxicity of TBT only to the amount of TBT present, nor is it valid to consider TBT detoxified if it has been degraded to DBT or MBT. Rather, the concentrations of these degradation products should also be considered.

Effect of environmental variables

Most of our ensuing studies were conducted on MBT because it was the organotin that caused the greatest loss of phage infectivity. To explore the nature of the reactivity of MBT with phage T4, several environmental parameters, i.e., pH, salinity, and temperature, were varied. Negligible phage inactivation occurred at pH 9 (Fig. 2A). As the pH was decreased, phage inactivation increased, and MBT had its greatest effect at pH 4, the lowest pH tested. In controls lacking MBT (Fig. 2B), insignificant decreases in infectivity occurred at all pH values except pH 4. Although infectivity decreased at pH 4, the extent and rate of inactivation were significantly greater when MBT was present (Fig. 2A). The increased inactivation of phage by MBT at low pH could have been the result of an increased concentration of the free MBT cation ($BuSn^{3+}$) at low pH. The cation is converted to simple neutral hydroxides [$BuSn(OH)_2^+$ and $BuSn(OH)_3$] and an unidentified carbonato species at neutral and higher pH values [6,26].

The effectiveness of MBT against the phage decreased with increasing salinity (Fig. 3). Phage present in suspensions without MBT (controls) showed little loss of infectivity over the period of the experiment. Laughlin et al. [26] determined that increasing the chloride concentration in TBT solutions caused increased stabilization of charged species resulting in an equilibrium shift toward TBTCl. This trend is probably similar for MBT, and this suggests that the cationic $BuSn^{3+}$ and the simple hydroxide species, but not the chloro complex, were potentially responsible for phage inactivation. Hence, MBT would probably be more active against bacteriophage T4 in fresh waters than in estuarine or marine waters.

The decrease in reactivity of MBT with increasing salinity (Fig. 3) coincides closely with the changes in speciation expected for MBT with increasing salinity but it does not coincide with the changes in hydrophobicity (K_{ow}) that MBT is expected to incur with increasing salinity [26]. The number of butyl groups bonded to the tin atom could influence the ability of an organotin to diffuse into the capsid of the phage and react with its nucleic acid. Accordingly, TBT might be excluded from or diffuse more slowly into the capsid, whereas MBT and Sn^{4+} may diffuse more readily. The strong adsorption of $SnCl_2$ to glass [20], such as the borosilicate tubes used in this reaction system, may be an important factor mediating its low activity. Decreases in chemical reactivity and toxicity with increasing chloride concentration (salinity) have also been observed for other metals [2–4,18].

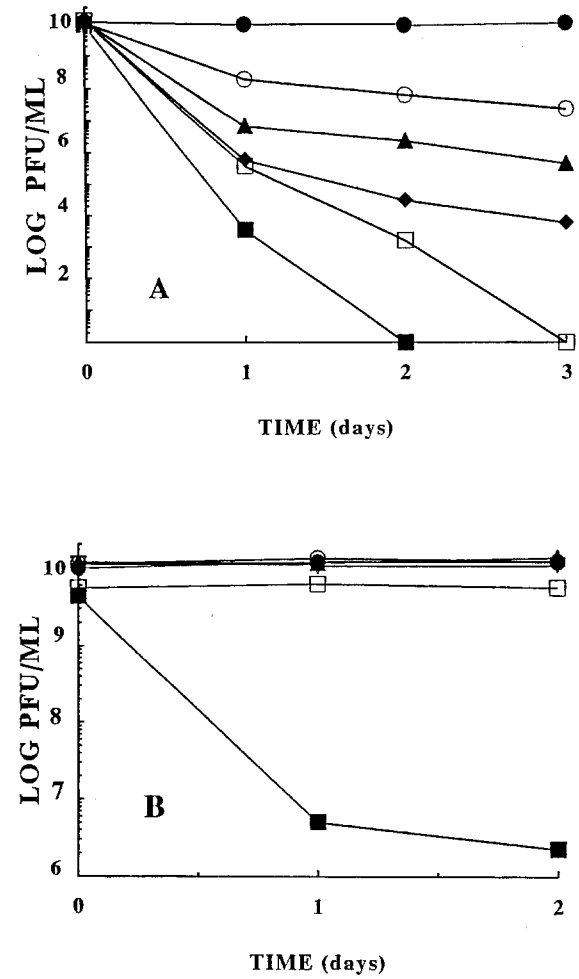


Fig. 2. Effect of pH on MBT-mediated loss of phage T4 infectivity. Phage were incubated with 84 μM MBT at 18 °C. (A) MBT present; (B) MBT absent. The absence of error bars indicates that the SEM was smaller than the symbol. ●, pH 9; ○, pH 8; ▲, pH 7; ◆, pH 6; □, pH 5; ■, pH 4. Note that the *y*-axes differ in panels A and B.

To determine whether phage T4 could be inactivated at concentrations of MBT lower than 84 μM, aqueous suspensions of phage at pH 6 were exposed to 8.4 and 0.84 μM MBT at 18 °C for a prolonged period of time. The results (Fig. 4) indicated that both of the latter concentrations of MBT could inactivate the phage at rates

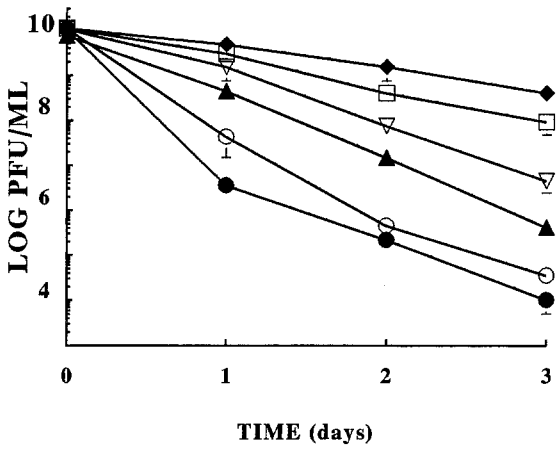


Fig. 3. Effect of salinity on MBT-mediated loss of T4 phage infectivity. Phage were incubated with 84 μM MBT at pH 6 and at 18 °C for the times indicated. Error bars indicate SEM. Where error bars are absent the error was smaller than the symbol. ●, no NaCl added; ○, 1 ppt NaCl; ▲, 2 ppt NaCl; ▽, 3 ppt NaCl; □, 10 ppt NaCl; ◆, 30 ppt NaCl.

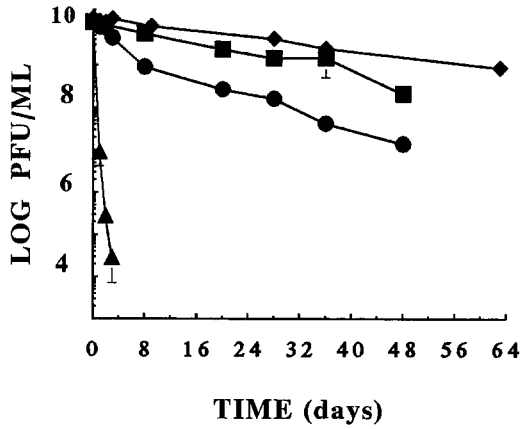
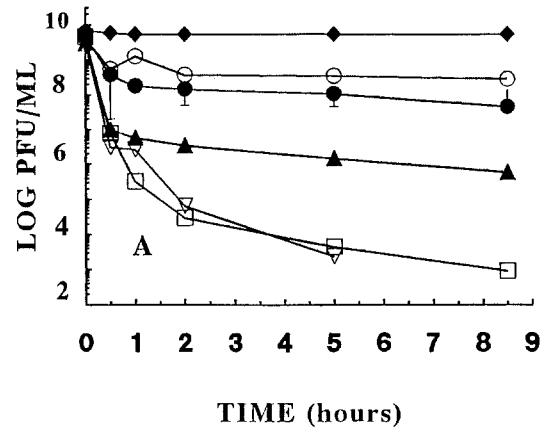


Fig. 4. Effect of varying MBT concentration on the loss of phage T4 infectivity. Phage were incubated with MBT at pH 6 and at 18 °C for times indicated. Error bars indicate the SEM. The absence of error bars indicates the error was smaller than the symbol. ◆, no tin added (methanol control); ▲, 84 μM MBT; ●, 8.4 μM; ■, 0.84 μM.



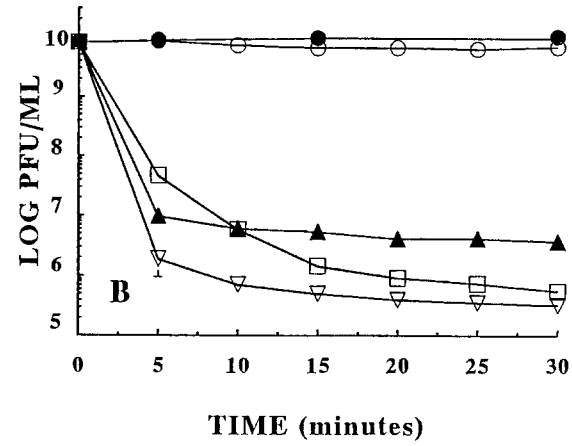


Fig. 5. Effect of tin compounds on phage T4 infectivity at 37 °C. Phage were incubated with 84 μM of the indicated tin compound at pH 7 for the times indicated. (A) Time-course over 9 h; (B) time-course over 30 min. Error bars indicate SEM. The absence of error bars indicates the error was smaller than the symbol. ◆, no tin added (methanol control); ●, Sn^{2+}; ○, TBT; ▲, DBT; ▽, MBT; □, Sn^{4+}. The no tin (methanol control) was omitted from panel B. It was not significantly different from the values for Sn^{2+}.

greater than background (no tin control) but at rates much slower than with 84 μM MBT.

In the beginning of these investigations, experiments to study the effects of pH, salinity, and MBT concentration on the rate of phage inactivation were conducted at 18 °C to simulate more closely ambient temperatures of natural waters. To shorten laboratory assay times and to study the kinetics of the reactivity of tin, the effects of tin compounds were compared at 37 °C. The same order of reactivity for the organotins was observed at 37 °C (Fig. 5A) and at 18 °C (Fig. 1), except that the rates of inactivation were greater at 37 °C, where phage titers decreased by two to three orders of magnitude within 5 min when exposed to DBT, MBT, or Sn^{4+} (Fig. 5B).

Nature of the interaction

After an initial steep decline in viable phage, a slower, more gradual decline was observed (Fig. 5A and B). This could have been the result of: (i) adsorption and/or volatilization of the organotin increased to a point where its concentration was below a reactive threshold; (ii) the presence of tin-resistant phage in the population; (iii) the tin compounds mediate a precipitation/flocculation reaction

in which crosslinking occurs between phage particles; or (iv) the presence of multiple sites of tin reactivity on each phage particle with only a single binding event being needed to inactivate the phage.

To determine if adsorption or volatilization influenced the inactivation kinetics, assays were conducted in which organotins were preincubated in the glass reaction tubes for 6 h before the addition of phage (data not shown). The kinetics and extent of phage inactivation closely resembled those without preincubation (Fig. 5A and B). Thus, adsorption to the glass vessel or volatilization did not decrease the MBT to a concentration insufficient to cause inactivation of the phage. In another experiment, multiple additions of phage were made to each reaction mixture at 30-min intervals, to observe whether the inactivation kinetics changed. After every phage addition, an initial exponential decline in phage numbers was observed, followed by a more linear decline (Fig. 6). These results suggest that there was sufficient nonadsorbed, nonvolatilized MBT present in solution to inactivate each new addition of phage.

Attempts to isolate stocks of $SnCl_4$- or MBT-resistant phage T4 were unsuccessful. Viable phage plated from reaction mixtures in which most of the phage had been inactivated by exposure to MBT or $SnCl_4$ were as sensitive to MBT or $SnCl_4$ as the original stocks. When $\sim 10^{10}$ phage T4 were exposed to 84 μM MBT for 6 h at 37 °C, the titer decreased below detectable levels (10 PFU/ml, Fig. 6) suggesting that all phage in the reaction mixture were tin sensitive. Thus, the PFUs remaining when the slopes of the MBT or $SnCl_4$ inactivation curves approach zero represent single phage or flocs of phage with ≥ 1 viable phage and not tin-resistant mutants. The biphasic kinetics of inactivation might be the result of two types of competing events: (i) crosslinking (flocculation) reactions between phage; and (ii) multiple reactions with individual phage particles causing inactivation. MBT may react with and crosslink, but perhaps not inactivate, phage T4 causing them to flocculate. This would decrease the apparent infectivity, because a clump of phage particles would be counted as a single PFU or be defective in plating assays. When the concentration of unreacted phage decreases below a certain threshold, further flocculation may decrease until either more tin compound or phage is added.

Increasing the initial concentration of 84 μM MBT, present at time zero, to 168 μM MBT after 30 min increased the rate and extent of phage inactivation beyond the rate observed in reactions containing only 84 μM MBT (Fig. 7). The increases in rates of inactivation when more phage (Fig. 6) or additional MBT was added (Fig. 7) after 30 min suggests that both phage and MBT concentrations are rate-limiting during the secondary, linear decline in phage infectivity (Fig. 7). This is in contrast to the primary exponential decline in phage viability which appears to be limited by phage concentration.

To test this hypothesis, both the concentration of MBT and phage were varied 10–100-fold to yield different tin-to-phage (Sn/P) ratios. At 84 μM, MBT inactivated phage at all initial phage concentrations (Fig. 8). When the initial phage concentration was decreased to 5×10^8 or 6×10^7 PFU/ml, inactivation occurred at 84 or 8.4 μM MBT (Fig. 8). At 0.84 μM MBT, inactivation did not occur at any of the initial phage concentrations during the

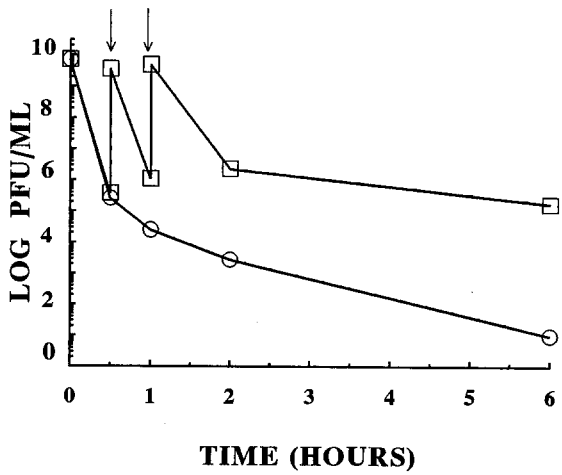


Fig. 6. Effect of adding additional phage during incubations with MBT. Phage were incubated in the presence of 84 μM MBT at pH 6 and at 37 °C for the times indicated. ○, phage was added at T_0; □, phage was added at T_0 and again at 30 min (arrow) and at 1 h (arrow).

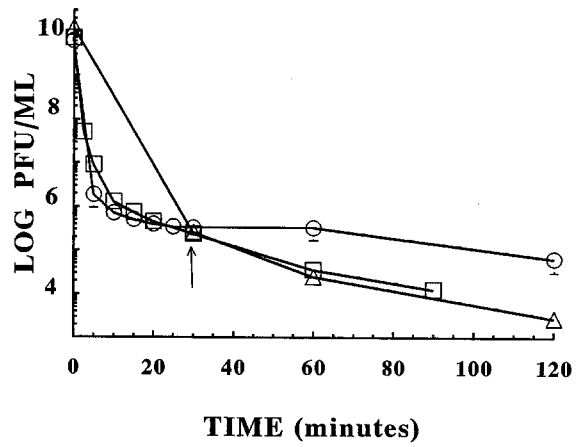


Fig. 7. Effect of adding additional MBT during incubation. Phage were incubated at pH 6 and at 37 °C. ○, 84 μM MBT present at T_0; △ and □, 84 μM MBT present at T_0, then at 30 min (arrow) additional MBT added to increase MBT to a total concentration of 168 μM.

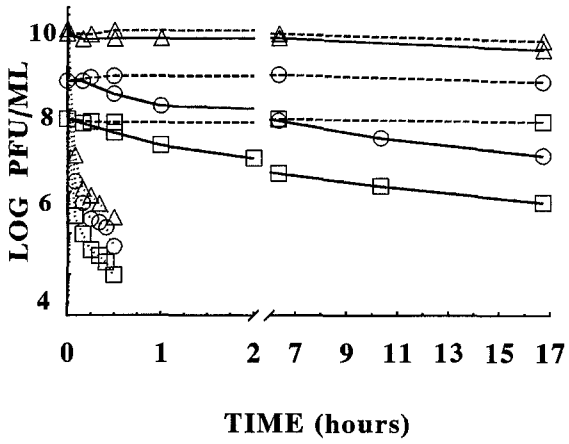


Fig. 8. Effect of varying initial phage concentration and MBT on inactivation kinetics. Phage were incubated in the presence of MBT at 37 °C for the times indicated. Concentration of MBT in reaction: – – – 0.84 μM MBT; ——8.4 μM MBT; . . . . , 84 μM MBT. Initial phage concentration in PFU/ml: △, $7–9 \times 10^9$; ○, 5×10^8; □, 6×10^7. Note the break in the *x*-axis and change in time scale.

17-h incubation period. At TBT concentrations ≤1 μM, adsorption to glass becomes a significant problem [28] and results in lower dissolved concentrations of TBT. If adsorption to glass is as significant for MBT as it is for TBT and other organotins, the threshold concentration of MBT causing phage inactivation may actually be lower than 1 μM. The initial exponential decline in PFUs present in reaction mixtures containing 84 μM MBT is typical of chemical reactions that exhibit pseudo-first-order kinetics where one of two reactants is in vast excess. Apparently, MBT was in excess at 84 μM, with phage concentration being the limiting 'reactant'. The reactions containing 8.4 μM MBT exhibited more linear rates of inactivation, suggesting second-order (bimolecular) kinetics.

The ratios of Sn to phage used in experiments shown in Fig. 8 are presented in Table 1. MBT concentration and not the Sn/phage ratio was apparently the controlling factor in the precipitous decrease in phage titer in reactions 1, 2, and 3. Similarly, reactions 8 and 9 have similar Sn/phage ratios to reaction 1, yet the phage in reactions 8 and 9 were not inactivated.

Competition by zinc

Several metals inactivate coliphages [2–4,15,21–24,34]. Kozloff et al. [22] reported that $ZnCn^-$ and $CdCN^-$ inactivated phage T4 by reacting with the phage's short tail fiber, P12, which is a trimer composed of three gp12 monomers. The short tail fibers are responsible for irreversible binding to the host. They subsequently demonstrated that P12 and its homologues in the other T-even

TABLE 1

Tin-to-phage (Sn/P) ratios for reactions in Fig. 8

Reaction no.	MBT conc. (μm)	Phage (PFU/ml)	Sn/P ratio (MBT molecules/phage)	Relative rate of inactivation
1	84	9×10^9	5.6×10^6:1	exponential
2	84	5×10^8	1.0×10^8:1	exponential
3	84	6×10^7	8.4×10^8:1	exponential
4	8.4	7×10^9	7.2×10^5:1	~zero
5	8.4	5×10^8	1.0×10^7:1	linear
6	8.4	6×10^7	8.4×10^7:1	linear
7	0.84	7×10^9	7.2×10^4:1	zero
8	0.84	5×10^8	1.0×10^6:1	zero
9	0.84	6×10^7	8.4×10^6:1	zero

phages bound one molecule of Zn per trimer and that the phage possessed six of these trimers per baseplate [23–25]. They proposed that zinc-binding sites on the short tail fibers did not have 100% occupancy and that the fibers bound zinc and were activated upon the attachment of long tail fibers to their host's outer membrane. Zinc chloride inhibited the inactivation of phage T4 by MBT (Fig. 9). The affinity of MBT for sites on the phage particles appears to be an order of magnitude greater than the affinity of zinc. The association of tin compounds with 'lethal'

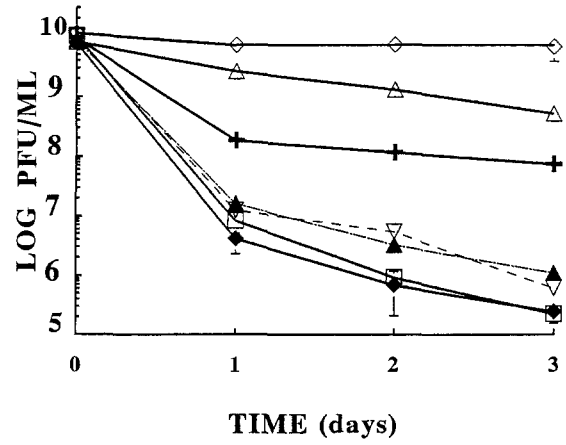


Fig. 9. Effect of zinc on MBT-inactivation of phage T4. Phage were incubated with 84 μM MBT (except where noted) at pH 6 and at 18 °C for the times indicated. Error bars indicated the SEM. The absence of error bars indicates the error was smaller than the symbol. ◇, no MBT added (control), zinc at the same concentrations as the other reactions containing MBT; ◆, 0 mM Zn; □, 0.15 mM Zn; ▽, 0.75 mM Zn; ▲, 1.5 mM Zn; +, 7.5 mM Zn; △, 15 mM Zn.

sites seems to be stable, if not irreversible, in binding to or disruption of phage structural components as all attempts to 'revive' the MBT-inactivated phage failed. Attempts to revive the phage by the addition of high concentrations of NaCl or by removing the MBT and adding $ZnCl_2$ failed to increase titers more than 10-fold (data not shown). It is unclear whether zinc competes with tin compounds for zinc-binding sites on P12. Competitive binding studies with a narrower range of zinc concentrations and other metals (e.g., $MgCl_2$) or chelators will help confirm the specificity of zinc's attenuation of tin's reaction with phage T4. This and other aspects of viral inactivation by tin compounds are the subjects of our continuing investigation.

ACKNOWLEDGEMENT

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