Characterization of an Inhibitor Causing Potassium Chloride Sensitivity of an RNA Polymerase from T4 Phage-Infected *Escherichia coli*†

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ABSTRACT: The nature of the inhibition by salt (KCl) of DNA-dependent RNA polymerase from T4 phage-infected *Escherichia coli* (T4 enzyme) was studied using holoenzyme preparations, core enzyme and σ fractions obtained by phosphocellulose column chromatography, and σ fractions further purified by gradient centrifugation in the presence and absence of 6 *M* urea. We showed with holoenzyme preparations that salt inhibits the formation of rifampicin-resistant preinitiation complexes. The inhibition was considerably reduced when a nonionic detergent (particularly of the Triton series) was included in the reaction mixtures. With T4 core enzyme and T4 σ fractions together with the same fractions from uninfected cells (host enzyme fractions) and different DNA templates, we showed that the T4 σ fraction plays a role in the salt-sensitive activity with T4 DNA. The salt sensitivity of the T4 σ fraction was antagonized by Triton; it was not a function of σ fractions isolated from phage cultures infected in the presence of chloramphenicol. As reported previously (Stevens, A. (1973), *Biochem. Biophys. Res. Commun. 54,* 488), the T4 σ fraction inhibited the activity of host σ when they were present together in reaction mixtures, particularly in the presence of salt. T4 σ further purified by centrifugation in glycerol gradients had the same properties as the cruder fraction, and the T4-specific polypeptide of mol wt 10000 (Stevens, A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 603) was found in the same fractions. If the glycerol gradients contained 6 *M* urea, the mol wt 10000 polypeptide was separated from the salt-stimulated σ. Fractions containing the small polypeptide could be added back to produce the salt-inhibitory effects. The inhibitory activity of both the crude σ fraction and the fractions containing the small polypeptide was inactivated at 65°C. The results suggest that the mol wt 10000 protein is a salt-promoted inhibitor, but the small amounts of it which are present in purified fractions of the T4 enzyme have not yet allowed its isolation in large enough quantities to permit a detailed study of its properties.

Highly purified RNA polymerase isolated from T4 phage-infected cells (T4 enzyme) in our laboratory contains small amounts (about 0.2 equiv) of the σ subunit (Stevens, 1973, 1974). Others have reported similar results (Travers, 1970; Khesin et al., 1972) or that the purified enzyme contains no σ (Seifert et al., 1971; Bautz and Dunn, 1969). The σ subunit is not newly synthesized after phage infection (Stevens, 1972). We have been interested in the activity of this subunit, and have previously shown that T4 σ is less stimulatory than host σ with core enzyme fractions and that the former inhibits the activity of the latter when they are present together in reaction mixtures, particularly in the presence of salt (Stevens, 1973, 1974). It seems reasonable that such a salt effect might be associated with the salt sensitivity of the overall reaction of the T4 enzyme with T4 DNA as a template. Crouch et al. (1969) first reported that an RNA polymerase fraction from T4-infected cells was inhibited by 0.2 *M* KCl. We also found (Stevens, 1974) that highly purified T4 holoenzyme was salt sensitive when assayed with T4 DNA as a template. The inhibitory effect of KCl was not found if the salt was added to the reaction mixture after the reaction began, suggesting that the effect was at the level of initiation of RNA chains. Kleppe (1975) recently reported that T4 core enzyme is inhibited by salt at the stage of initiation when T4 DNA or synthetic polymers containing purines on one strand are used as templates. The salt sensitivity of the T4 enzyme is in marked contrast to that of the host RNA polymerase, which is stimulated three- to fivefold by KCl at 0.2 *M*, predominantly at the level of initiation (for references, see Matsukage, 1972).

In this paper, detailed studies of the salt sensitivity of the T4 polymerase are reported. We found that nonionic detergents, particularly of the Triton series, antagonized the KCl effect. Other results suggest that the T4-specific polypeptide of mol wt 10000 (Stevens, 1972) is a σ-bound component which causes the salt sensitivity of the reaction with T4 DNA as a template. This sensitivity may be due to either direct inhibition or modification of the specificity of the enzyme. It was not possible to determine which was the case on the basis of RNA product analyses. The salt effect was called an inhibition since the activity of the T4 system was decreased considerably.

Materials and Methods

T4 holoenzyme, T4 core enzyme, and T4 σ were prepared from *Escherichia coli* cells infected with T4 phage *am* $42^- \times 47^-$ as described previously (Stevens, 1974). Host polymerase fractions were from uninfected *E. coli* cells. Cells infected with T4 phage in the presence of chloramphenicol were prepared as follows. Twenty-five liters of *E. coli* cells was grown at 30°C to a concentration of 3×10^8/ml. Chloramphenicol at a final concentration of 100 μg/ml was added; then 1 min later, T4 phage *am* $42^- \times 47^-$ was added at a multiplicity of 6. After 15 min the cells were collected and enzyme (called T4-CAP enzyme) was isolated.

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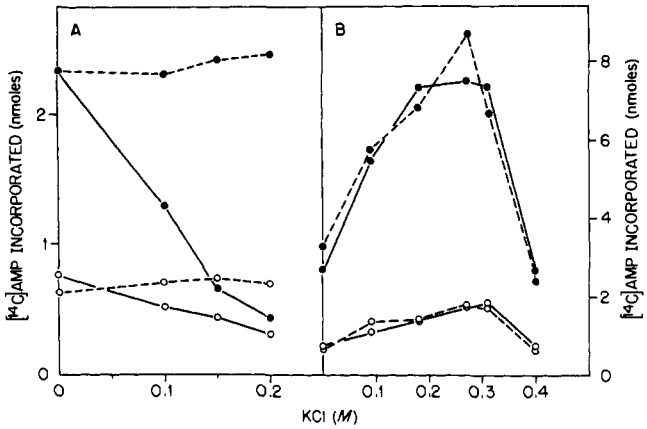

FIGURE 1: Effect of KCl and Triton X-405 on holoenzyme activity with T4 DNA as a template. Reaction mixtures were as described in Materials and Methods and contained the concentration of KCl shown. Triton X-405 (dashed lines) was present at a concentration of 1%. Both enzyme fractions were those purified through one glycerol density gradient. Reaction mixtures in (A) contained T4 holoenzyme at concentrations of 8 μg (○) and 32 μg (●). The reaction mixtures in (B) contained host holoenzyme at concentrations of 2.5 μg (○) and 10 μg (●).

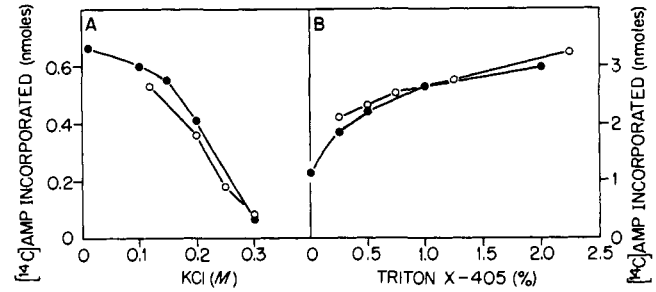

FIGURE 2: KCl and Triton X-405 concentration curves before and after preincubation of the T4 holoenzyme with KCl (A) and with Triton plus KCl (B). Reaction mixtures were as described in Materials and Methods and contained concentrations of KCl and Triton X-405 as shown; reaction mixtures in B also contained 0.2 *M* KCl. (A) The T4 enzyme used without preincubation (●) was a density gradient purified enzyme at a concentration of 4.4 μg. The preincubated enzyme (○) was the same enzyme that was subjected to a 10-min preincubation period at 37°C in a reaction mixture as follows: enzyme, 44 μg; bovine serum albumin, 400 μg; KCl, 0.3 *M*; all in a final volume of 0.8 ml. (B) The T4 enzyme used without preincubation (●) was a density gradient purified enzyme at a concentration of 6 μg. The preincubated enzyme (○) was the same enzyme that was subjected to a 10-min preincubation period at 37°C in a reaction mixture as follows: T4 enzyme, 60 μg; bovine serum albumin, 100 μg; KCl, 0.20 *M*; and Triton X-405 at 2%; all in a final volume of 0.25 ml.

For assay of the enzyme fractions, the reaction mixtures (0.2 ml) contained [^{14}C]ATP (Schwarz/Mann; 0.25 m*M*, 1500 cpm/nmol), UTP, CTP, and GTP (P-L Biochemicals, Inc.; 0.25 m*M* each), Tris buffer (pH 7.8, 20 m*M*), $MgCl_2$ (10 m*M*), 2-mercaptoethanol (10 m*M*), T4 DNA (isolated from T4D phage according to the procedure of Thomas and Abelson, 1966; 8–10 μg), bovine serum albumin (Schwarz/Mann, 100 μg), and enzyme. Incubation was for 10 min at 37°C, and determination of radioactivity incorporated into RNA was carried out as previously described (Stevens and Henry, 1964).

Ultra Pure urea was from Schwarz/Mann, rifampicin from Calbiochem, calf-thymus DNA from Worthington Biochemicals Corp., and poly(dA-dT) from Miles Laboratories, Inc.

Results

Effect of KCl and Triton X-405 on T4 and Host Holoenzymes. Figure 1A (solid lines) shows the effect of KCl on the enzyme activity with T4 DNA for two concentrations of T4 holoenzyme; Figure 1B shows the same experiment for two concentrations of host holoenzyme. As previously described for T4 holoenzyme (Stevens, 1974), the salt inhibition was greater at the higher (fourfold) enzyme concentration. Host enzyme was stimulated 2.5-fold by the optimal KCl concentration at both enzyme concentrations. Triton X-100 was found to antagonize the KCl inhibition of the T4 enzyme, and further studies with different detergents showed that Triton X-405 was best at maintaining the activity of the T4 enzyme in the presence of KCl. Figure 1 (dashed lines) shows the effect of Triton X-405 on the enzyme activities of T4 and host holoenzymes at different KCl concentrations. With T4 holoenzyme, in the presence of 1% Triton X-405, KCl had little inhibitory effect, even at 0.2 *M*. Triton X-405 had little or no effect on the host enzyme at different KCl concentrations.

Figures 2A and 2B show that the effects of salt and Triton X-405 on the T4 holoenzyme are not the direct result of irreversible inactivation or activation of a protein. A portion of T4 enzyme was preincubated with 0.3 *M* KCl; then the KCl concentration was lowered by dilution into reaction mixtures. The activity of the enzyme before and after preincubation (Figure 2A) was studied at increasing KCl concentrations. The activity of the enzyme responded essentially the same way to increasing KCl concentration either before or after preincubation with KCl. A portion of the T4 enzyme was also preincubated with 0.2 *M* KCl in the presence of 2% Triton X-405. The concentration of Triton was lowered, also by dilution into reaction mixtures, and the activity of the enzyme before and after preincubation was studied. KCl was present at 0.2 *M* in all the reaction mixtures. The same Triton concentration curve was obtained for preincubated enzyme as for that without preincubation (Figure 2B). Thus, Triton has little or no direct activating effect on the enzyme, but affects its activity when assayed in the presence of salt.

Rifampicin inhibits RNA synthesis with RNA polymerase by binding to the enzyme (Wehrli et al., 1968; diMauro et al., 1969). If RNA polymerase–DNA complexes are formed first by incubating the reaction mixtures in the absence of ribonucleoside triphosphates, the enzyme is highly protected from rifampicin (Hinkle et al., 1972). Thus, rifampicin-resistant RNA polymerase–DNA complexes are a measure of the amount of preinitiation complex formation which has occurred. With the T4 enzyme the number of rifampicin-resistant RNA polymerase–DNA complexes formed during a 5-min incubation period was measured in the presence and absence of KCl. Figure 3 (solid lines) shows the effect of KCl concentration on preinitiation complex formation using two concentrations (one being four times the other) of T4 enzyme; KCl was very inhibitory even at 0.1 *M*. The same reaction mixtures are also shown with Triton X-405 present during the preincubation period (dashed lines). Its presence protected the enzyme to a considerable degree from the KCl inhibition. KCl was also added to reaction mixtures with rifampicin and ribonucleoside triphosphates *after* preinitiation complex formation. It was *not* inhibitory when added together with rifampicin and the ribonucleoside triphosphates.

The effect of temperature of incubation on the KCl inhibition was studied. KCl inhibited little at 25°C and less at 30°C than at 37°C, the usual incubation temperature. The

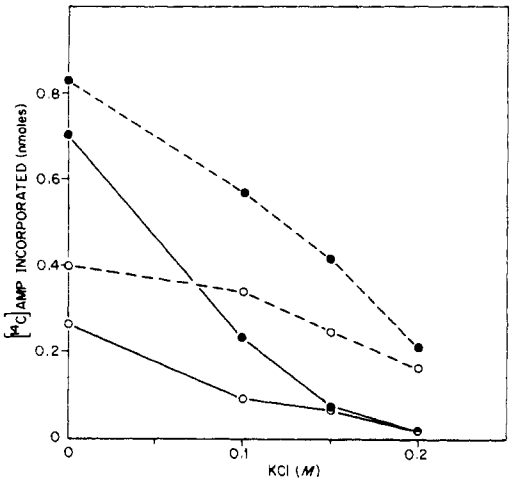

FIGURE 3: Effect of KCl concentration on the formation of rifampicin-resistant preinitiation complexes in the presence and absence of Triton X-405. Reaction mixtures for preinitiation complex formation contained Tris buffer, $MgCl_2$, mercaptoethanol, T4 DNA, and bovine serum albumin as described in Materials and Methods. Enzyme was present at a concentration of 6 μg (○) or 24 μg (●); Triton X-405 was absent (solid lines) or present (dashed lines) at a concentration of 1%; and KCl concentration was varied as shown. The mixtures were incubated for 5 min at 37°C. Rifampicin (2 μg) and ribonucleoside triphosphates as described in Materials and Methods were then added, and the mixtures were incubated for an additional 10 min at 37°C.

Table I: Effect of KCl and Triton X-405 on T4 and Host σ and Core Enzyme Fractions.[a]

Enzyme Fraction	[14C]AMP Incorpd (nmol) for Additions			
	None	KCl	Triton	Both
Host core	0.16	0.52	0.18	0.42
Host core + host σ	0.97	4.80	0.98	2.92
Host core + T4 σ	0.22	0.57	0.48	1.94
T4 core	0.33	0.26	0.18	0.23
T4 core + host σ	1.63	2.00	1.51	1.93
T4 core + T4 σ	0.27	0.09	0.70	0.69
T4-CAP core	0.10	0.20	0.10	0.16
T4-CAP core + T4-CAP σ	0.55	1.15	0.53	0.90

[a] The reaction mixtures were as described in Materials and Methods. When KCl was added it was present at a final concentration of 0.15 *M*; when Triton X-405 was added it was present at a concentration of 1%. The protein concentrations of the enzyme fractions were as follows: host core enzyme, 7.5 μg; T4 core enzyme, 8.5 μg; T4-CAP core enzyme, 7.9 μg; host σ, 1.1 μg; T4 σ, 0.9 μg; and T4-CAP σ, 0.60 μg.

results suggest that KCl is not affecting the melting of regions of the DNA template, since in such a case one would expect the inhibition to be relieved by a temperature increase.

Effect of Salt and Triton X-405 Using σ and Core Enzyme Fractions. σ and core enzyme fractions derived from T4 holoenzyme, host holoenzyme, and a T4 holoenzyme prepared from cells infected with phage in the presence of chloramphenicol (called T4-CAP enzyme; see Materials and Methods) were prepared by phosphocellulose column chromatography. The effects of KCl and Triton were studied further using these enzyme fractions. Table I shows the effects of KCl, Triton, and KCl plus Triton on the activity of different enzyme fractions and combinations of fractions. KCl at 0.15 *M* stimulated the activity of host core enzyme about threefold. When host core enzyme and host σ were

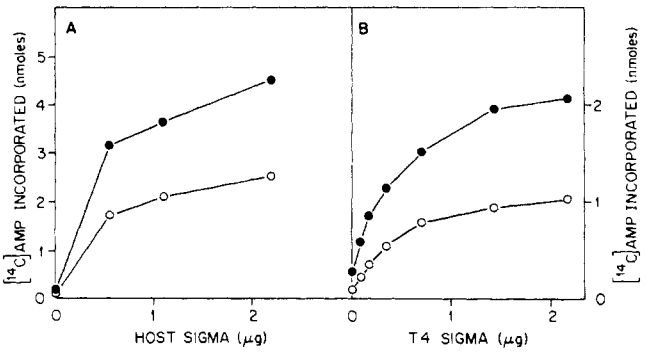

FIGURE 4: Host and T4 σ concentration curves with both host and T4 core enzymes. The reaction mixtures were as described in Materials and Methods. KCl at 0.15 M and Triton X-405 at 1% were present in all the reaction mixtures. Protein concentrations of the core enzyme fractions were as follows: host core enzyme, 7.5 μg (●); T4 core enzyme, 8.5 μg (○).

used together, the activity was stimulated about fivefold in the presence of KCl; Triton alone had no effect, and it slightly inhibited the host fractions in the presence of KCl. With host core enzyme and T4 σ, KCl stimulated about 2.5-fold; Triton alone stimulated about twofold, and KCl plus Triton stimulated maximally (ninefold). With T4 core enzyme alone, the activity was decreased about 20% by KCl alone and 50% by Triton alone. With the T4 core enzyme and host σ, the activity was stimulated about 25% by KCl; addition of Triton had little effect. With T4 core enzyme and T4 σ, the activity was reduced by two-thirds in the presence of KCl and increased 2.5-fold in the presence of Triton, and also in the presence of KCl plus Triton. With T4-CAP core enzyme, the reaction was stimulated by salt. With both T4-CAP core enzyme and T4-CAP σ, the reaction was also stimulated by KCl, as were the host enzyme fractions. These results show that the T4 σ fraction plays a role in the salt sensitivity of the T4 enzyme, since its activity is quite different from that of host σ. The results also suggest that T4 core alone is salt sensitive, but it is possible that the core fraction contains small amounts of residual σ. Neither T4 core enzyme alone nor T4 core enzyme plus T4 σ is inhibited by KCl when calf-thymus DNA or poly(dA-dT) is used as template instead of T4 DNA. The calf-thymus DNA and poly(dA-dT) do not require σ activity for a high reaction rate (Bautz et al., 1969; Berg et al., 1971).

Since all the enzyme fractions were active in the presence of both KCl and Triton, host and T4 σ concentration curves were determined with the two core enzymes in the presence of both KCl and Triton X-405 (Figure 4). Host σ (Figure 4A) and T4 σ (Figure 4B) gave similar concentration curves, with the optimal σ concentration being about 1.5 μg when the core enzyme concentration was about 8 μg. Host core enzyme plus host σ was the most active combination; T4 core enzyme plus host σ was about 60% as active; host core enzyme plus T4 σ was slightly less active than the latter combination; and T4 core enzyme plus T4 σ was about one-half as active as host core enzyme plus T4 σ.

That T4 σ inhibits the activity of host σ in combination with either core enzyme at high salt concentration (0.15 *M* KCl) was described previously (Stevens, 1973). In those studies T4 σ was only slightly inhibitory to host σ plus host core enzyme at low salt concentration (0.05 *M* KCl). Those findings also suggested that the salt sensitivity of the T4 holoenzyme is due to the presence of an inhibitor or modifier of σ activity which is found in the σ fraction after phospho-

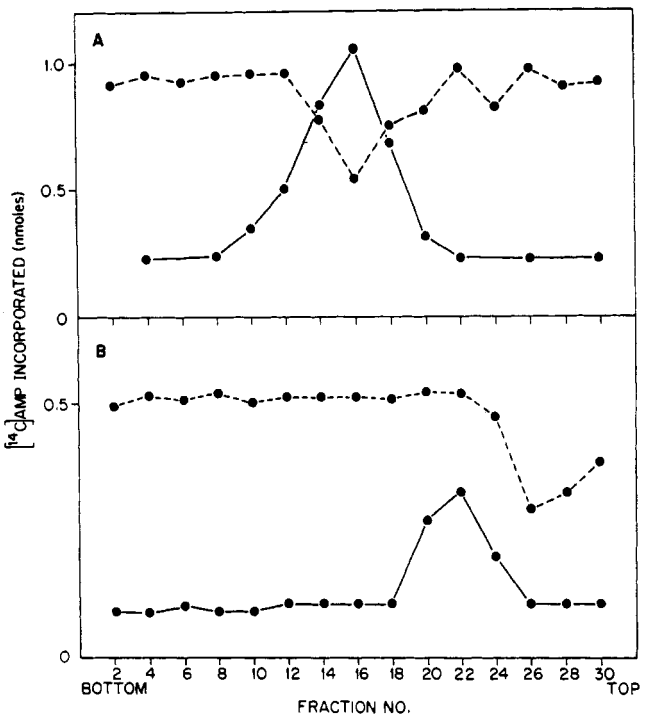

FIGURE 5: Sedimentation profiles of T4 σ fractions in glycerol density gradients in the absence (A) and presence (B) of 6 *M* urea. (A) T4 σ (25 μg) in 0.2 ml of a solution containing 50 m*M* KCl, 20 m*M* Tris buffer (pH 7.8), 1 m*M* EDTA, 1 m*M* dithiothreitol, and 5% glycerol was layered onto 5 ml of a 12.5–30% glycerol gradient containing the same components. The tube was centrifuged for 16 hr at 44000 rpm (SW 50 head, Spinco L2). Fractions (0.15 ml) of the gradient were collected and assayed. For determination of T4 σ stimulation of T4 core enzyme (solid lines) the reaction mixtures were as described in Materials and Methods and also contained 0.15 *M* KCl and 1% Triton X-405. T4 core enzyme was present at a concentration of 7 μg. For determination of T4 σ fraction inhibition of host σ activity (dashed lines), the reaction mixtures were as above with no Triton X-405. T4 core enzyme was present at a concentration of 7 μg and host σ at a concentration of 1 μg. (B) T4 σ (20 μg) purified by gradient centrifugation as described above was precipitated with ammonium sulfate and dissolved in 0.2 ml of the following buffer: 5% glycerol containing 10 m*M* Tris buffer (pH 7.8), 10 m*M* $MgCl_2$, 0.1 m*M* EDTA, 100 m*M* KCl, 10 m*M* dithiothreitol, and 6 *M* urea. The sample was layered on 5 ml of a linear glycerol gradient solution (12.5–30% glycerol) containing the same buffer. Centrifugation was for 40 hr at 43000 rpm as above. Fractions (0.15 ml) of the gradient were collected and assayed as described above in A.

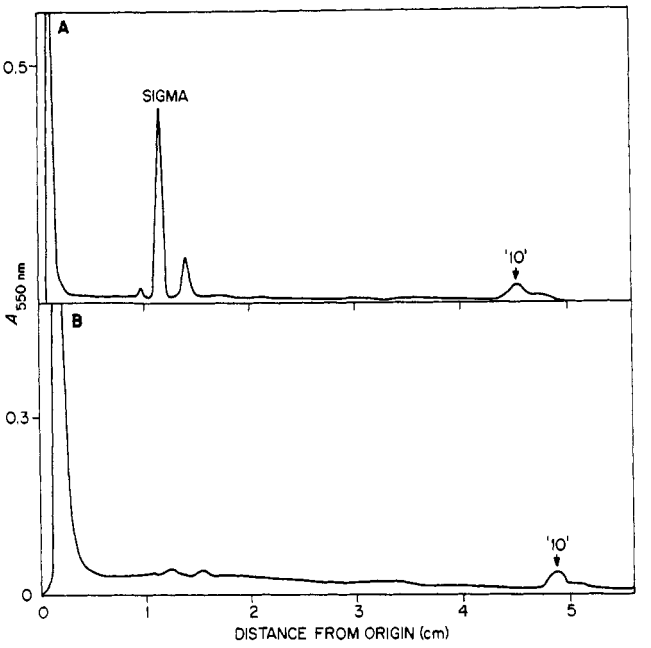

FIGURE 6: Densitometer scans of stained gels of a T4 σ fraction after purification by centrifugation in a glycerol density gradient (A) and of an inhibitor fraction from a 6 *M* urea gradient (B). "10" refers to the mol wt 10000 polypeptide. (A) An aliquot of a T4 σ fraction from the gradient of Figure 5A was electrophoresed. (B) A fraction (no. 27) obtained from the gradient of Figure 5B was electrophoresed. Polyacrylamide (12%) gels containing sodium dodecyl sulfate were used as previously described (Stevens, 1972). Also, staining and scanning of the gels were carried out as described previously (Stevens, 1972).

cellulose chromatography. Further studies were made to see the effect on the inhibitor of increasing the concentration of T4 DNA. Increasing the DNA concentration fourfold did not decrease the inhibition. Triton relieves the inhibition almost completely.

Centrifugation of T4 σ in Glycerol Density Gradients in the Absence and Presence of 6 M Urea. T4 σ was purified further by glycerol gradient centrifugation. Fractions obtained were assayed both for stimulation of T4 core enzyme (in the presence of both KCl and Triton X-405) and for inhibition of T4 core enzyme plus host σ in the presence of 0.15 *M* KCl. The results are shown in Figure 5A. The major fraction of the inhibitory activity of T4 σ fractions was found at the same position as the σ stimulatory activity. The results suggest that the inhibitor is closely associated with T4 σ.

Gel electrophoresis of the most active σ fraction from the gradient shown in Figure 5A was carried out. A densitometer tracing of the gel is shown in Figure 6A. Two protein components are present in addition to the σ band: one has a molecular weight slightly smaller than that of σ, while the other is the T4-specific binding protein of mol wt 10000 which has been described previously (Stevens, 1972, 1974).

We attempted to remove the other proteins by centrifuging T4 σ in 1% Triton X-405 and in 2 or 3 *M* urea. The results were the same as those shown in Figure 5A. When T4 σ was centrifuged in 6 *M* urea, however, the inhibitory activity separated from the stimulatory σ activity. These results are shown in Figure 5B. Figure 6B is a densitometer scan of a gel run of the peak inhibitory fraction from Figure 5B. The main protein component is the T4-specific protein of mol wt 10000. It is the only protein which shifts position relative to σ on 6 *M* urea centrifugation. The results suggest that it is the inhibitory component and that it may be closely associated with T4 σ under usual isolation conditions.

Heat-inactivation curves were determined, using both the crude σ fraction from phosphocellulose columns and a fraction of the 6 *M* urea gradient containing the mol wt 10000 protein. Inhibition of host σ activity was measured. As shown in Figure 7, both the crude σ fraction and the mol wt 10000 protein fraction are inactivated at the same temperature. The results further suggest that the mol wt 10000 protein is the inhibitory component of the T4 σ fraction.

The inhibitor separated in the presence of 6 *M* urea was tested to see if its inhibition of host σ activity was enhanced by KCl (see Table II). The first experiment shows the effect of the inhibitor fraction on the activity of host core enzyme plust host σ. In the absence of KCl, there is little (about 17%) inhibition of the activity of the enzyme fractions. In the presence of salt and inhibitor, the activity of host core enzyme plus host σ is reduced almost to the level of host core enzyme alone. The second experiment shows the effect of the inhibitor on T4 core enzyme plus host σ. The inhibitor fraction decreases the activity by more than 50% at low

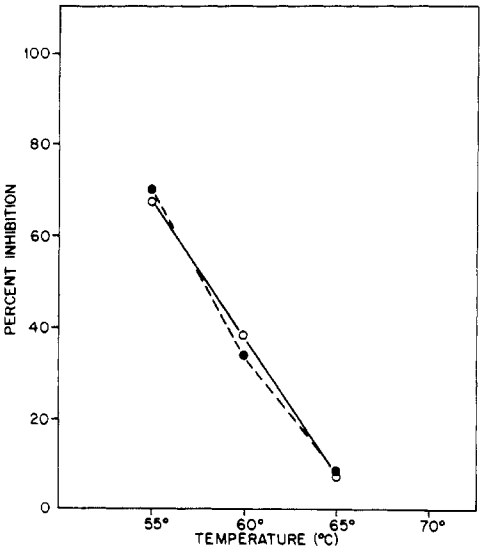

FIGURE 7: Heat inactivation of the inhibitor in crude σ fractions and in a fraction from a 6 *M* urea gradient. A crude σ fraction from a phosphocellulose column and a fraction similar to no. 27 of the 6 *M* urea gradient shown in Figure 5B were heated at the indicated temperature for 10 min. Both samples were heated in the 6 *M* urea buffer described in Figure 5. They were then assayed for their inhibition of host σ activity using reaction mixtures similar to those described in Figure 5: (○) crude σ fraction; (●) 6 *M* urea gradient fraction.

Table II: Effect of KCl on the Inhibitor Isolated from 6 *M* Urea Gradients.[a]

Enzyme Fraction	[^{14}C]AMP Incorpd (nmol) for Additions	
	None	0.15 *M* KCl
Experiment 1		
Host core	0.13	0.41
Host core + host σ	0.53	1.83
Host core + host σ + inhibitor	0.44	0.49
Experiment 2		
T4 core	0.63	0.34
T4 core + host σ	1.80	2.26
T4 core + host σ + inhibitor	1.0	0.34

[a] The reaction mixtures were as described in Materials and Methods. The protein concentrations of the enzyme fractions were as follows: host core enzyme, 7.9 μg; T4 core enzyme, 12 μg; host σ, 0.5 μg; inhibitor fraction, 0.12 μg.

salt and to that of core enzyme alone in the presence of salt. The results are thus analogous to those described previously for the crude σ fraction (Stevens, 1973). In addition, it was found that both the inhibition by crude σ fractions and that by the isolated inhibitor fraction were overcome by Triton X-405.

Table III shows the activity of T4 σ isolated by centrifugation in the 6 *M* urea gradient. Its activity was measured in the presence of host core enzyme. The results can be compared with those in Table I. T4 σ centrifuged in 6 *M* urea has activity similar to that of host σ, that is, it is stimulated by KCl, and Triton inhibits slightly when it is also added.

Discussion

Our investigations show that T4 enzyme is salt sensitive primarily because the enzyme contains an inhibitory component present in purified σ fractions. Several lines of evidence suggest that the T4-specific protein of mol wt 10000

Table III: Effect of KCl and Triton X-405 on T4 σ Isolated from a 6 *M* Urea Gradient.[a]

Enzyme Fraction	[^{14}C]AMP Incorpd (nmol) for Additions			
	None	KCl	Triton	Both
Host core enzyme	0.12	0.31	0.10	0.29
Host core + T4 σ (crude)	0.17	0.34	0.27	1.33
Host core + T4 σ (purified in a 6 *M* urea gradient)	0.47	1.93	0.58	1.53

[a] The reaction mixtures were as described in Materials and Methods. The concentration of KCl was 0.15 *M* and the Triton X-405 was 1%. The concentration of host core enzyme was 5 μg. The concentration of T4 σ in both cases was approximately 1 μg.

is the inhibitor. First, it is present in the purified σ fraction and separated only on 6 *M* urea treatment. Second, the inhibitor is not present when the phage infection occurs in the presence of chloramphenicol. Heat-inactivation curves suggest that the inhibitor is a protein. Until the protein can be isolated in reasonable amounts from cruder fractions, the exact mechanism of its action will remain uncertain. Enzyme preparations have been examined for DNase activity; they contain no endonuclease or exonuclease that might inhibit the reaction with T4 DNA. Increasing the amount of T4 DNA does not affect the extent of inhibition, so it is not likely that the inhibitor acts by binding to DNA.

Several questions arise from this study for which we as yet have no answers. Can the inhibitor be an artefact of isolation of the enzyme? If not, what is the function of the inhibitor, i.e., is it really an inhibitor or is it a modifier of the specificity of the T4 enzyme? To answer the first question is not easy. It is possible that the small protein has a high affinity for acidic proteins and binds very tightly to σ, which is quite acidic. The answer to the second question depends to a large degree on knowing the nature of the RNA product formed when σ containing the small protein is active. We have examined the nature of the product formed using both Triton X-405 and KCl in the reaction mixtures. The product was assayed for symmetry and for its content of chloramphenicol RNA as described by Brody and Geiduschek (1970). The RNA formed is slightly more symmetric (5%) than RNA formed in the presence of host σ (1–2%), as measured by RNA duplex formation with early T4 messenger RNA. It is difficult to interpret the fact that the RNA product is slightly (10–15%) less competitive with chloramphenicol RNA for duplex formation with denatured DNA than the RNA product formed with host σ. Either the greater symmetry leads to less measurable chloramphenicol RNA or there is more delayed early, quasi-late, or late RNA in the product. Bogdanova et al. (1970) and Khesin et al. (1972) first described an anti-σ activity in crude protein fractions of T2 or T4 phage-infected *E. coli*. Inhibitors of the σ activity of *E. coli* holoenzyme also have been described for two other phage-infected systems: T3 and T7 (Hesselbach et al., 1974; Mahadik et al., 1972, 1974). The T3 inhibitor has yet to be purified, but its action appears to be quite complicated, since it does not bind to the holoenzyme–DNA complex except in the presence of a particular combination of ribonucleoside triphosphates. The T7 inhibitor has been identified as a protein with a mol wt of approximately 66000; it also functions at the level of the holoenzyme by inhibiting the σ activity of the enzyme.

Nonionic detergents of the Brij and Tween series were in-

vestigated as well as Triton detergents for their capacity to antagonize the KCl inhibition of activity. In both series, detergents with an HLB number (see Umbreit and Strominger, 1973) of about 14–15 were active. Their activity was not as strong as that of similar detergents in the Triton series or Triton X-305 and Triton X-405 with higher HLB numbers. When Triton X-405 was used together with a less active Brij or Tween detergent, the stimulation of activity was less than when using Triton alone, which suggests that Brij and Tween detergents inhibit the Triton effect.

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Alkyl Isocyanates as Active Site-Specific Reagents for Serine Proteases. Location of Alkyl Binding Site in Chymotrypsin by X-Ray Diffraction†

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ABSTRACT: The structure of octylcarbamoyl-α-chymotrypsin to a resolution of 3.0 Å is described. The *n*-octyl side chain of the active site directed irreversible inactivator octyl isocyanate is bound exclusively in the hydrophobic substrate binding pocket. The *n*-octyl isocyanate forms a planar urethane bond with the Ser-195 O_γ and extends approximately 1 Å deeper into the hydrophobic pocket than the indolyl group of indoleacryloyl-α-chymotrypsin (Henderson, R. (1970), *J. Mol. Biol. 54,* 341). All the structural changes are essentially identical with those observed in indoleacryloyl-α-chymotrypsin including the observation of a hydrogen bonded water molecule between the carbonyl oxygen of the octylcarbamoyl group and the imidazole group of His-57. The observed mode of *n*-octyl alkyl binding to chymotrypsin is consistent with the hypothesis proposed earlier (Brown, W. E. and Wold, F. (1973), *Biochemistry 12,* 828).

The previous papers in this series (Brown and Wold, 1973a,b) have shown that octyl and butyl isocyanates are active site directed irreversible inactivators of chymotrypsin and elastase, respectively. Based on chemical data (Brown and Wold, 1973a), it was postulated that the inactivation reaction proceeded in a two-step manner. First, the enzyme binds the *n*-alkyl side chain of the isocyanate to form a noncovalent intermediate, analogous to an enzyme–substrate complex. This specific binding is followed by a covalent linkage of the isocyanate group to some functional group in the active site of the enzyme. In support of the second step, it was later shown that the modified functional group in each case was the active site serine, serine-195 in chymotrypsin and serine-188 in elastase (Brown and Wold, 1973b).

On the other hand, the first step of this reaction sequence is based on the assumption that the *n*-alkyl side chain of the

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