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The Reaction of Indole and T2 Bacteriophage*

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Many aromatic compounds bind to bacteriophage T2 and prevent adsorption of the virus to its host cell. Indole and iodobenzene are the most active of a large number of substances tested. All active compounds are either π- or n-electron donors, and their ability to inhibit adsorption is correlated with their ability to form molecular charge-transfer complexes. Indole and other inhibitors cannot react with all of the alternative forms which T2 particles can assume in solution. The phage particles normally participate in a rapid equilibrium between a state in which they can adsorb to *E. coli* B ("active state"), and a state in which they cannot adsorb ("inactive state"). Indole reacts only with the nonadsorbing form of phage, and fixes the particles in the inactive form. The change from active to inactive phage results from a change in the tail-fiber configuration of the T2 particle from an extended to a nonextended state. This alteration of the fibers also affects the hydrodynamic behavior of T2. Bacteriophage T2H in the active state has a velocity sedimentation coefficient 10–15% smaller than it has in the inactive state.

Anderson (1945, 1948) has shown that a medium containing L-tryptophan is required for the attachment of T4 bacteriophage to its host, *Escherichia coli*. The ability of a number of tryptophan derivatives and analogs to substitute for tryptophan was studied (Anderson, 1946), and it was concluded that the NH_3^+, COO^-, and indole moieties of the molecule were all involved in the reaction with T4. Delbrück (1948) subsequently found that the activation of T4 by L-tryptophan was prevented if indole were also present, presumably by competition of the indole with the corresponding part of the tryptophan molecule.

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Bacteriophage T2 does not require tryptophan for adsorption, but indole was observed to inhibit adsorption of a strain of T2 known as T2H (Hershey and Davidson, 1951). We have found that indole will also inhibit T2L, another strain of T2.

The reaction of T2 with indole (like that of T4 with tryptophan) affects the initial attachment of the phage to *E. coli* B. The indole reaction must involve the tail fibers of the phage, since these have been shown in two types of experiments to contain the sites of attachment to the host: (1) Isolated fibers were found to adsorb to host cells and retain their host specificity (Williams and Fraser, 1956); and (2) adsorption of T2 to *E. coli* B was found to be interrupted by a serum which was largely antifiber in activity (Franklin, 1961).

The phage-indole or phage-tryptophan reaction also has been shown by immunological experiments to involve another component of the virus, the contractile sheath-protein. After reaction of phage with sheath antisera, T4 no longer has a tryptophan requirement for adsorption (Jerne, 1956), and indole no longer inhibits the adsorption of T4 or T2 (Jerne, 1956, Brenner *et al.*, 1962).

These observations on the role of both sheath and tail fibers have led to the hypothesis by Brenner *et al.* (1962) that: (a) T2 particles in their normal state and T4 particles which have reacted with tryptophan

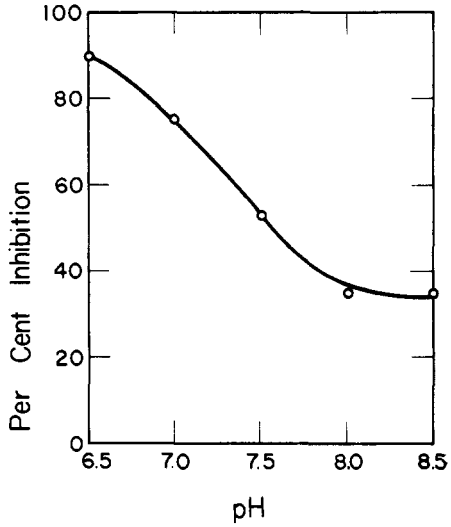


FIG. 1.—Per cent inhibition of T2H adsorption as a function of *p*H. Phosphate or Tris buffer at ionic strength 0.15 was used. Standard procedures given under Methods and Materials were employed.

can adsorb because their tail fibers are extended and available for attachment to the host cell; and (b) T2 particles which have reacted with indole and T4 particles with no tryptophan added (or with tryptophan *and* indole added) cannot adsorb because their tail fibers are nonextended, i.e., bound to a component of the virus itself.

The experiments described in this paper are concerned with three main aspects of phage-cofactor interaction: (a) the effect of the T2-indole reaction on the biological properties of the phage, i.e., on its ability to adsorb to *E. coli* B, and its susceptibility to $Cd(CN)_3^-$ inactivation; (b) the effect of indole on the velocity sedimentation coefficient of T2H; and (c) the chemical nature of the binding of indole and other inhibitors to T2.

METHODS AND MATERIALS

Purification of Compounds Used.—The aromatic substances used (mostly obtained from commercial sources) were purified by recrystallization and/or distillation so that their spectra agreed with those given in the literature. Pyridyl pyridinium chloride·HCl was decolorized in hot, dilute HCl solution using acid-washed Norit A, evaporated to dryness, dissolved in warm ethanol, and precipitated by addition of ethyl ether. Benzothiophene was a gift of W. E. Parham.

Bacteriophage Preparation.—Genetically homogeneous T2H was isolated from a single plaque from a stock of A. D. Hershey, and was prepared and assayed by standard procedures (Adams, 1959a). The T2L used was initially received from S. E. Luria several years ago. Anti-T2 serum was the gift of O. Maaløe. As used in the standard inhibition experiment described below it inactivated T2 at a rate corresponding to $K = 1.7\ \text{min}^{-1}$.

Standard Inhibition Experiment: Determination of the Effectiveness of Various Compounds as Inhibitors.—A concentrated stock of purified bacteriophage T2H was diluted with a preincubation mixture of diluting fluid (0.025 M sodium diethylbarbiturate, *p*H 7, + 0.125 M NaCl + 0.001 M $MgSO_4$), or inhibitor dissolved in diluting fluid, to a concentration of 2×10^8 T2H/ml. After several minutes, 1 volume (usually 0.1 ml) from this preincubation mixture was mixed with 19 volumes of a solution which had the same inhibitor concentration and also contained 2×10^7 *E. coli* B/ml. Adsorption of the phage to the bacteria began at the time of mixing. The bacteria had been previously grown to about 5×10^8/ml in nutrient broth at 37°, sedimented and washed in diluting fluid four times, concentrated and added to the incubation mixture 1 minute before the phage was added from the preincubation mixture. The temperature of the preincubation and incubation mixtures was 30°. A maximum of 8% of the phage was adsorbed in 5 minutes. At 1 and 2 minutes after adsorption began aliquots were removed from the incubation mixture and put into solutions of T2 antisera. The sera irreversibly inactivated nonadsorbed phage particles so that they could not subsequently infect cells, but did not damage cells which had already been infected. After 10 minutes further dilutions were made, the samples were plated on nutrient agar with *E. coli* B, and the number of infected cells formed during the incubation period was determined from the number of plaques.

A compound was tested as an inhibitor in this way at several concentrations, and the effect of at least two concentrations of indole was determined at the same time. Although there was a small variation from experiment to experiment in the amount of indole required for 50% inhibition, the *relative* effectiveness of an inhibitor was reproducible; i.e., the ratio: $[\text{inhibitor}]_{50\%\ \text{inhibition}}/[\text{indole}]_{50\%\ \text{inhibition}}$ remained constant.

Known amounts of the relatively water-insoluble inhibitors, such as benzene and its derivatives, were added to the diluting fluid several hours before the addition of virus or bacteria. The tightly stoppered containers were shaken at room temperature until the organic compounds were completely dissolved. It was possible to prepare solutions containing, for example, 5×10^{-4} M toluene (monomethyl benzene) and 10^{-4} M xylene (dimethyl benzene).

Determination of Association Constants with Chloranil as the Acceptor.—Association constants for the formation of complexes by aromatic electron donors with π-electron acceptor compounds were determined by measurement of the light absorption of the complexes (Benesi and Hildebrand, 1949) in the following manner. Solutions of the donor compound were made in 0.002 M chloranil in butyl ether. The donor concentration was varied (at least three concentrations were used) but was always in great excess over 0.002 M, the concentration of the chloranil. All solutions were made up under a red safelight; under these conditions the colors of the complexes formed remained stable for several hours. The absorbance of each chloranil-donor solution was read at a wavelength greater than 480 mμ, where the 0.002 M chloranil does not absorb appreciably. The association constant K_{assoc} was calculated from:

$$K_{\text{assoc}} = \frac{[\text{complex}]}{[\text{donor}]([\text{chloranil}]_{\text{initial}} - [\text{complex}])}$$

Substituting [complex] = absorbance/ϵ, where ϵ is the molar absorbance of the complex, and rearranging this expression, one obtains:

$$\frac{1}{[\text{donor}]} = \frac{1}{\text{absorbance}}(K_{\text{assoc}}\,\epsilon\,[\text{chloranil}]_{\text{initial}}) - K_{\text{assoc}}$$

A graph of 1/[donor] as a function of 1/absorbance gave a straight line, and its vertical intercept gave the negative of K_{assoc}.

Determination of Association Constants with Pyridyl Pyridinium Chloride·HCl as the Acceptor.—An aqueous medium was used as solvent: 0.025 M sodium diethyl-

barbiturate + 0.125 M NaCl + 10^{-3} M $MgSO_4$; this was the diluting fluid of the standard absorption experiments. The procedure was that described above for the butyl ether systems, except that the acceptor was present in excess, and its concentration was varied, while the donor was kept at a low fixed concentration. The association constant was then determined by the equation given above, but with the role of donor and acceptor reversed.

The complexes were yellow, and absorbances were therefore read in the 400–500 mμ region. Blanks were subtracted for the substantial absorbance of the pyridinium compound at these wavelengths. The donor was present in 0.02 M concentration, giving a blank value for absorbance of 0.00–0.02 at the wavelengths indicated; no correction was made for it.

Velocity Sedimentation-Coefficient Determination.— Velocity sedimentation coefficients for T2H were obtained using a Spinco Model E analytical ultracentrifuge and employing standard procedures given by Schachman (1959).

Results

Although the effect of indole was first observed in a system containing the bacterial host as well as the virus particles, it has since been established that indole reacts with the T2 particles themselves. Brenner *et al.* (1962) showed that the T2H-indole reaction protects T2H from $Cd(CN)_3^-$ inactivation. Further, the sedimentation coefficient of T2H increased in the presence of indole (see below) at concentrations which would also have been effective in inhibiting phage adsorption. Therefore inhibition is believed to be entirely due to the phage-indole reaction, not to an interaction of indole with *E. coli* B.

Addition of indole to an incubation mixture reduced the number of infectious centers formed by T2H and its host cell by reducing the extent of initial attachment of the phage to the cell. No subsequent reaction necessary for infection (Kozloff, 1960) was affected; the number of infected cells formed during the incubation of T2H with a large excess of bacteria, plus the number of phage remaining in the supernatant after removal of the bacteria by centrifugation, remained constant in the presence or absence of indole, and equaled the total number of T2H particles added.

Effect of Indole on Adsorption and Inactivation of T2H and T2L

Conditions Affecting the Inhibition Reaction of T2H.— Indole and other inhibitors became more effective in preventing the adsorption of T2H to *E. coli* B when the incubation conditions were altered by one of the following procedures: increasing the divalent cation concentration, decreasing the monovalent cation concentration, lowering the temperature, and lowering the *p*H. All of these environmental changes also caused the adsorption rate to decrease, even with *no* inhibitor added. This is illustrated by the effect on adsorption and inhibition of the addition of Mg^{++} to the incubation medium. At *p*H 7, 27°, ionic strength 0.15, and in the absence of divalent ions, the adsorption rate of T2H to the host cell was 2.6×10^{-9} $cm^3 min^{-1}$. The addition of 10^{-3} M Mg^{++} caused the rate to drop to 1.6×10^{-9} $cm^3 min^{-1}$. The effect of an inhibitor on the reaction rate was very small under the conditions of rapid adsorption, but greatly increased with the addition of Mg^{++}. Table I shows the inhibition obtained with two inhibitors, indole and benzene, with or without Mg^{++} added.

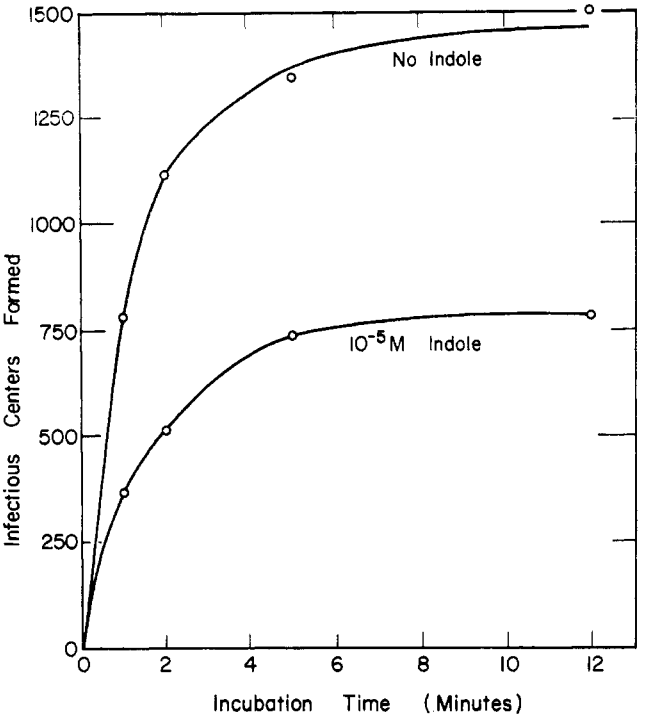


FIG. 2.—Adsorption of T2H to *E. coli* B in the presence and absence of indole. Incubation mixture: 0.125 M NaCl, 0.025 M sodium diethylbarbiturate, 0.001 M $MgSO_4$, HCl to *p*H 7; *E. coli* B at 5×10^8/ml, T2H at 10^8/ml.

TABLE I
EFFECT OF ADDED Mg^{++} ON INHIBITION OF T2H ADSORPTION[a]

Inhibitor	$[Mg^{++}]$	Inhibition (%)
5×10^{-6} M indole	0	0
	10^{-3} M	41
5×10^{-3} M benzene	0	18
	10^{-3} M	75

[a] Incubation mixture: 0.125 M NaCl, 0.025 M sodium diethylbarbiturate, Mg^{++} as indicated, HCl to *p*H 7; *E. coli* B at 2×10^7/ml; T2H at 10^7/ml.

Figure 1 shows that lowering the *p*H similarly increased the effectiveness of a given concentration of indole.

Kinetics of T2H Adsorption in the Presence of Indole.— We had expected the reason for the decrease in the number of *E. coli* B infected with T2H in an indole solution to be a decrease in the rate of adsorption of the phage. However, this was not the case. Instead it was found (see Figure 2) that some of the phage particles (about 48% in the case illustrated) did not adsorb, regardless of the interval in which they were exposed to the cells, while most of the remainder adsorbed at about the same rate as they would have adsorbed in the absence of indole. The particles which could not adsorb were not inhibited by a component formed in the reaction medium during the 12 minutes of incubation. Figure 3 shows that when a new aliquot of T2H was added to the incubation mixture 6 minutes after the first aliquot, the adsorption curve was repeated. Further, when an aliquot was removed from the incubation mixture in which adsorption had stopped, into an identical bacteria-indole mixture, adsorption still did not occur. The inability of some of the phage particles to adsorb in an indole solution

TABLE II

HETEROGENEITY OF T2H WITH RESPECT TO SENSITIVITY TO LOW INDOLE CONCENTRATIONS[a]

Experiment	Indole Concentration ($\times 10^{-5}$ M)	Incubation	Phage Concentration in Incubation Mixture (per ml)	Phage Unadsorbed after 5 Minutes (%)
I	1	(1) Initial	1.1×10^8	58
		(2) With nonadsorbed phage from (1)	2.6×10^3	81
II	1	(1) Initial	1×10^8	56
		(2) With nonadsorbed phage from (1)	2.4×10^3	80
III	0.7	(1) Initial	8×10^8	31
		(2) With nonadsorbed phage from (1)	8.2×10^5	78
		(3) With nonadsorbed phage from (2)	2.1×10^3	66
IV	0.7	(1) Initial	1.2×10^6	38
V	0.7	(1) Initial	3×10^3	36

[a] *E. coli* B at 5×10^8/ml in exper. I and II; at 2×10^9/ml in exper. III, IV, and V.

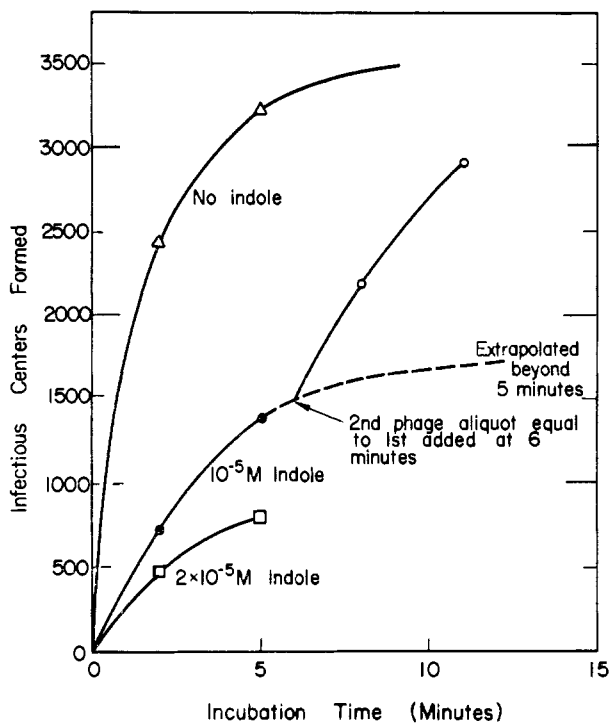


FIG. 3.—Adsorption of T2H to *E. coli* B with 0, 10^{-5} M, and 2×10^{-5} M indole. Incubation mixture: 0.125 M NaCl, 0.025 M sodium diethylbarbiturate, 0.001 M $MgSO_4$, HCl to *p*H 7; *E. coli* B at 5×10^8/ml, T2H at 3×10^7/ml.

was thus a property of these phage particles themselves, and not of the adsorption medium.

It was not entirely surprising that almost all of the T2H was, in the presence of indole, divided into two stable populations, one containing phage particles which can adsorb (active phage), and the other phage particles which cannot adsorb (inactive phage). The fact that there is very little interconversion of these forms in the presence of indole is confirmed by the observation that T2H particles sediment in two distinct peaks in indole solution. However, when the indole concentration was altered the fraction of the phage which was active or inactive was instantaneously altered. Since no simple equilibrium existed between these forms, the response could not be explained by a "mass action" effect on a single equilibrium. The following alternative explanation was therefore considered. The number of inactive phage particles increased when the indole concentration increased because the phage particles varied in their sensitivity to indole. To investigate whether T2H are heterogeneous, as the foregoing implies, a "successive-incubation" experiment was used; phage particles which did not adsorb in one incubation were separated from the host cells and diluted out of indole, then put back into another indole-bacteria mixture to see if they were more susceptible than the original population to inhibition by the same concentration of indole. The results are summarized in Table II. Using 10^{-5} M indole, the fraction of inactive phage (phage in the supernatant) increased in experiment I from 58% in the first incubation to 81% in the second, and in experiment II from 56% to 80%. Using a lower indole concentration, three successive incubations were performed (experiment III), yielding 31% inhibition in the first, 78% in the second, and 66% in the third. Control experiments (experiments IV and V) showed that the increase in inhibition obtained in the two final incubations of experiment III was not an artifact due to the use of a lower phage concentration. Since the phage stock used was prepared from a single-plaque isolation, it is unlikely that the heterogeneous response to indole found in these experiments is of genetic origin. The nature of the heterogeneity is not clear.

Inhibition of T2L by Indole.—The adsorption of the strain T2L has been reported to be unaffected by indole (Adams, 1959b), and we have confirmed this in adsorption experiments using typical conditions for the incubation of phage with host cells. However, when these conditions were altered in the directions which had been found to increase the inhibition of T2H, inhibition of T2L could also be observed. For example, T2L adsorption was inhibited by indole at temperatures in the range of 4–6°. Table III shows that inhibition also became greater as the *p*H was lowered: 2×10^{-3} M indole was effective at *p*H 6.7, and 2×10^{-5} M indole at *p*H 5.85. It should be noted, however, that all experiments reported in this paper, except those given here, were performed using T2H.

Indole Protection of T2H from $Cd(CN)_3^-$ Inactivation.—We have found that indole is less effective in protecting T2H against $Cd(CN)_3^-$ inactivation (Brenner *et al.*, 1962) when $Mg(NO_3)_2$ is omitted from the standard $Cd(CN)_3^-$ inactivating solution (Kozloff and Lute 1957). The addition to the reagent of either 10^{-3} M $Mg(NO_3)_2$ or 10^{-3} M $CaCl_2$ restored the protective action of the indole, an effect parallel to the divalent ion requirement for indole inhibition of adsorption. In Figure 4 the action of Mg^{++} on inactivation and protection is shown; Mg^{++} did not significantly affect

TABLE III
EFFECT OF *p*H ON THE INHIBITION OF T2L ADSORPTION BY INDOLE[a]

*p*H	Indole	Infectious Centers Formed in 1 Minute
5.58	0	30
	2×10^{-5} M	10
	2×10^{-3} M	1
6.7	0	632
	2×10^{-5} M	514
	2×10^{-3} M	117
7.55	0	652
	2×10^{-5} M	724
	2×10^{-3} M	513

[a] Incubation mixture: phosphate buffer at ionic strength 0.1; $[Mg^{++}] = 0.67 \times 10^{-4}$ M; $T = 21°$.

the inactivation rate, but increased the protection afforded by indole to the phage.

Effect of Indole on the Sedimentation of T2H

Velocity sedimentation coefficients were obtained for T2H at *p*H 7.5 and 5.9, in phosphate buffer alone in buffer containing 3.3×10^{-5} M indole. Single peaks were obtained in both cases. At both *p*H values this concentration of indole caused 100% inhibition of T2H adsorption to *E. coli* B. The sedimentation coefficient at *p*H 7.5 increased in the presence of indole from 903 to 1012, while at *p*H 5.9, both with and without indole, the "fast" form ($s_{20,w} \approx 1040$) was obtained (Table IV).

TABLE IV
SEDIMENTATION OF T2H IN THE PRESENCE AND ABSENCE OF INDOLE[a]

*p*H	Indole ($\times 10^{-5}$ M)	Sedimentation Coefficient ($s_{20,w}$)
7.5	0	903
	3.3	1012
5.9	0	1049
	3.3	1038

[a] T2H used was a preparation dialyzed for 2 days against 0.15 M NaCl + 10^{-5} M $MgSO_4$, adjusted to *p*H 7. Sedimentation conditions: T2H at 2×10^{12}/ml in phosphate buffer at indicated *p*H, ionic strength 0.10; $[Mg^{++}] = 0.67 \times 10^{-5}$ M; $T = 24.5°$.

Figure 5 shows the sedimentation of T2H at *p*H 7.4 in buffered NaCl solution alone, or in 10^{-5} M or 10^{-4} M solutions of indole. The comparable adsorption experiment for 10^{-5} M and 10^{-4} M indole gave 46% and 100% inhibition, respectively. At a low indole concentration the T2H can be seen in the figure to have sedimented in two forms; in the absence of indole only the slower-sedimenting form was present ($s_{20,w} = 945$), and in the presence of sufficient indole to inactivate all of the phage for adsorption only the fast-sedimenting form ($s_{20,w} = 1091$) was present.

The Chemical Nature of the Binding of Indole to T2

Examples of inhibitors and noninhibitors of T2H adsorption are given in Table V. No common functional group could be identified as necessary for the reaction with the phage particle. Further, benzene, with no functional groups, was an effective inhibitor. It was noted that these aromatic inhibitors had in

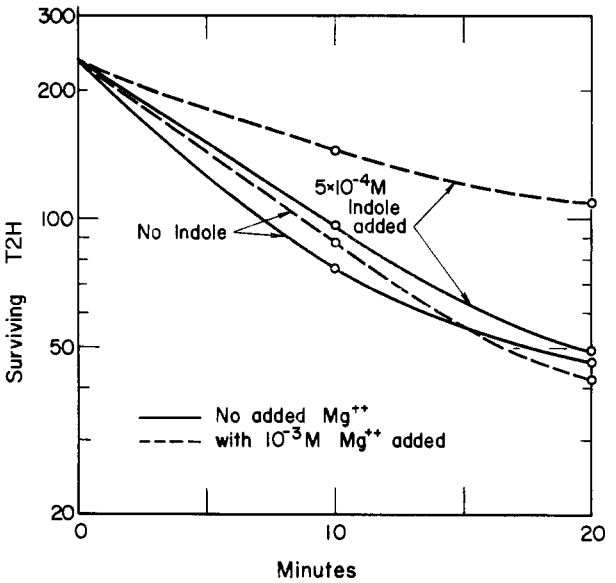


FIG. 4.—Effect of addition of Mg^{++} and indole on Cd-$(CN)_3^-$-inactivation of T2H. The $Cd(CN)_3^-$ reagent contained: 0.138 M $Cd(NO_3)_2$, 40.0 ml; 0.20 M NaCN, 86.4 ml; 0.30 M $NaNO_3$, 200 ml; 0.025 M $Mg(NO_3)_2$, 20 ml; when the $Mg(NO_3)_2$ was omitted an additional 5 ml of 0.30 M $NaNO_3$ was added. The solutions were adjusted to *p*H 7.7 with HNO_3 and diluted with H_2O to a total volume of 500 ml. The phage were diluted 1–10 with the $Cd(CN)_3^-$ reagent.

TABLE V
ACTIVITY OF AROMATIC COMPOUNDS AS INHIBITORS OF T2H ADSORPTION

Inhibitors		
Single-Ring Compounds	Indole Derivatives	Larger Fused-Ring Heterocycles
Benzene	D- or L-Tryptophan	Chlorpromazine
Toluene	5-Hydroxy tryptamine (serotonin)	Lysergic acid diethylamide tartrate (LSD-25)
Bromobenzene	*N*-Acetyl indole	
Aniline	2-Methyl indole	
Noninhibitors		
Pyrrole		Purine
Pyridine		Folic acid
	DPN^+	

common the ability to act as electron-donors in the formation of molecular charge-transfer complexes (Mulliken, 1952). That is, each has the property of reacting reversibly with an electron acceptor to form a complex of the two molecules which is stabilized by the transfer of a nonbonding electron from an orbital of the electron-donor to an overlapping vacant orbital of an electron-acceptor molecule. Compounds are classified as π-donors or *n*-donors, according to whether the electron transferred is a π-electron of the donor ring-system or a localized *p*-electron of one atom of the donor molecule. If the formation of a charge-transfer complex is responsible for the inhibition reaction, it must also be true that in order to bind to the acceptor molecule on the phage only one orientation of the aromatic ring system of the donor, relative to the phage surface, is permitted. In this orientation the donor may act as a π- or *n*-electron donor, but only if the π- or *p*-orbital containing the electron to be transferred is oriented so that it overlaps the vacant orbital of the acceptor molecule on the phage. Pyridine

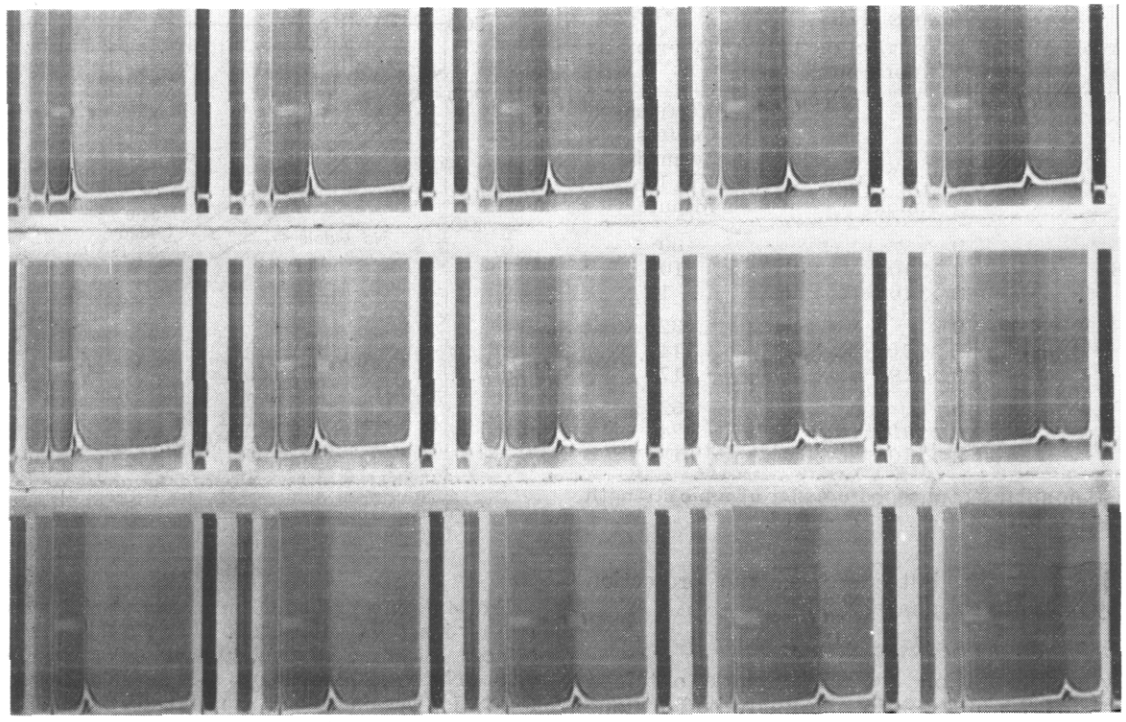


FIG. 5.—Sedimentation of T2H in the presence and absence of indole. Upper frames: [indole] = 0, $s_{20,w}$ = 942; middle frames: [indole] = 10^{-5} M, $s_{20,w}$ = 949, 1122; lower frames: [indole] = 10^{-4} M, $s_{20,w}$ = 1091. Sedimentation conditions: T2H at 2 × 10^{12}/ml (by phosphorus determination); 0.09 M NaCl + phosphate buffer at *p*H 7.4 and ionic strength 0.01; [Mg^{++}] = 0.6 × 10^{-5} M; T = 24.5°.

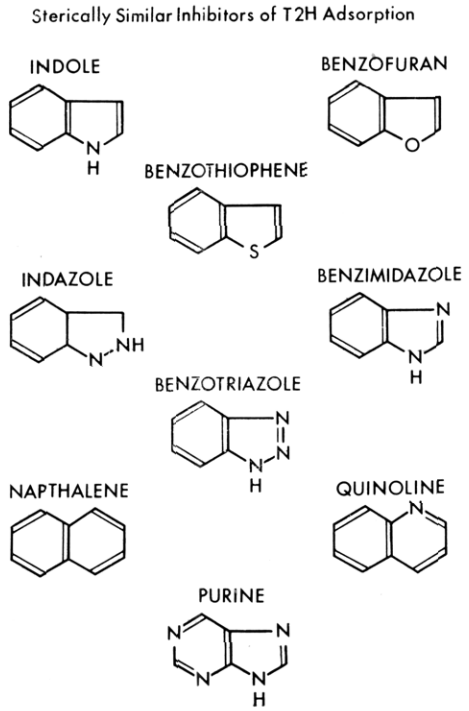


FIG. 6.—Sterically similar heterocyclic compounds which were tested to determine their effectiveness as inhibitors of T2H, and ability to act as π-electron donors.

and benzene provide an example of the effect of this steric requirement. Benzene is an inhibitor, and can be one only by virtue of π-electron donation, since it contains no mobile *n*-electrons. Pyridine, on the other hand, is not an inhibitor, though it is a good *n*-donor. Given the same "fit" to the phage particle as benzene, the *p*-orbital of its nitrogen atom is not oriented so as to permit charge transfer. Although π-electron donation *is* sterically permissible, pyridine is too weak a π-electron donor to be an inhibitor.

To test the hypothesis that charge-transfer complexes are responsible for the binding of inhibitor molecules to T2, three series of compounds were examined to ascertain whether a correlation exists between their effectiveness as inhibitors of T2 adsorption and their effectiveness as electron donors.

Comparison of Inhibitor and Donor Properties of π-Electron Donors.—Compounds which are sterically similar to each other were selected for comparison since it was found from experiments with substituted indoles that all side chains reduced inhibitor effectiveness. Figure 6 shows the nine compounds which were compared as adsorption inhibitors and as donors. Their inhibitor effectiveness was determined as a function of concentration (Figure 7). Using the concentration for 50% inhibition as the criterion, the activity of a substance relative to indole was determined (Table VI). Extent of association with a common acceptor was measured for the same group of compounds, using chloranil as the acceptor, with butyl ether as solvent, for all the donor compounds which were soluble in this system at sufficiently high concentration (Table VI). These included indole, benzothiophene, benzofuran, naphthalene, and quinoline. Indazole is too polar to be measured in this way, but an association constant was obtained in an aqueous system, using pyridyl pyridinium chloride·HCl as acceptor. The aqueous system was also used for indole, the reference compound, and for benzofuran. Benzotriazole, benzimidazole, and purine are also too polar to use in the butyl ether system. However, in the aqueous system it was impossible to determine their association constants since the absorption spectra of the complexes (if any) were in the ultraviolet where the pyridinium compound itself absorbed very strongly.

Inhibition of T2H Adsorption by n-Electron Donors.—Halogenated benzenes can be expected to be excellent *n*-donors since their ionization potentials are low, rela-

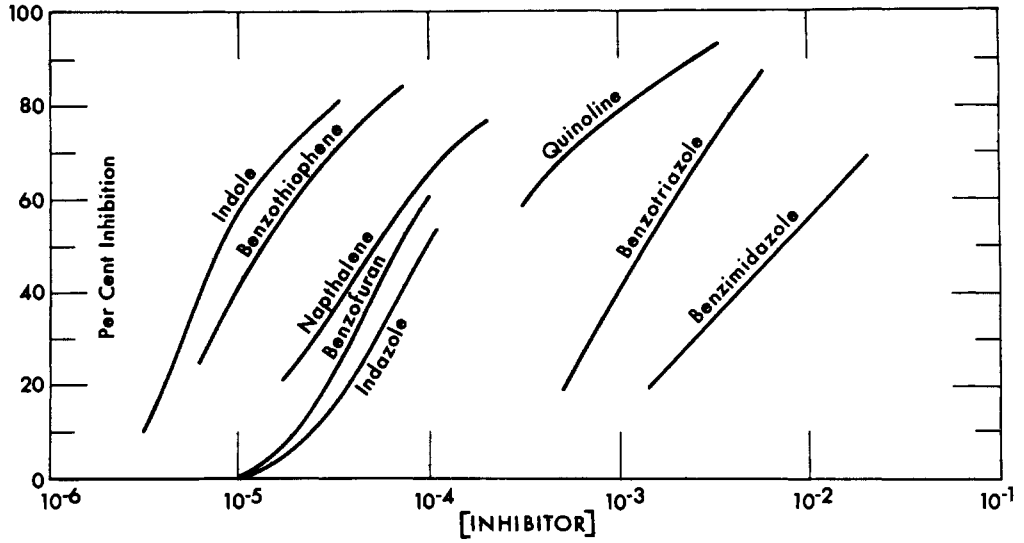


FIG. 7.—Inhibition of adsorption of T2H to *E. coli* B as a function of inhibitor concentration for the nine sterically similar heterocycles given in Figure 6.

TABLE VI

INHIBITOR EFFECTIVENESS AND ASSOCIATION CONSTANTS FOR STERICALLY SIMILAR COMPOUNDS

Compound	Relative Inhibitor Effectiveness[a]	Relative Association Constant	Color in Butyl Ether
Indole	1.0	1.0	Purple
Benzothiophene	0.4	0.33 ± 0.06	Yellow
Napthalene	0.2	0.31 ± 0.03	Orange
Benzofuran	0.1	0.10 ± 0.05 0.06 ± 0.03[b]	Yellow
Indazole	0.08	0.26 ± 0.05[b]	—
Quinoline	0.04	0.31 ± 0.05	Yellow
Benzotriazole	0.006		
Benzimidazole	0.001		
Purine	<0.0001		

[a] Accurate to the significant figure shown. [b] Aqueous system.

tive to benzene. Further, ease of electron donation increases as the molecular weight of the halogen substituent increases and the ionization potential becomes progressively lower (Watanabe, 1957). Table VII shows that the extent of inhibition of T2H also follows this order: bromobenzene was very effective relative to benzene, while iodobenzene was even more active, and was, in fact, the only compound tested which was as effective as indole as an inhibitor.

Effect of Steric Hindrance on Inhibition of Adsorption.—When an aromatic donor and an acceptor molecule form a complex, any substituent on the donor which increases the distance of closest approach to the donor ring by the acceptor will reduce the extent of π-electron transfer. A methyl substituent will not alter the distance of closest approach to a benzene ring; this remains the same for methylbenzene as for benzene, 3.5 A. But ethylbenzene has a larger distance of closest approach, about 5 A (Merrifield and Phillips, 1958). Therefore, complex formation is impeded for ethylbenzene relative to methylbenzene, and the effect is magnified if there is more than one ethyl group on the aromatic ring. (Merrifield and Phillips [1958] found that the association constant for hexamethyl benzene was fifty-one times greater than that for hexaethylbenzene using a common acceptor, tetracyanoethylene).

A comparison was made of the inhibitory effect on T2H adsorption of (a) methyl- and ethylbenzene, and (b) *m*-dimethyl- and *m*-diethylbenzene (Table VIII). Inhibition was found to decrease with increasing size of the alkyl substituent, as would be expected on the basis of the steric interference discussed above: methylbenzene is three times more effective than ethylbenzene, and *m*-dimethylbenzene is about ten times more effective than *m*-diethylbenzene. (The inductive effect of one or two methyl or ethyl substituents is also evident; the alkyl groups are electron-repelling, and their substitution in a benzene ring therefore increases its electron density. Methylbenzene is thus six times more effective than benzene, while *m*-dimethylbenzene is five times more effective than methylbenzene, or thirty times more effective than benzene.)

TABLE VII

RELATIVE INHIBITOR EFFECTIVENESS OF BENZENE AND HALOGENATED BENZENES[a]

Compound	Concentration for 50% Inhibition ($\times 10^{-5}$ M)	Relative Effectiveness
Benzene	210	1
Bromobenzene	6	35
Iodobenzene	0.5	400

[a] The standard inhibition procedures and media given in the text were used.

TABLE VIII

THE INHIBITION OF T2H ADSORPTION BY ALKYL-SUBSTITUTED BENZENES[a]

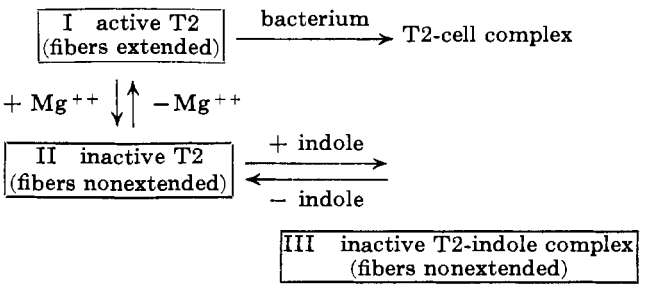
Compound	Concentration for 50% Inhibition ($\times 10^{-4}$ M)	Relative Effectiveness
Benzene	21	1
Methylbenzene (toluene)	3.5	6.0
m-Dimethylbenzene (xylene)	0.7	30
Ethylbenzene	10	2.1
m-Diethylbenzene	7.0	3.0

[a] The standard inhibition procedures and media given in the text were used.

Discussion

Two reactions of bacteriophage T2 are affected by certain environmental conditions such as high divalent cation concentration, low monovalent cation concentration, low *p*H, and low temperature: (1) the adsorption rate of the T2 to *E. coli* B is reduced; and (2) the phage particle reacts more readily with indole, and is then fixed in a configuration in which it cannot adsorb to *E. coli* B, and cannot be inactivated by $Cd(CN)_3^-$.

A relationship between these reactions is evident if the equilibria shown below are assumed to govern the interconversion of T2 which can adsorb ("active T2"), and T2 which cannot ("inactive T2"). (In these equilibria the effect of Mg^{++} on the interconversion of the forms is shown. However, increased $[H^+]$ or lowered temperature could equally well have been used to illustrate the shifting of the equilibrium I $\rightleftharpoons$ II toward the inactive form of the phage.)



Since the reaction of the T2 sheath with antiserum eliminates the inhibition of T2 adsorption by indole (Brenner *et al.*, 1962), it appears that these particles cannot assume the inhibited state unless certain sheath sites are free. From this, Brenner *et al.* (1962) hypothesized that a T2 particle which is inactive because of reaction with indole is one which has its tail fibers bound to the sheath (form III in the equilibria). We now assume, further, that phage particles which are inactive, not because of reaction with indole but because of ionic conditions or low temperature, also have nonextended fibers (form II), and that the phage must have this form in order to react with indole.

The relationship between the three T2 states given in the equilibria and the sedimentation behavior which we have observed for T2H (Table IV) appears to be as follows: (1) At *p*H 7.5, under the conditions indicated in Table IV, T2H particles adsorb rapidly and therefore are predominantly in the form with extended fibers (form I). The phage sediments with $s_{20,w} \approx 900$ at this *p*H; therefore this is the sedimentation coefficient which characterizes active phage. (2) At *p*H 5.9 T2H particles adsorb at a greatly reduced rate, and are predominantly in the inactive form (form II), so that the sedimentation coefficient at this *p*H of $s_{20,w} \approx 1025$ is characteristic of inactive phage. (3) Inactive phage which has reacted with indole has been assumed to have a configuration similar to inactive phage in the absence of indole, and so should show similar sedimentation behavior. In agreement with this, at both *p*H 7.5 and 5.9, phage particles in a solution of high indole concentration were found to sediment with $s_{20,w} \approx 1025$.

It is assumed in the equilibria given that only inactive phage can react with indole. The basis for this assumption is the very poor inhibition obtained with indole under conditions when the phage particles have a rapid rate of adsorption, i.e., when the particles are essentially all active.

One perplexing phenomenon is that at an indole concentration giving partial inhibition, the T2H population appears to be heterogeneous with respect to adsorption properties. Some phage particles adsorb to *E. coli* B at a rate approximately the same as that of phage in the absence of indole, while the remaining particles adsorb very slowly, if at all (Figures 2 and 3). This indicates that inactive phage particles are not all identical, and that their differences are reflected in the rate at which they react with indole (i.e., the rate of the reaction II → III). The existence of such differences is not surprising in view of the fact that there are six tail fibers which probably can be bound to the main portion of the virus in a variety of ways. Those inactive particles for which the reaction with indole is very fast compared to the rate at which they become active (II → III fast compared to II → I) remain inactive all of the time (in form II or III) and sediment at the rate characteristic of these forms, i.e., $s_{20,w} \approx 1025$. For those which bind indole poorly, the reaction II → III must be slow relative to II → I. These phage particles adsorb to bacteria at about the same rate in the presence and absence of 10^{-4} M indole and they are predominantly in the active form and sediment with $s_{20,w} \approx 900$. These assumptions can account for the dual sedimentation of T2H in the presence of a low concentration of indole, some phage sedimenting with $s_{20,w} \approx 900$, some with $s_{20,w} \approx 1025$. (It must be added that the division of the phage population into two categories, particles highly sensitive to indole at a certain concentration and particles totally insensitive, is obviously an oversimplification, in that there must be a small fraction of the population having intermediate reactivity with indole. In adsorption experiments these are probably responsible for residual adsorption at a slow rate, too slow to be seen, for example, in Figure 2. Similarly, in sedimentation experiments in indole solution, only the highly indole-sensitive fraction and the highly indole-insensitive fraction of the T2H population sediment as distinct forms with essentially no interconversion, but phage particles of intermediate sensitivity would most likely also be present, though they would be too few and inhomogeneous to be seen.)

It is not possible to compute an association constant from adsorption experiments for the binding of indole to T2H, since it cannot be determined what fraction of the inactive phage particles have indole bound to them. Attempts at direct chemical measurement of an association constant have been unsuccessful. However, the equilibrium II $\rightleftharpoons$ III goes measurably to the right, as detected in inhibition and sedimentation experiments, at concentrations of indole (or iodobenzene) of the order of 10^{-5} M. This means that the phage surface at the site of reaction must be a region highly suitable to complex formation, and possibly is a nonpolar region, since the energy for charge-transfer is greatly reduced in such an environment (Kosower, 1962).

The equilibria proposed for the forms I, II and III also explain the absence of inhibition for T2L under conditions (*p*H 7, 30°, $\mu = 0.10$, 10^{-3} M Mg^{++}) which are favorable for T2H inhibition. The adsorption rate of T2L is not decreased by this concentration of Mg^{++}, presumably because the active form is less readily converted to the inactive form than in the case of T2H. The absence of the inactive form under these conditions accounts for the lack of response to indole. When conditions of reduced adsorption do occur, and presumably the inactive form exists, then inhibition is observed.

Sedimentation data bearing on various forms of the phage particle have been previously reported for T2L bacteriophage. Cummings and Kozloff (1960, 1962)

have shown that T2L sediments in two distinct stable forms, a slow one with $s_{20,w}$ of about 700, and a fast one with $s_{20,w}$ of about 1000. This 40% change in sedimentation rate has been shown to be due to a change in phage head-shape and to a simultaneous alteration in porosity of the phage head. For T2H, under the conditions used in this investigation, all variation in sedimentation coefficients is believed to be due to tail-fiber effects. While the inactive form of T2H appears to correspond in sedimentation behavior to the fast form of T2L ($s_{20,w}$ = 1000), no evidence for a slow form of T2H with sedimentation coefficient of 700 S, like that noted for T2L, was observed. Bendet *et al.* (1957) have claimed that the entire 40% change in the sedimentation coefficient of T2L is due to a change in tail-fiber configuration. Their main evidence comes from electron micrographs of T2 (presumably T2H, not T2L) with tail fibers extended or not extended. In view of the convincing evidence that for T2L head changes are the major cause of the 40% change (Cummings and Kozloff, 1960, 1962; Cummings, 1963), and the data in this paper that a change in tail-fiber configuration of T2H involves only a 10–15% change in sedimentation constant, it appears that this latter phenomenon was the one actually studied by Bendet and his colleagues.

The hypothesis has been proposed that the inhibition of T2H adsorption occurs as a result of the formation of a complex of an aromatic donor (inhibitor) with an acceptor (on the phage particle), and that, further, complex formation requires a specific orientation of the ring-system relative to the phage surface and the acceptor molecule on it. Indole, benzofuran, benzothiophene, and napthalene are good inhibitors; this is to be expected, since they are good π-donors (have a high ring electron density). Indole, benzofuran, and benzothiophene might also be anticipated to have n-donor properties, since the hetero atoms of these compounds have p-orbitals normal to the ring. However, the extent of orbital overlap with the acceptor would be poor compared to the overlap of their π-electron orbitals. Inhibitor effectiveness and association constants for these compounds are in reasonable agreement.

The validity of comparing effectiveness as inhibitor and association constant for members of a series of compounds depends upon consideration of at least three factors which influence the measurements: (1) Relative association constants change when the solvent changes (Merrifield and Phillips, 1958, Smith, 1955), so that a comparison of degree of molecular association in butyl ether solution with that at the reactive site of the bacteriophage can only be qualitative. (2) The effectiveness of a donor compound as an inhibitor depends not only on a high association constant for binding to an electron acceptor but also on a low energy requirement for electron transfer. Moreover, these factors do not entirely determine each other. The relative association constants are almost the same for the napthalene and benzothiophene, but the energy requirement for electron transfer to chloranil is smaller for napthalene than for benzothiophene, as evidenced by the longer wavelength of absorption of the napthalene-chloranil complex. (3) An inhibitor may have both n-donor and π-donor properties. (This is true for benzothiophene, but not for napthalene.) The energy for n-electron transfer, as well as for π-electron transfer, is thus relevant to the effectiveness of an inhibitor.

Indazole and quinoline are somewhat poorer inhibitors than the first four compounds discussed above, probably because of the lowered π-electron density which results from substitution of electron-withdrawing nitrogens in their ring-systems. Although the association constants for indazole and quinoline are large compared to inhibitor effectiveness, these constants reflect the formation of complexes, stabilized by n-electron donation, which require an orientation not permitted in the inhibition reaction of these compounds with the phage acceptor.

Benzotriazole, benzimidazole, and purine had little or no inhibitor activity; again, this is predictable on the basis of the lowered π-electron density due to tertiary nitrogen substitution.

It can be concluded that the chemical and biological properties of this series of nine sterically similar compounds (and of the other two series tested) support the proposal that the formation of a charge-transfer complex is responsible for the binding of the inhibitor molecule to the phage particle.

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