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Chelating Agent Shock of Bacteriophage T5

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When two strains of phage T5 (heat-susceptible form T5*st*$^+$ and its heat-resistant mutant T5*st*) were placed in solutions containing various high concentrations of chelating agents (sodium citrate and ethylenediaminetetraacetic acid) at room temperature, they could be effectively inactivated by rapid dilution in distilled water of relatively low temperatures (2 to 37 C). This phenomenon has been termed "chelating agent shock" (CAS). The susceptibility of phage T5 to CAS increased with an increase in the concentration of chelating agents and with an increase in temperature of the water used for rapid dilution. Under any given condition, T5*st*$^+$ was much more sensitive to CAS than was T5*st*. Phage T5 was protected against inactivation by the addition of monovalent or divalent metal salts, but not by the addition of nonionic solutes, to the shocking water prior to CAS treatment. This finding is compatible with the view that cations combined with the phage protein are removed by the chelating agent, although no metal ion has been identified in the phage protein. Alternatively, since the chelating agents used are polyanions, they may bind relatively tightly to the protein subunits in the head of T5, thereby distorting the structure of the phage head. Rapid dilution of these distorted particles could lead to loss of phage DNA. No evidence for recovery of phage activity could be obtained by the addition of metal salts to the inactivated phage after CAS. The morphological properties of phage inactivated by CAS are similar to those of heat-inactivated T5 phage. Electron micrographs showed that most of the phage particles consisted of empty head membranes; some of the particles had lost their tails. Both heritable and nonheritable resistance to heat was accompanied by resistance to CAS in phage T5. The sensitive element detected by each test seemed to be the same.

Anderson (4) reported that rapid dilution of concentrated salt solutions containing T2 and other T-even phages resulted in a loss of phage activity. This phenomenon was called "osmotic shock." In contrast to T-even phages, T5 and other T-odd phages are resistant to osmotic shock with monovalent metal salts (6, 9).

We have found that when the two strains of phage T5 [T5*st*$^+$ and T5*st* (3)] are placed in a 2 M solution of sodium citrate at room temperature, they can be effectively inactivated by rapid, but not by slow, dilution in distilled water at 15 C.

The action of chelating agents on heat inactivation of phage T5 was studied extensively by Lark and Adams (6). They reported that the presence of a chelating agent accelerates the heat-inactivation rate of phage T5, and they concluded that the chelating agents decrease the heat stability of phage T5 by breaking some of the coordinate bonds linking a metal cation to the phage particle without removing the cation completely from its site. In this paper, the inactivation of concentrated mixtures of chelating agent and phage T5 by rapid dilution is described.

MATERIALS AND METHODS

Wild-type T5*st*$^+$, its heat-resistant mutant T5*st*, and their host, *Escherichia coli* strain B, were employed in this study. Clonal stocks of the heat-susceptible T5*st*$^+$ and heat-resistant T5*st* forms of phage T5 were prepared in the following way. The susceptible clonal stock was started from a single plaque from an assay plate of the unheated laboratory stock of T5. The heat-resistant stock originated from a single plaque from an assay plate of the heated (55 C for 4 hr in 0.15 M NaCl) laboratory stock of phage T5. In each case, a single plaque was picked, resuspended, and cultured in a broth culture of *E. coli* B. The heat susceptibility of these prepared stocks was tested. Routine methods of phage technique have been described by Adams (2). The saline buffer diluent contained 0.85% NaCl and 0.025% $MgSO_4$; it was buffered at *p*H 7.0 with an appropriate mixture of 0.067 M Na_2HPO_4 and 0.067 M KH_2PO_4.

The chelating agents used, sodium citrate, ethylenediaminetetraacetic acid (EDTA), and sodium oxalate,

were saturated in a 60 C water bath to 2 M, 0.3 M, and 0.2 M, respectively. For experiments these saturated solutions were appropriately diluted to various concentrations.

Immediately prior to an experiment, phage was suspended in the saline solution containing 0.85% NaCl and 10^{-4} M $MgSO_4$ at room temperature (about 20 C), the phage concentration being about 5×10^8 particles/ml. A sample containing 0.3 ml of this saline phage stock was added to 2.7 ml of various high concentrations of chelating agent (citrate or EDTA) at room temperature. After about 20 min, 1 ml of this chelating agent-phage mixture was rapidly diluted into 100 ml of distilled water in 200-ml Pyrex bottles; the bottles were then placed in a water bath at various temperatures. The distilled water used for rapid dilution was designated "shocking water." To complete rapid dilution, the Pyrex bottles were vigorously rotated by hand immediately after dilution. Then the samples were appropriately diluted in buffered saline diluent for assay by the agar layer method. The same procedure in the absence of chelating agent gave no significant inactivation.

Modifications of these techniques will be discussed in connection with specific experiments.

RESULTS

Stability of phage T5 in the saturated solutions of the chelating agents. The two forms of phage T5 (T5*st*$^+$ and T5*st*) were incubated in 2 M saturated solutions of citrate at various temperatures. After various time intervals, the mixtures were diluted into 0.4 M NaCl solution and then into buffered saline, and were assayed by the agar layer method. At temperatures below 37 C, no appreciable inactivation of either form of phage T5 was observed for 2 hr. Saturated solutions of other chelating agents, i.e., EDTA (0.3 M) and oxalate (0.2 M), were also tested. Again, no significant inactivation was observed.

Inactivation of phage T5 by "chelating agent shock." The primary experiment was performed under the same condtions used in osmotic shock experiments. The two forms of phage T5 (T5*st*$^+$ and T5*st*) were suspended in a 2 M solution of citrate and were incubated at room temperature. After 20 min, the phage strains could be inactivated by rapid, but not by slow, dilution in distilled water at 15 C (*see* Table 1). In the rapid dilution, the survival of T5*st*$^+$ was about 3.5% and the survival of T5*st* was about 10%. A control, slowly diluted into 1 M citrate, equilibrated for 20 min in 0.5 M citrate, and then equilibrated for 20 min in the buffered saline diluent, showed no significant inactivation. A similar result was also obtained by the control titration method described in Materials and Methods. Thus, we have called this phenomenon "chelating agent shock" (CAS). The other chelating agents, EDTA and oxalate, were also studied. These data are summarized in Table 1. In each instance, T5*st*$^+$ was very sensitive to CAS, whereas T5*st* was less sensitive. With the other six phages of the T-series and with λ phage, no appreciable inactivation could be demonstrated.

Stent (8), Adams (1), and Adams and Lark (3) reported that phage T5 is rapidly inactivated in dilute saline at 38 C. For comparison with the inactivation in dilute saline at 37 C, the kinetics of inactivation after CAS treatment was studied. As shown in Fig. 1, a striking decrease in survival is observed immediately after rapid dilution. No further decrease in survivors was observed with further incubation in water for 60 min. Therefore, inactivation by CAS (with 2 M citrate) occurs simultaneously with rapid dilution. Rapid 100-fold dilution of phage T5 from the saline (containing 0.85% NaCl and 10^{-4} M $MgSO_4$), in the absence of chelating agent, to 0.1 M NaCl or to distilled water gave no significant inactivation.

Effect of the concentration of chelating agents on CAS. T-even phages are sensitive to osmotic shock, whereas T-odd phages are resistant. Anderson (4) interpreted osmotic shock as follows. The envelope of the virus particle is somewhat permeable to solute, but much more permeable to water. The susceptibility of a phage particle to osmotic shock depends on the magnitude of the difference in permeability of the head protein to water and solute. Since the molecules of the chelating agents used are relatively large, it was thought that these agents may have an osmotic effect on phage T5. A number of experiments were performed to test this possibility.

TABLE 1. *Inactivation of T5st$^+$ and T5st by chelating-agent shock with saturated solutions of chelating agents*

Chelating agent	Survival (%) with shocking water at									
	37 C		25 C		20 C		15 C		2 C	
	T5*st*$^+$	T5*st*	T5*st*$^+$	T5*st*	T5*st*$^+$	T5*st*	T5*st*$^+$	T5*st*	T5*st*$^+$	T5*st*
Sodium citrate (2 M)	0.02	12	1.2	11	2.6	11	3.5	10	1.7	8.4
EDTA (0.3 M)	0.018	0.073	11	30			55	89	86	69
Sodium oxalate (0.2 M)	5.6		71	91						

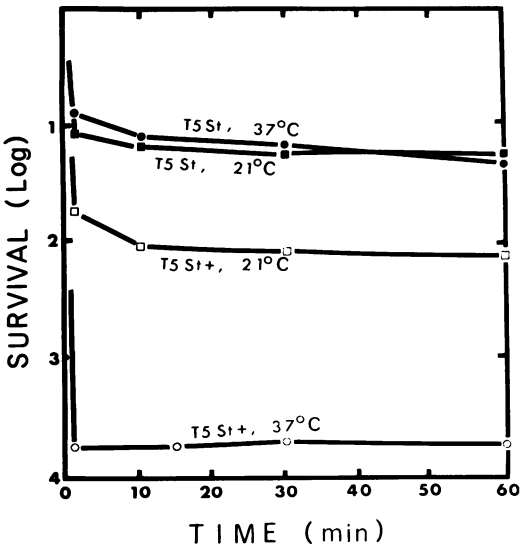

FIG. 1. *Kinetics of the inactivation of T5st+ and T5st phages by incubation after CAS. The incubation temperature was the same as the temperature of the shocking water. The chelating agent used was 2 M citrate.*

First, the concentration effect of chelating agents on CAS was investigated. When preincubated in the various concentrations of chelating agents at room temperature, T5*st* was stable. However, when preincubated in 0.075 and 0.1 M EDTA solutions, T5*st*+ was inactivated to a survival of 10^{-2} in 1 hr, though it was stable in 0.3 M EDTA solution. Therefore, the effect of the EDTA concentration on the sensitivity of T5*st*+ to CAS could not be tested.

As shown in Fig. 2, the sensitivity of phage T5 to CAS increased as the concentration of chelating agent increased. Furthermore, high inactivation efficiencies were observed with relatively low concentrations of chelating agent (e.g., 0.15 M citrate on T5*st*+ and 0.075 M EDTA on T5*st*) at 37 C. These results suggested that CAS results from (i) removal of cations from phage rather than from an osmotic effect, or (ii) distortion of the coat structure by the chelating agents due to their polyanionic character.

Effect of temperature on CAS. Tubes of "shocking water" were placed in an ice bath at 2 C and in water baths at various temperatures above 10 C. After equilibration at these temperatures, CAS was carried out. The results are shown in Fig. 3.

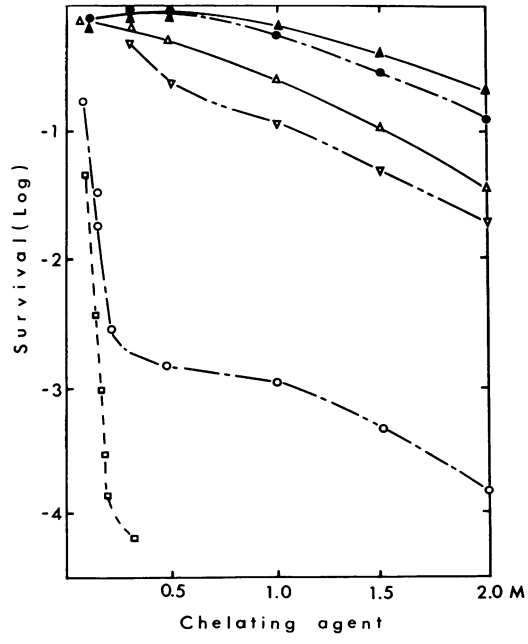

FIG. 2. *Effect of the concentration of chelating agent on CAS. The temperature listed for each symbol is the temperature of the shocking water. Symbols: ▲, T5st with citrate at 15 C; ●, T5st with citrate at 37 C; △, T5st+ with citrate at 15 C; ▽, T5st+ with citrate at 21 C; ○, T5st+ with citrate at 37 C; □, T5st with EDTA at 37 C.*

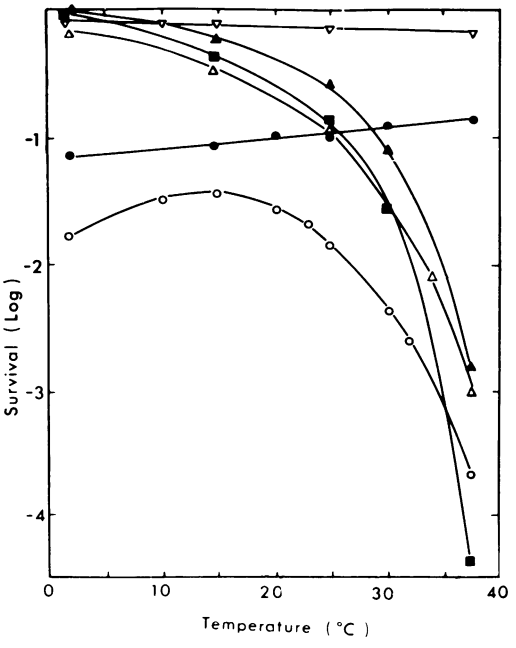

FIG. 3. *Effect of temperature on CAS. The chelating agent used and its concentration are listed for each symbol. Symbols: ○, T5st+ with 2.0 M citrate; △, T5st+ with 1.0 M citrate; ▲, T5st+ with 0.5 M citrate; ●, T5st with 2.0 M citrate; ▽, T5st with 1.0 M citrate; ■, T5st+ with 0.3 M EDTA.*

Under the appropriate conditions, both forms of phage T5 (T5*st*$^+$ and T5*st*) were susceptible to CAS, even at 2 C. In general, however, both forms were much more susceptible at high temperatures than at low temperatures. The increase in sensitivity of phage T5 to CAS with increasing temperature is the reverse of the temperature effect of osmotic shock of T-even phages (4), indicating that osmotic effect is not involved.

Protection against CAS by ionic solution. An experiment was designed to determine whether the nature of the shocking medium, i.e., distilled water, ionic solution, or nonionic solution, had any effect on the inactivation efficiency of phage T5 by CAS.

Various ionic and nonionic solutions (NaCl, $MgCl_2$, glucose, and lactose) were used as a substitute for distilled water in CAS treatment. Figures 4 and 5 show that the inactivation of phage T5 by CAS was reduced by ionic solutions. On the other hand, there was no protection by nonionic solutions; tests with lactose or glucose solutions (ranging from 0.15 to 1.5 M) showed

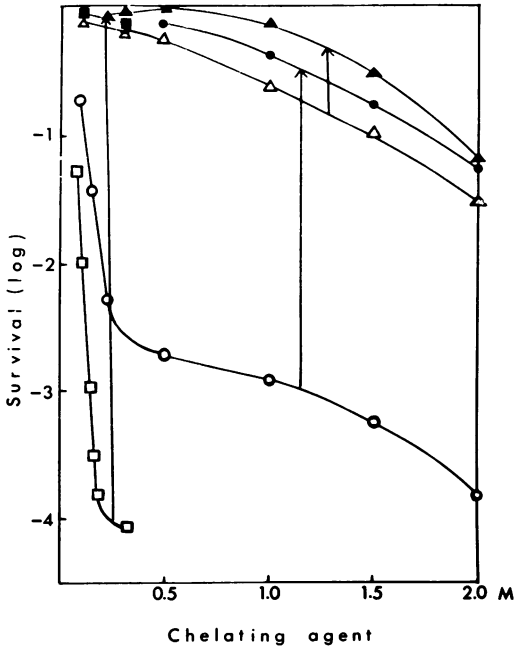

FIG. 4. *Protection against CAS by 0.85% NaCl in shocking medium. The relationship between the constant concentration of NaCl and the concentration of chelating agent is shown. The conditions for CAS are indicated for each symbol; the temperature listed is the temperature of the shocking water. Symbols: ↑, protection by addition of NaCl (0.85%) to shocking water; ○ and ●, T5st$^+$ with citrate at 37 C; △ and ▲, T5st$^+$ with citrate at 15 C; □ and ■, T5st with EDTA at 37 C.*

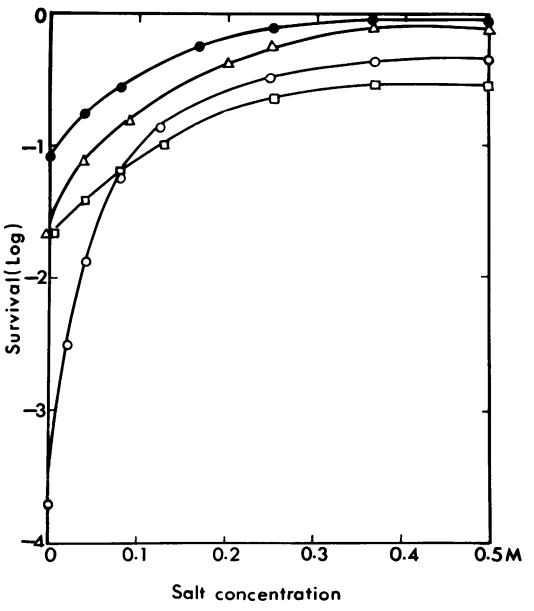

FIG. 5. *Protection against CAS by various concentrations of ionic solutes in the shocking medium. The chelating agent was citrate (2 M). The salt used and the temperature of the shocking medium are indicated for each symbol. Symbols: ○, T5st$^+$ by NaCl at 37 C; □, T5st$^+$ by NaCl at 15 C; ●, T5st by NaCl at 37 C; △, T5st$^+$ by $MgCl_2$ at 15 C.*

the same survival after CAS as with water. Thus, the possibility that the inactivation of phage T5 by CAS is due to an osmotic effect of the chelating agent on phage T5 was excluded. It seemed apparent that inactivation of phage T5 by CAS is the result of removal of cations.

When monovalent or divalent cations were added to the inactivated T5 phage after CAS treatment, no increase in phage titer was observed.

Morphological changes in phage T5 with CAS. For morphological study, phage T5*st*$^+$ (5×10^{10} particles per ml) was inactivated by CAS at 37 or 24 C (to a survival of about 5×10^{-4}) and was concentrated by centrifugation at 70,000 × *g* for 1 hr. Electron micrographs of this material showed that most of the observable particles consisted of empty head membranes (Fig. 6). When CAS was performed at 37 C, about 40% of these particles lost their tails. However, the majority of particles retained their tails with similar treatment at 24 C. At concentrations above 10^{11} particles per ml, phage T5 shows a strong blue Tyndall effect. CAS inactivation was accompanied by loss of the Tyndall effect. These findings demonstrated that the CAS inactivation of phage T5*st*$^+$ results in production of phage "ghosts" similar to those obtained by heat treatment (6).

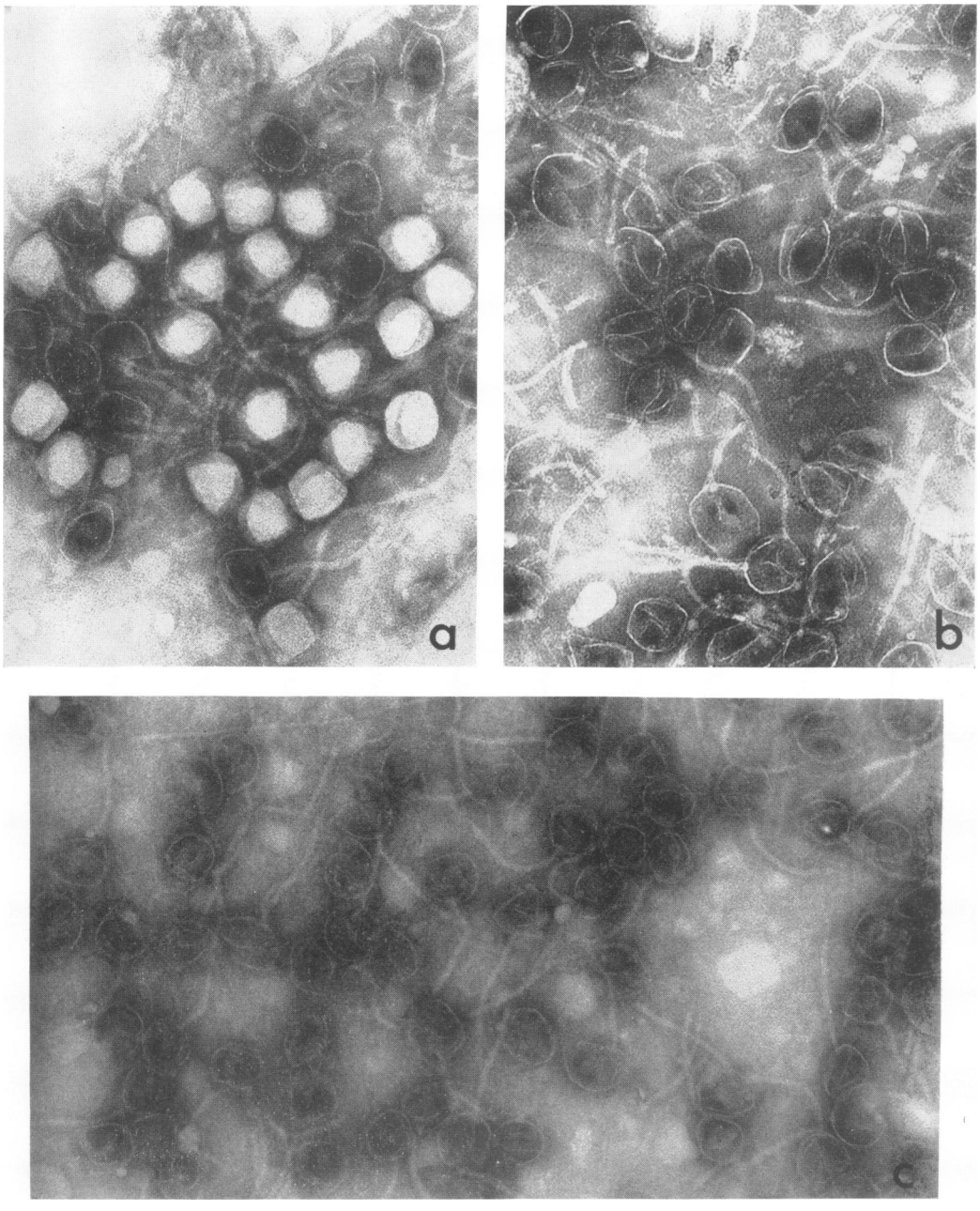

FIG. 6. *Electron micrographs of T5 phage. For CAS treatment, 1.5 M sodium citrate was used. (a) Untreated control of T5st+. (b) T5st+ ghost after CAS treatment at 37 C. (c) T5st+ ghost after CAS treatment at 24 C. ×67,000.*

Comparison of properties of T5st+ and T5st. As shown in Fig. 1, 2, and 3, phage T5*st*+ was much more sensitive to CAS than was T5*st*. The difference in sensitivity increased with temperature and with the concentration of chelating agent. With 1 M citrate and shocking water at 37 C, the heat-resistant T5*st* was resistant to CAS, whereas the heat-susceptible T5*st*+ was inactivated to a survival of about 10^{-3} (*see* Fig. 2). The survival ratio is of the same order of magnitude as that

found by Adams and Lark (3) for heat inactivation of T5*st*$^+$ and T5*st*. When the CAS treatment was repeated on the primary shocked T5*st*$^+$ suspension, the titer of surviving particles did not decrease further; these CAS-resistant survivors also survived heat treatment in 0.4 M NaCl at 50 C for 2 hr. Thus, the T5*st*$^+$ particles which survived CAS treatment were resistant to both CAS and heat. A stock of phage T5*st*$^+$ was inactivated to a survival of about 10^{-3} by heat; the survivors were likewise resistant to CAS and to heat.

Plaques picked from heat-resistant stocks either before or after CAS treatment have invariably been typical of the CAS-resistant variant. With the heat-susceptible clones, the proportion of CAS-resistant particles was about 10^{-3}, a value similar to the proportion of heat-resistant phage particles in heat-susceptible plaque clones (3). The CAS-resistant fraction survived for at least 4 hr in 0.4 M NaCl at 50 C and was also resistant to CAS. However, when this fraction was plated and the plaques were picked and analyzed, the results were typical of the CAS- and heat-susceptible form (Table 2). Hence, the CAS-resistant phage particles present in single plaque clones of the heat-susceptible form are phenotypically resistant but genotypically susceptible to CAS and heat. We have no explanation for the nonhereditary CAS and heat resistance of this small proportion of virus particles in the CAS- and heat-susceptible clones.

TABLE 2. *Properties of single plaque clones isolated from survivors after CAS treatment of heat-susceptible single plaque clones*

Clonal stock	Survivors of CAS treatment (%)[a]	Survivors of heat treatment (%)[b]	
		10 min	20 min
Clone			
1	0.03	1.29	0.22
2	0.11		0.52
3	0.11	0.45	0.25
4	0.08		3.04
5	0.03	1.57	0.30
6	0.11	2.92	0.64
7	0.04		0.94
8	0.26	0.99	0.40
9	0.21		0.61
10	1.43		0.14
11	0.04	2.77	0.32
Stock[c]			
Laboratory stock of T5	0.19	1.72	0.82
T5*st*$^+$ stock	0.10	1.16	0.43
T5*st* stock	82.1	75.1	61.0

[a] Treated with 1 M citrate and shocking water at 37 C.
[b] Incubated in 0.4 M NaCl at 50 C.
[c] To compare with single clones.

The laboratory phage stock of T5 was inactivated to a survival of about 10^{-3} by CAS. From a plate of survivors, 20 plaques were picked at random and were tested by CAS and heating; 8 were typical of the resistant mutant and 12 were typical of the susceptible form.

The foregoing experiments indicated that CAS resistance is always accompanied by heat resistance.

DISCUSSION

One interesting aspect of CAS is that T5 ghosts are formed by CAS treatment. Lark and Adams (6) suggested that the effect of chelating agents on the heat stability of phage T5 may be explained by assuming that some metal cation is firmly bound to the heat labile site, contributing to the structural stability of the site. There are two known cases of firmly bound metal cations in animal viruses, iron in mouse encephalomyelitis virus (7) and copper in vaccinia virus (5).

Although no metal ion has been identified in the phage protein, the chelating agent may react with a metal cation which is a structural component of the phage particle, thus breaking the bonds that hold parts of the phage particle in the rigid "native" structure. In the course of rapid dilution, the chelating agent may remove the cations from the phage particle. It seems reasonable to assume that temperature influences the effect of the removal of cations. Alternatively, since the chelating agents used are polyanions, they may bind relatively tightly to the protein subunits in the phage head, thereby distorting the structure. Rapid dilution of these distorted particles could lead to the loss of phage DNA. Such a distortion could also make the particles more sensitive to heat. Both of these possibilities are supported by our findings that the heat-sensitive strain of T5 (T5*st*$^+$) is more sensitive to CAS and that an increase in the temperature of the shocking water increases the efficiency of CAS.

The shocking media containing ionic solutes prevented CAS of phage T5, but the nonionic solutes did not. This indicates that the removal of cations from phage T5 and the distortion of phage structure can be prevented by the presence of cations in the shocking medium, because of the association of the added cations and the chelating agent.

The heat-susceptible form of phage T5, T5*st*$^+$, is very sensitive to CAS, whereas the heat-resistant form, T5*st*, is resistant. Thus, heat-resistant

mutants can be selected from laboratory stocks by CAS.

These findings suggest that the element of phage T5 which is sensitive to CAS may be similar or identical to the element which is sensitive to heat inactivation.

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