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Chemically Induced Cofactor Requirement for Bacteriophage T4D[1]

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The treatment of bacteriophage T4D with 2-hydroxy-5-nitrobenzyl bromide, a specific reagent for alkylating the indole ring of tryptophan residues, converts these particles from a cofactor-independent form to a cofactor-sensitive form. These treated T4D particles phenotypically resemble T4B particles in certain respects. Their ability to form plaques on minimal medium plates is increased by the addition of L-tryptophan and is inhibited by the addition of indole. In liquid medium, their rate of adsorption is dependent on the presence of the cofactor L-tryptophan. L-Tryptophan-requiring phage have been produced by in vitro assembly of treated tail-fiberless particles of a T4D amber mutant plus untreated tail fiber preparation. When treated tail fibers were used with untreated tail-fiberless particles, the newly assembled particles did not require cofactor. A model of the tail structure of all the T-even bacteriophages is presented which postulates that the active configuration of the tail fibers requires that there be either (i) an endogenous tryptophan residue of the phage particle itself or (ii) an exogenously added L-tryptophan molecule complexed with a specific tryptophan receptor site, most likely on the phage base plate.

Anderson reported in 1945 (3–5) that certain strains of T4 bacteriophage required L-tryptophan as a cofactor for adsorption; since then, this phenomenon has attracted considerable interest (7–9, 12, 15). In 1950, a kinetic study by Wollman and Stent (28) showed that approximately 5 or 6 cofactor molecules are needed to activate a T4B bacteriophage particle. Since the phage tail has sixfold symmetry, including a hexagonal-shaped base plate with a tail fiber at each apex of the hexagon, it seems likely that there are six receptor sites on the phage tail. It has been shown recently that L-tryptophan is able to convert a population of inactive phage particles to active particles by causing a conformational change in the phage tail structure (10, 20, 21). Active phage particles have their six tail fibers extending downward from their tail plate and can adsorb to host cells, whereas inactive particles have their tail fibers oriented from the base plate toward the head of the virus particle.

Some of the work in the past 10 years has suggested that this phenomenon depends not on some peculiarity of T4 phage particles but on a common structural feature found in all the T-even bacteriophages (1) and possibly in another coliphage, "OX6" of Fildes and Kay (14). For example, T2H strains (20) can be converted into an inactive form (tail fibers are not extended) by the addition of indole. Further, some strains of T4B (12) appear to be both tryptophan-requiring and indole-sensitive. The properities of the indole-binding reaction indicate that this reaction is similar to the binding of tryptophan to T4B phages.

This report is concerned with the chemical induction of cofactor sensitivities in a cofactor-independent strain of T4D bacteriophage. The results obtained by the use of specific reagents to modify amino acid residues on the intact T4D particle indicate that an endogenous tryptophan residue, i.e., one present already in phage tail structure, and exogenously added tryptophan act equivalently in producing the active forms.

MATERIALS AND METHODS

Bacteriophage preparations and assays. Purified and concentrated stocks of *E. coli* B bacteriophage T4D, a strain which does not require the cofactor tryptophan, and T4B01, a cofactor-requiring strain, were prepared by standard procedures (21). All phage assays, except where specifically stated, were done at 23 C on glucose-NH_4Cl-salts minimal medium plates [M-9 of Adams

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(2), adjusted to *p*H 6.6 with HCl] to which various cofactors at a final concentration of 60 μg/ml were added when required. When used, cofactor was also added to the soft agar tubes. A multiple T4D amber mutant X4E, defective in tail fiber genes 34, 35, 37, and 38, was obtained from R. S. Edgar (13). A stock of this mutant was prepared which was 99% inactive, as determined by a comparison of its titer and optical density with that of viable phage. Electron micrographs confirmed that the majority of these phage particles had no visible tail fibers. Cell extracts of *E. coli* B containing phage tail fibers were prepared by the technique of Edgar and Wood (13) with the use of the T4D amber mutant B17, defective in gene 23 for the formation of phage heads.

Chemicals. HNB-Br, 2-hydroxy-5-nitrobenzyl bromide (6, 17, 19, 22), was obtained from Calbiochem, Los Angeles, Calif. It was stored at −20 C in a desiccator, dissolved in alumina-dried absolute methanol at 4 C to a concentration of 0.22 M, and then used immediately. When phage were treated with this reagent, the final methanol concentration was usually 10% or less by volume. This concentration of methanol at 4 C and *p*H 4.2 for periods of 10 min or less did not inactivate virus particles. After several months, the solid reagent lost activity and it was necessary to obtain fresh reagent.

N-bromosuccinimide was obtained from Matheson, Coleman and Bell, East Rutherford, N.J. Fresh aqueous stock solutions at 5×6^{-4} M were prepared for each experiment and used at a final concentration of 3×10^{-5} M in the reaction mixture. *N*-1-(4-Pyridyl)-pyridinium hydrochloride was obtained from Eastman Chemical Co., Rochester, N.Y. A fresh aqueous stock solution of 0.1 M reagent was prepared immediately before use and was added at a final concentration of 0.015 M to phage 10 min before the addition of any indole-attacking reagent. Tetranitromethane (24, 26), obtained from Mann Research Corp., New York, N.Y., was dissolved in 95% ethyl alcohol at a final concentration of 0.84 M. The final concentration of the tetranitromethane was 0.0084 M, and the ethyl alcohol was 1% or less by volume in the reaction mixture.

RESULTS

Effect of tryptophan reagents on cofactor requirements of T4D. When T4D particles, a cofactor-independent phage strain, were treated with HNB-Br, the plaque-forming ability of the surviving particles was enhanced by the addition of L-tryptophan and was inhibited by the addition of indole (Table 1 and Fig. 1). The conditions of the treatment were critical, and the results were dependent on the *p*H, temperature, and method of addition of this reagent. HNB-Br is a highly specific reagent for alkylation of the indole ring of tryptophan residues (6, 17, 19, 22) at acid *p*H values; it also reacts with cysteine (20% of the rate with tryptophan) and with tyrosine, especially at neutral or higher *p*H values, and it is rapidly hydrolyzed in water. Purified T4D preparations were dialyzed at 4 C overnight against 0.2 M acetate buffer, *p*H 4.2 (the lowest *p*H allowing viability), and diluted in this buffer at 4 C to a concentration of 5×10^{11} particles per ml. Three

TABLE 1. *Cofactor requirements of T4D after treatment with various reagents*

Reagent	Relative titer (tryptophan medium/ minimal medium)
1. None	1.0
2a. 2-Hydroxy-5-nitrobenzyl bromide.	2.4
2b. Pyridyl-pyridinium chloride, then 2-Hydroxy-5-nitrobenzyl bromide	1.0
3. *N*-bromosuccinimide	1.0
4. Tetranitromethane	1.0

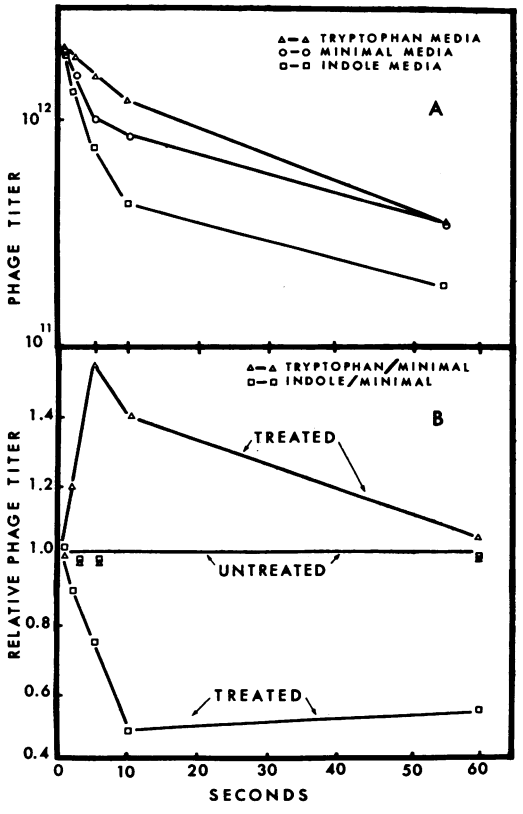

FIG. 1. *Rate of T4D inactivation after treatment with HNB-Br. T4D, at a concentration of* 5×10^{11} *phage particles per ml in acetate buffer at pH 4.2 at 4 C, was treated with HNB-Br at final concentration of* 1.1×10^{-2} M, *rapidly diluted, and plated on tryptophan, indole, and minimal medium plates. Figure 1A shows the decrease in phage titer as assayed on each type of plate; Figure 1B shows ratios of titers on tryptophan plates and indole plates to titers on minimal plates.*

equal samples of 0.22 M HNB-Br reagent in absolute methanol at 4 C were added to a final concentration of 0.016 M at 15-sec intervals, and the reaction was stopped by 1,000-fold dilution in saline-gelatin at the end of 45 sec. This procedure consistently gave about 30% survivors, as assayed on either tryptophan-containing plates or broth plates. When the total amount of HNB-Br was added rapidly as a single sample to the phage, there was usually only 20% survival (*see* Fig. 1), and the effect on cofactor requirements was reduced.

The typical results given in Table 1 show that the plaque titer of treated phage in the presence of tryptophan was 2.4 times higher than on minimal plates. In other experiments with different batches of HNB-Br, the ratio of plaques on tryptophan medium to minimal medium was occasionally as high as 3.8. To confirm that a tryptophan residue was being attacked by the HNB-Br, a reversible tryptophan masking reagent (11), pyridyl pyridinium chloride hydrochloride (PPCl), was added to the T4D 5 min before treatment with HNB-Br. The results in Table 1 indicate that, under these conditions, HNB-Br had no effect on cofactor requirements; the phage particles gave the same number of plaques in the presence and absence of L-tryptophan. PPCl also protected the phage against HNB-Br inactivation; there were 50% survivors in these experiments, as compared to the usual 30% survivors.

A second reagent thought to be specific for tryptophan residues, *N*-bromosuccinimide (27), was used to treat T4D. *N*-bromosuccinimide, at a final concentration of 3×10^{-5} in 0.2 M acetate at *p*H 4.2, inactivated 80% of the T4D particles in 45 sec. The survivors showed no tryptophan requirement (Table 1). It has been reported that *N*-bromosuccinimide oxidizes rather than alkylates the indole ring (22, 28). The results suggest that only certain alterations of an endogenous tryptophan residue(s) of the phage structure lead to the formation of a tryptophan-requiring particle.

Effect of a tyrosine reagent on T4D. Tetranitromethane (24, 26), which specifically alters the hydrophobic aromatic residue tyrosine, was used to treat T4D. Phages were incubated at 30 C for 10 min with 0.008% tetranitromethane in acetate buffer at *p*H 4.7, and the reaction was stopped at various times by dilution with 1,000 volumes of saline-gelatin at *p*H 4.7. The phage particles were over 90% inactivated by this reagent in 10 min. T4D particles treated with tetranitromethane, in marked contrast to the effect of HNB-Br, never gave higher titers on plates containing tryptophan (Table 1) than on minimal medium. These results are consistent with the view that only specific alterations of indole rings on T4D particles are responsible for the requirement for exogenously added tryptophan.

Rate of formation of cofactor-sensitive T4D. HNB-Br hydrolyzes in water with a half-life of less than 30 sec (17, 19, 22), which means that all reactions in aqueous systems are really competition reactions between tryptophan (and cysteine and tyrosine) and water. This makes a detailed kinetic analysis of the action of HNB-Br on aqueous suspensions of phage particles difficult. In the experiment shown in Fig. 1, methanolic HNB-Br was added to give an initial concentration of 1.1×10^{-2} M to T4D phage in 0.2 M acetate (*p*H 4.2) buffer at 4 C. Samples were taken at various times and diluted with saline-gelatin. Figures 1A and 1B show the inactivation of T4D and the effect of tryptophan and indole on its plating characteristics. The maximal tryptophan requirement was reached within 5 sec and then declined. This experiment was repeated 10 times with similar results. Apparently the alkylation of a phage indole ring makes the particle not only tryptophan-requiring but also more sensitive to a second attack by the HNB-Br, either on another indole ring or on a cysteine or tyrosine residue.

HNB-Br treatment also induces an indole sensitivity to plaque formation which the original T4D particles do not have. It should be mentioned in this connection that one strain of T4B studied by Delbrück (12) was both tryptophan-requiring and indole-sensitive. In these experiments, it is not clear whether particles which require tryptophan are also always indole-sensitive. However, after 60 sec of treatment, there appear to be many particles which are still indole-sensitive but which no longer can be activated by tryptophan.

Reaction of treated T4D with cofactors: specificity, reversibility, and temperature dependence. The specificity of other substances, in addition to L-tryptophan, in enhancing plaque formation by HNB-Br-treated T4D was examined in a manner similar to that used by Anderson (3–6). The titer of treated phage on nutrient broth plates was always found to be identical to that obtained on minimal medium plates supplemented solely with L-tryptophan. D-Tryptophan added to minimal plates was ineffective as a cofactor for treated T4D. Similarly, L-tyrosine was inactive, but surprisingly L-phenylalanine at 60 μg/ml was almost as active as L-tryptophan (or broth) cofactors for plaque formation by treated phage. The effect of these compounds on treated T4D is similar but not identical to their activities as cofactors for T4B (3–6).

The reaction of L-tryptophan with treated T4D was found to be completely reversible. In three separate experiments, a portion of treated T4D was exposed to 50 μg/ml of L-tryptophan for 10 min. The tryptophan was diluted $1:10^4$ in saline-gelatin, and the phage was plated on various media. This preincubation of treated T4D with L-tryptophan had no effect on the number of plaques later formed on minimal, tryptophan, or broth plates.

Temperature is another parameter affecting cofactor requirements of T4B strain, originally described by Anderson (5). Tryptophan dependence decreases with increasing temperature. The effect of temperature on the plaque-forming ability of treated T4D is shown in Fig. 2. It is clear that the tryptophan requirement of treated T4D particles showed a marked increase at lower temperatures. The specifity, reversibility, and the increased cofactor requirements at low temperatures support the view that alkylation of certain tryptophan residues in T4D converts this phage into particles phenotypically like T4B strains.

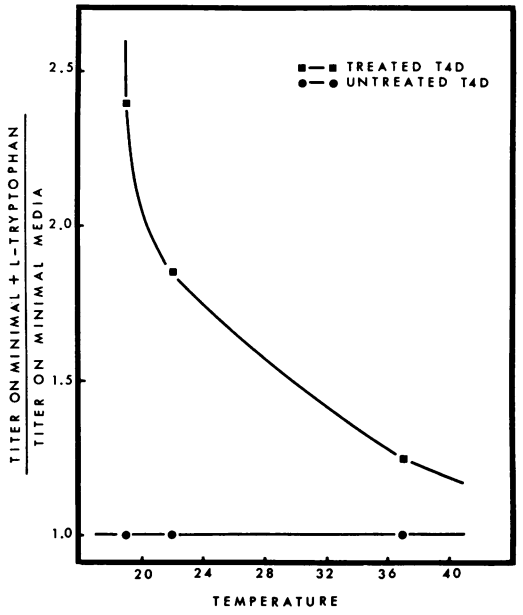

FIG. 2. *Relative phage titers of untreated T4D and HNB-Br treated T4D as a function of temperature. T4D at a concentration of* 5×10^{11} */ml in acetate buffer at pH 4.2 was treated at 4 C with HNB-Br (at a final concentration of* 1.6×10^{-2} *M), by adding three equal amounts once every 15 sec. The reaction was stopped at the end of 45 sec. The phage were then diluted in saline-gelatin and plated on minimal and tryptophan plates which were incubated at three different temperatures. The relative phage titers represent the ratio appearing on tryptophan media to those on minimal media.*

Rate of adsorption of treated and untreated T4D. The rates of adsorption of T4D were determined by standard procedures in which the fraction of unadsorbed phage was measured at various times (Fig. 3). Untreated T4D under these conditions had an adsorption rate constant of 10^{-9} ml/min, both in the presence and in the absence of L-tryptophan. The rate of adsorption of T4D after treatment with HNB-Br was greatly decreased. The adsorption rate constant [assuming that 5×10^8 bacteria per ml gave the maximal rate (29)] was 8×10^{-11} ml/min, or only 8% that of the untreated particles. The addition of L-tryptophan

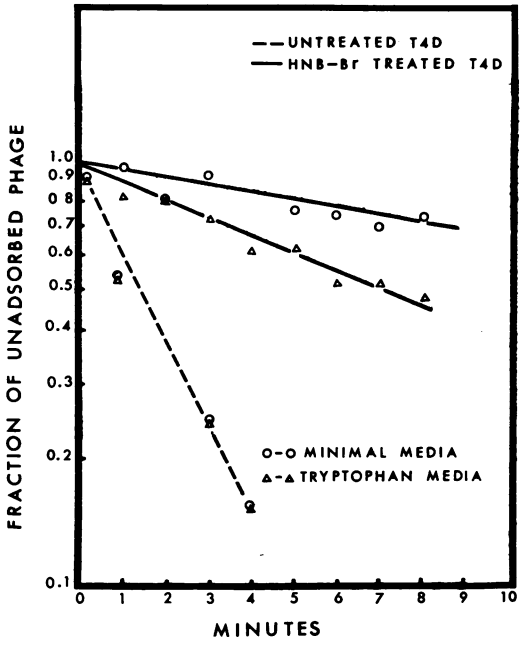

FIG. 3. *Adsorption of untreated and HNB-Br treated T4D. T4D, at a concentration of* 5×10^{11} *phage particles per ml in acetate buffer at pH 4.2 at 4 C, was treated with HNB-Br (at a final concentration of* 1.6×10^{-2} *M), by adding three equal amounts, one every 15 sec, and stopping the reaction at the end of 45 sec. The treated phage were then added to Escherichia coli B at* 2×10^9*/ml to a multiplicity of infection of 0.01 at 23 C in glycerol Casamino Acids medium (17), either free of cofactor or containing 60 μg/ml of L-tryptophan. This high bacterial concentration was necessary since very little adsorption occurs at lower bacterial concentrations, with or without cofactor. At various times after infection, samples were removed and treated with chloroform to destroy infective centers; the free phage were then assayed on broth plates. Similarly, the adsorption characteristics of untreated T4D (at a bacterial concentration of* 5×10^8*/ml) were examined in the presence and absence of tryptophan.*

increased the rate of adsorption of treated particles to 2×10^{-10}, or 2.5-fold. It is apparent that the tryptophan requirement for plaque formation by treated phage on minimal medium plates is a function of the effect of tryptophan on the rate of adsorption. However, these experiments show that HNB-Br treatment kills some particles and damages practically all the particle so that their ability to adsorb is greatly reduced. Even though 30 to 40% of the surviving particles are able to form plaques on minimal media in the absence of tryptophan, these particles still do not adsorb (*see* Fig. 3) at the rate of untreated particles. There is no evidence from these adsorption studies that treated phage consists of two different populations of particles; one which is relatively normal and one which requires cofactor. Rather, it appears that all are injured by HNB-Br and that, depending upon the time allowed (and the temperature), a varying fraction will be activated by tryptophan.

Although the effect of L-tryptophan on the adsorption rate of treated T4D is qualitatively similar to the effect of this cofactor on T4B, quantitatively the effect on T4D is dramatically less. T4B in the presence of L-tryptophan under these conditions has an adsorption rate constant of about 10^{-9} ml/min, similar to that of T4D. In the absence of tryptophan, the adsorption rate constant is too low to be measured precisely but is of the order of 10^{-12} ml/min, a decrease of about 10^3.

Site of alkylation by HNB-Br. Experiments have been carried out to determine which phage substructure is being altered by the HNB-Br treatment. The phage assembly experiment described by Edgar and Wood (13) permits the separate testing of two different phage components.

Figure 4 shows one of three such experiments where 8×10^{10} purified fiberless phage particles were first treated with 0.03 M HNB-Br for 30 sec. The reaction was stopped by dilution, and then a bacterial cell extract containing B17 phage tail fibers was added. This mixture was assayed for phage immediately and then was incubated at 30 C. Samples were removed during this incubation and plated on minimal and tryptophan plates. (Treatment of the tail-fiberless particles decreased the ability of these particles to participate in the assembly reaction only slightly, from 125-fold increase in the control to 100-fold.) Although the ratio of plaques found on tryptophan plates as compared with minimal plates was 1 at zero-time, it increased to over 2 in the first 20 min. Further, it was found that the tryptophan requirement of the assembled particles was specific for L-tryptophan and that D-tryptophan was ineffective.

The converse experiment was also performed. The cell extract containing tail fibers was treated with HNB-Br, and these tail fibers were used for phage assembly. The tail fiber extract treated with HNB-Br showed a 40% decrease in assembly capacity from that of the untreated control (only 60-fold as compared to 125-fold in 40 min); however, none of the assembled particles required tryptophan. This finding supports the conclusion that the site of endogenous tryptophan, which affects tail fiber configuration, is not on the tail fibers themsleves.

Effect of HNB-Br treatment on cofactor requirements of T4B. T4B was also treated with HNB-Br at *p*H 4.2 in the absence of tryptophan, and its ability to form plaques on minimal and tryptophan medium at 23 C was examined. Before treatment, the titer of the T4B preparation was 3.0×10^{12}/ml as assayed on tryptophan plates and 2.0×10^{8}/ml on minimal medium plates. After treatment, the titer on tryptophan plates was reduced to 1.1×10^{12}, and the titer on minimal plates was 3.0×10^{7}. Treatment with this reagent increased the ratio of titers on tryptophan to minimal media from 1.5×10^{4} to 3.7×10^{4}. Similar results were found in five separate experiments. Although the fraction of the total

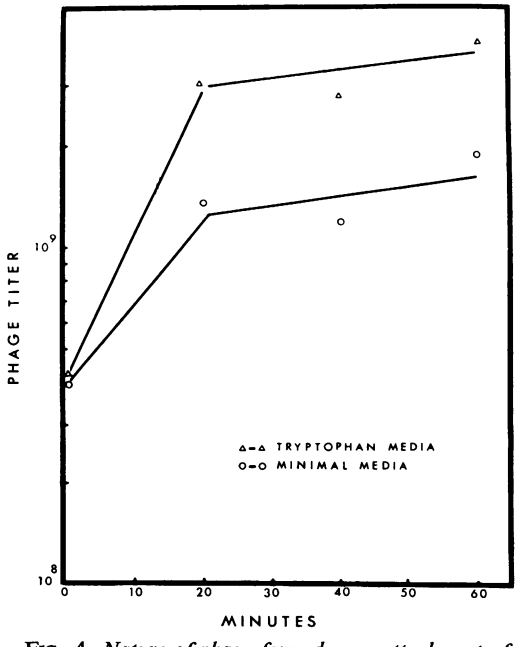

FIG. 4. *Nature of phage formed upon attachment of tail fibers to tail-fiberless particles treated with HNB-Br. Purified T4D amber mutant X4E (14) tail-fiberless particles were treated with HNB-Br at a final concentration of* 3.6×10^{-2} M. *An extract of bacteria infected with the amber mutant of T4D, B17, containing tail fibers was added, and the assembled phage was assayed on minimal and tryptophan plates.*

number of T4B particles affected is very small, it is likely that, similar to the effect on T4D, alkylation of a tryptophan residue of T4B increases its tryptophan dependence.

T4B was also treated with the tyrosine reagent, tetranitromethane, as was T4D. T4B was rapidly inactivated (90% in 10 min), but there was no change in the surviving population of the relative titers on minimal as compared to tryptophan media.

DISCUSSION

The results reported clearly implicate an endogenous tryptophan residue, natural to the phage structure, as critical in maintaining the active tail fiber configuration of T4D. The identification of this feature of phage structure by HNB-Br treatment rests on (i) the specificity of HNB-Br in alkylating tryptophan residues; (ii) the blocking of its action by PPCl, a substance which reversibly complexes with tryptophan residues; and (iii) the increase in both the adsorption rate and the ability to form plaques upon the addition of L-tryptophan.

Although HNB-Br treatment irreversibly inactivates a fraction of the virus particles and damages all the particles to some extent, most of the 30% which survive adsorb faster in the presence of L-tryptophan. The finding that as many as 15 to 20% of the original particles only form plaques in the presence of tryptophan argues against the possibility that the HNB-Br treatment selects only a very small fraction of the original population which now behave uniquely. In many respects, the treated T4D particles resemble T4B, but it should be emphasized that these damaged T4D particles only adsorb 2.5 times faster in the presence of the cofactor, whereas the adsorption rate of T4B is increased several orders of magnitude by the cofactor. It should also be noted that these treated particles, upon reproduction, behave like the original T4D.

The endogenous tryptophan cannot be on the tail fibers; the base plate is the most likely site, although a site on the tail sheath (7) cannot be excluded. Presumably, the endogenous tryptophan binds to the acceptor site, also probably on the base plate, and induces a conformational state in which the tail fibers extend outward from the phage particle rather than toward the head (*see* Fig. 5a). Upon alkylation by HNB-Br, a bulky nitrobenzyl group (∅) is substituted on the indole ring, preventing its binding to the acceptor site (Fig. 5b). This allows, or causes, this portion of the base plate to assume an alternate configuration in which the tail fibers extend toward the virus head. The acceptor site can then bind an exogenous L-tryptophan molecule, which results in the active configuration (Fig. 5c). Exogenous tryptophan is not as efficient in this regard as the endogenous tryptophan.

A similar endogenous tryptophan residue can be pictured on T4B particles. Presumably, this residue normally is in a much less favorable position to bind to the acceptor site but can do so occasionally. Upon treatment with HNB-Br, the chance that the endogenous tryptophan could react with the acceptor is reduced even further, and the T4B phage population appears more tryptophan-dependent.

The model also offers an explanation of the inhibitory effect of indole on plaque formation by

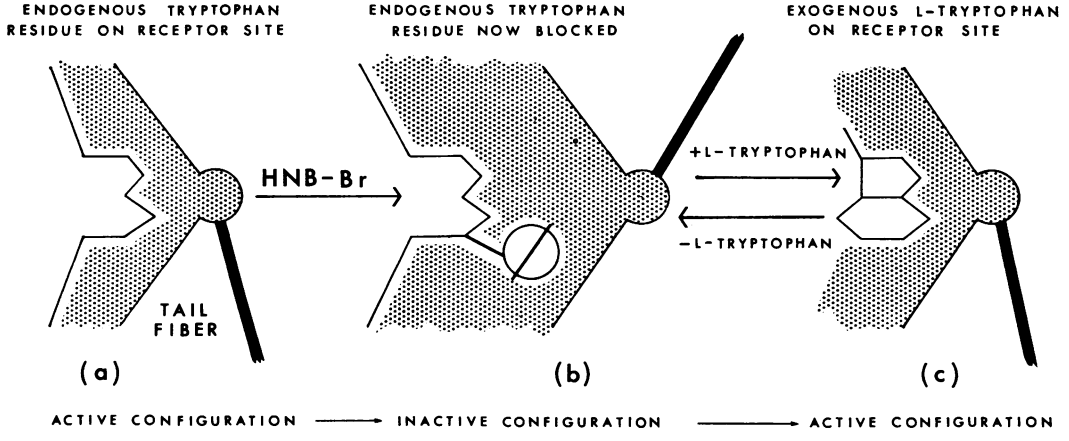

FIG. 5. *Schematic representation of the effect of HNB-Br on the endogenous tryptophan residue in one apex of the hexagonal tail plate of T4D bacteriophage. The binding of an exogenous tryptophan molecule after treatment results in a change in tail fiber configuration.* [*The exact site of the alkylation by HNB-Br, signified by* ∅ (6), *on the indole ring is not certain.*]

treated T4D [and also by T2H (20)]. Indole apparently competes for the acceptor site with the endogenous tryptophan residue. However, indole lacks the necessary three-carbon side chain of the endogenous L-tryptophan residue, which is required to induce the formation of the active configuration. Untreated T4D is insensitive to indole (presumably because of the efficient binding of the endogenous tryptophan residues with the acceptor site), and indole can bind to the acceptor site only after the endogenous tryptophan is alkylated.

Since the endogenous tryptophan residue is still present in extensively dialyzed and highly purified phage preparations, it seems more likely that it is a residue in one of the phage tail proteins (Fig. 5) rather than a tightly complexed noncovalently bonded molecule. Tryptophan complexes, either due to hydrophobic binding or electron sharing, are readily dissociated by dialysis. Although this point is not established, a reasonable suggestion can be advanced both for the nature of the tryptophan acceptor site and the endogenous tryptophan residue. Kanner and Kozloff (20) concluded that indole binds to the T2H phage tail because of its ability to form a molecular complex with an electron acceptor compound. T-even phages have been found to contain an unusual folic acid in their tail structures, which might well be the electron acceptor compound (24). Recently, this folate has been identified as a high molecular weight dihydropteroylpolyglutamate (Kozloff, Lute, Crosby, and Verses, *unpublished observations*). Further, it has been found that highly purified preparations of tail plates of T4D contain the enzyme dihydrofolate reductase. Studies on this enzyme from mammalian sources by Hillcoat, Perkins, and Bertino (18) show both that there is a tryptophan residue near the active site and that this enzyme undergoes conformational changes upon binding substrate and cofactors. The corresponding tryptophan residue of the dihydrofolate reductase in the phage tail plate is an attractive possibility for the critical endogenous tryptophan residue found in this study.

ACKNOWLEDGMENTS

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