MOLECULAR PHARMACOLOGY, 13, 706-718

The Inhibitory Effect of Phenanthridines on the Synthesis of Ribonucleic Acid Catalyzed by Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase

STELIOS AKTIPIS AND NIKOS PANAYOTATOS

Department of Biochemistry and Biophysics, Stritch School of Medicine, Loyola University of Chicago, Maywood, Illinois 60153

(Received November 16, 1976) (Accepted February 10, 1977)

SUMMARY

AKTIPIS STELIOS & PANAYOTATOS, NIKOS (1977) The inhibitory effect of phenanthridines on the synthesis of ribonucleic acid catalyzed by deoxyribonucleic acid-dependent ribonucleic acid polymerase. *Mol. Pharmacol.*, 13, 706-718.

The interaction of RNA polymerase with DNA prior to the initiation of RNA synthesis involves the formation of a specific complex (I) which is in equilibrium with a rapidly starting (RS) complex necessary for the rapid initiation of RNA chains. Phenanthridinium derivatives, including ethidium bromide, are effective inhibitors of RNA synthesis. These inhibitors do not affect either the rate of transformation of the I complex to the RS complex or the rate of transformation of the RS complex to the initiation complex. Phenanthridines, which intercalate into the DNA template and modify its conformation, act instead by limiting the number of enzyme molecules which can recognize and interact productively with the promoter regions of the template to form I complexes. This explanation is also consistent with the observed variation of the effectiveness of various phenanthridinium derivatives as inhibitors of RNA polymerase. Derivatives with a bulky phenyl group at C-6 of the phenanthridinium ring are decidedly better inhibitors of the enzyme than derivatives which carry a small aliphatic group in the same position.

INTRODUCTION

Phenanthridines exhibit a variety of biochemical and pharmacological properties. Ethidium bromide, a well-studied member of this class, is characterized by antitrypanosomal (1), antibacterial (2), and antiviral (3) properties.

At the molecular level, ethidium bromide has been found to inhibit mitochondrial RNA synthesis (4) and mitochondrial DNA replication (5). It also inhibits the appearance of closed circular viral DNA in cells infected by avian sarcoma virus (6). These properties of ethidium bromide appear to be related to its inhibitory effect on the processes of nucleic acid synthesis. Studies *in vitro* have shown that ethidium bromide is a strong inhibitor of RNA polymerase (7), deoxyribonuclease I (8), reverse transcriptase (9), and DNA polymerase I (10, 11). The last enzyme is also strongly inhibited by a number of other phenanthridines (12).

The inhibitory effect of ethidium bromide, at least on the synthesis of nucleic acids, can be attributed to the interaction of the inhibitor with the DNA template. On the basis of X-ray diffraction data it has been postulated that DNA forms complexes in which the phenanthridinium

This research was supported by Grant CA 10346 and a General Research Support Grant to Loyola University from the National Institutes of Health.

ISSN 0026-895X

ring is intercalated; i.e., it is inserted between adjacent base pairs (13). This notion has been strengthened by the results of studies of the intrinsic viscosity and sedimentation coefficient of DNA–ethidium bromide complexes (14) as well as the results of examination of such complexes by electron microscopy (15). Furthermore, the intercalation model has been found to be consistent with the other known physicochemical properties of these complexes, including spectral properties (16), temperature–optical density profiles (17), circular dichroism properties (18–20), and fluorescence properties (21).

In the present study we have examined in some detail the mechanism of inhibition of RNA polymerase by ethidium bromide and have concluded that inhibition occurs primarily at an early step in the process of initiation of RNA synthesis. Furthermore, a number of structural analogues of ethidium bromide were found to inhibit the same step in RNA synthesis. These findings have provided an opportunity to compare a number of phenanthridines and suggest a relationship between the specific structures of these compounds and their effectiveness as inhibitors of RNA polymerase.

MATERIALS AND METHODS

Nucleoside triphosphates (sodium salts) were purchased either from Sigma or from P-L Biochemicals, specified as at least 98% pure. Tritium- or ^{32}P-labeled nucleotides (ammonium salts) were purchased from Amersham/Searle. Calf thymus DNA was purchased from Worthington. Bacteriophage T2 was obtained from Miles Laboratories at a concentration of 5–10 $\times$ 10^{12} virus particles/ml and a titer of 2.1 $\times$ 10^{12} plaque-forming units/ml.

The method of Thomas and Abelson (22) was used for the isolation of T2 DNA. The purified T2 DNA, in 0.01 M Tris-HCl, 0.01 M KCl, and 50 μM EDTA, pH 7.9, was stored at −20°. The concentration of the product was determined spectrophotometrically, using a molar extinction coefficient of 6500 at 260 nm and a nucleotide mean molecular weight of 357 (23). The A_{260}:A_{280} ratio of the product was 1.80, and the A_{260}:A_{230} ratio was 2.1, indicating very low protein contamination (22).

Escherichia coli K-12 DNA-dependent RNA polymerase (EC 2.7.7.6) was purchased from Sigma in a 50% glycerol solution. The solution exhibited a specific activity of 800 units/ml of solution (2.1 mg of protein per milliliter of solution) with calf thymus DNA as template, using the unit definition of Burgess *et al.* (24). A higher enzymatic activity of 1600 units/ml of solution was noted when a T2 DNA template was used, which is indicative of an enzyme with a high content of σ-subunit (24). Indeed, the rifampicin experiments described below indicate that the RNA polymerase had 65% of the activity expected if all enzymatic protein were active holoenzyme (25).

Rifampicin was obtained from Sigma. Ethidium bromide was purchased from Calbiochem. Ethidium bromide analogues were kindly provided by Dr. T. I. Watkins, Boots Company, Ltd., Nottingham, England. The chemical formulae, molecular weights, and molar extinction coefficients of these compounds are given in Table 1.

Binding parameters. Binding parameters for the various DNA-phenanthridinium complexes were determined spectrophotometrically. Calf thymus DNA was dissolved at 5° in buffer consisting of 0.2 M KCl, 0.04 M Tris-HCl, 0.01 M $MgCl_2$, 0.1 mM EDTA, and 0.32 mM K_2HPO_4, pH 7.9. The solution was dialyzed overnight and filtered. The DNA concentration was determined at 260 nm, using a molar extinction coefficient of 6600 (26).

Solutions of increasing DNA to drug ratio were prepared by adding increasing amounts of DNA to constant amounts of drug solution in the same buffer and adjusting it to a final volume of 10 ml. After mixing, each solution was transferred to a water-jacketed optical cell of 10-cm path length kept at 37°, and the difference absorption spectrum was recorded against a corresponding solution which did not contain drug.

The absorption spectra of the DNA complexes of all drugs examined displayed sharp isosbestic points in the 380–400 nm and 460–500 nm regions.

TABLE 1

Chemical structures and spectrophotometric properties of some phenanthridinium derivatives

The molar extinction coefficients are based on the formula weight of the compounds and were determined from the absorption spectra of solutions prepared at a concentration of 1-2 mg/25 ml in 0.04 M Tris-HCl, pH 7.9, at room temperatures.

Systematic name	Abbreviation	Mol wt	E_{max}
3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide	EB	394	5600_{479}
3,8-Diamino-5-ethyl-6-methylphenanthridinium bromide	DMEB	332	3900_{463}
3,8-Diamino-6-ethyl-5-methylphenanthridinium bromide	DEMB	332	4700_{463}
3,8-Diamino-5-methyl-6-nonylphenanthridinium chloride	DMNC	386	4900_{467}

The absorbance of each complex ratio, A_r, and that of the "total" drug, A_0, was obtained for each drug complex at the wavelength for which a maximum $A_0 - A_r$ value was noted. The absorbance of bound drug, A_b, was calculated by the method of Li and Crothers (27). A plot of $1/(A_0 - A_r)$ vs. $1/(\text{DNA} - I_0)$, where I_0 stands for total phenanthridinium concentration, yields a straight line with an intercept on the ordinate equal to $1/(A_0 - A_b)$. From the value of this intercept, A_b may be calculated.

The fraction of DNA bound drug (b) was calculated using Eq. 1:

$$b = \frac{A_0 - A_r}{A_0 - A_b} \quad (1)$$

The molar ratio of inhibitor bound to DNA per nucleotide (r_b) and the concentration of "free" (i.e., unbound) (I_f) and bound (I_b) inhibitor were calculated using Eqs. 2 and 3, respectively:

$$r_b = b\,\frac{I_0}{\text{DNA}} \quad (2)$$

$$I_f = I_0 - I_b = I_0 - b \cdot I_0 = I_0 \cdot (1\text{-}b) \quad (3)$$

The values of the apparent association constant (K_{app}) and the maximum number of binding sites per DNA nucleotide (n) were obtained from Scatchard plots as described by Eq. 4:

$$\frac{r_b}{I_f} = n \cdot K_{app} - K_{app} \cdot r_b \quad (4)$$

RNA synthesis was determined by measuring the incorporation of radioactivity from labeled nucleoside triphosphates into material insoluble in 5% TCA.[1]

For an examination of the effect of inhibitor on the rate of RNA synthesis in the presence of a constant amount of template, 30 μM T2 DNA was used. This concentration was chosen so that, under the conditions described below, it would correspond to the minimum amount required to saturate the activity of 4 μg of RNA polymerase.

Buffer (37 μl) was mixed with appropriate amounts of T2 DNA to yield final concentrations of 0.2 M KCl, 0.04 M Tris-HCl, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.32 mM K_2HPO_4, 0.1 mM dithiothreitol, and 0.4 mg/ml of BSA at pH 7.9 and 25°. Drug (if any) was added, and the solution was brought to 84 μl with deionized water at 0-5°. The mixture was then transferred to a 37° bath, and after 6 min the enzyme (4 μg) was added in 8 μl of a solution consisting of 3 μl of buffer (at 2.5 times the final concentration), 3 μl of deionized water, and 2 μl of the enzyme solution. The reaction was initiated 7 min later by the rapid addition of a solution consisting of ATP, GTP, and CTP at 0.8 mM each and 0.4 mM [8-^{3}H]UTP (0.01 or 0.1 μCi/nmole) in 8 μl of water adjusted to pH 7.9 with 1.0 M Tris base. The reaction was allowed to proceed for exactly 7.0 min and was terminated by the rapid addition of 0.5 ml of an ice-cold solution containing 0.1 M sodium pyrophosphate and 2 mM UTP (or 0.02 M ATP or GTP for the initiation experiments), followed by 0.5 ml of ice-cold 11% TCA-1.0 M

[1] The abbreviations used are: TCA, trichloroacetic acid; BSA, bovine serum albumin; EB, 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide); DMEB, 3,8-diamino-5-ethyl-6-methylphenanthridinium bromide; DEMB, 3,8-diamino-6-ethyl-5-methylphenanthridinium bromide; DMNC, 3,8-diamino-5-methyl-6-nonylphenanthridinium chloride.

KCl-0.01 M sodium pyrophosphate solution.

After mixing and standing in ice for at least 10 min, the insoluble material was collected in a Whatman GF/C filter and rinsed with 15 ml of ice-cold 5% TCA-1.0 M KCl-0.01 M sodium pyrophosphate solution, followed by 30 ml of 95% ethanol. After drying under suction at 105° for 3 min, the filter was counted under 0.4% 2,5-diphenyloxazole in toluene.

RNA chain initiation. This was measured exactly as described above, with γ-^{32}P-labeled ATP or GTP (specific activity, 1.0 μCi/nmole) and final concentrations of 0.2 mM ATP, GTP, CTP, and [8-^{3}H]UTP (0.01 μCi/nmole).

The filters were washed with 100 ml of the TCA-KCl-sodium pyrophosphate solution, followed by 60 ml of 80% ethanol, and were subsequently stirred before drying and counting. Identical ^{3}H counts were recovered after this treatment, indicating that no product had been lost.

Simultaneous counting of samples containing both ^{32}P and ^{3}H was effected with the Beckman Isoset system with a gain setting of 3.0. Under these conditions no tritium spillover could be detected in the ^{32}P-above-^{3}H window, while less than 2% spillover from ^{32}P was detectable in the ^{3}H-below-^{14}C window.

Experiments with rifampicin. These experiments were conducted in buffer consisting of 0.04 M Tris-HCl, 0.02 M $MgCl_2$, 0.1 mM EDTA, 0.32 mM K_2HPO_4, 0.1 mM dithiothreitol, and 0.8 mg/ml of BSA, pH 7.9, at 25°.

For measuring the rate of RNA synthesis as a function of preliminary incubation time, appropriately concentrated buffer (37 μl) was mixed with drug, template (50 μM T2 DNA), and deionized water to a final volume of 72 μl. After the mixture had stood in ice for at least 10 min, the enzyme (4 μg) was added in 8 μl of a solution consisting of 3 μl of concentrated buffer, 3 μl of deionized water, and 2 μl of the commercial enzyme preparation. After an additional 10 min at 0°, the mixture was transferred to a 30° bath for the desired period of time. The reaction was then initiated by the rapid addition of an equimolar mixture of ATP, UTP, CTP, and GTP containing rifampicin in 20 μl of 0.04 M Tris-HCl, pH 7.9, which had been equilibrated at room temperature and prepared so as to result in final assay concentrations of 30 μg/ml of rifampicin and 0.4 mM nucleotide (0.01 μCi/nmole of [8-^{3}H]ATP). For the control reaction, rifampicin was replaced by an equal volume of deionized water. After exactly 1.5 min of incubation at 30°, the reaction was terminated and each mixture was processed in the same manner as in the absence of rifampicin.

In experiments measuring the rate of RNA synthesis as a function of rifampicin concentration, the buffer, inhibitor, DNA (90 μM), and deionized water were mixed as described above. Then the enzyme (2 μg in 8 μl) was added, and the mixture was preincubated for 7 min at 30°. The reaction was initiated by the addition of a nucleotide-rifampicin mixture prepared so as to result in 0.4 mM nucleotide (0.10 μCi/nmole of [8-^{3}H]ATP) while maintaining the appropriate final concentration of rifampicin. After 1.5 min at 30° the reaction was terminated, and each mixture was processed as described above.

RESULTS

The effect of ethidium bromide on the rate of RNA polymerase-catalyzed synthesis of RNA can be compared with the effects of the three structural analogues of ethidium bromide shown in Table 1. The inhibitory effectiveness of these phenanthridinium derivatives (Fig. 1) appears to fall into two distinct groups. DMNC and especially EB are the strongest inhibitors, while DEMB and DMEB are less effective, especially at the lower range of inhibitor concentrations.

The inhibitors compared in Fig. 1 have closely related chemical structures. They are 3,8-diamino derivatives of the phenanthridinium ring system and differ only in the nature of the substituents in positions 5 and 6 of the ring. These inhibitors are expected to form similar complexes with DNA, since, by analogy with ethidium bromide, other phenanthridinium derivatives also appear to interact with DNA by intercalation (28).

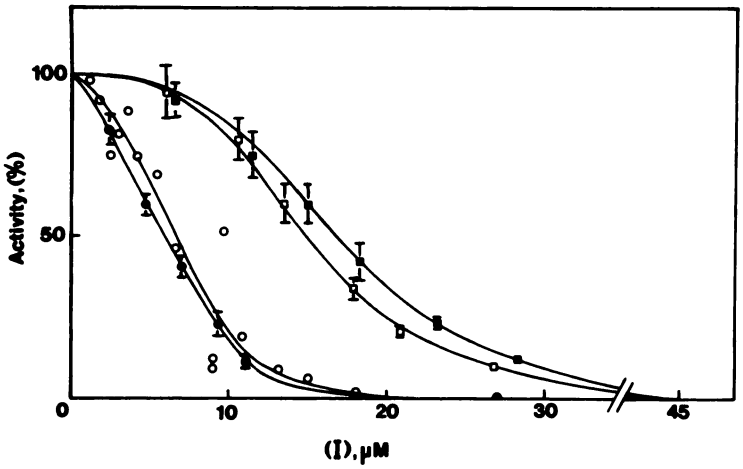

FIG. 1. *Effect of inhibitor concentration on rate of RNA synthesis relative to uninhibited reaction*

RNA polymerase was assayed in the presence of 30 μM T2 DNA and 4 μg of enzyme as described under MATERIALS AND METHODS. In the absence of inhibitor, 2×10^4 cpm were incorporated from [8-^{3}H]UTP (specific activity, 0.025 μCi/nmole) in 7 min at 37°. Each bar represents the standard deviation from the mean of 12 determinations. ●——●, EB; ○——○, DMNC; □——□, DMEB; ■——■, DEMB.

The similarities in the circular dichroism, equilibrium binding parameters, temperature–optical density profiles (20), and fluorescence properties of the complexes formed between some 3,8-diaminophenanthridinium derivatives and DNA provide additional evidence that these complexes have similar conformations. This conclusion is reinforced by the observation that 3,8-diaminophenanthridinium derivatives which carry a phenyl group in position 6 of the ring, such as ethidium, dimidium, and propidium, are characterized by identical angles of DNA unwinding regardless of the nature of the group present in position 5 of the ring (28).

In view of the existing similarities in structure and conformation of the complexes formed between DNA and the 3,8-diaminophenanthridinium derivatives, the reason for the differences noted in the inhibitory properties of these complexes toward RNA synthesis is not immediately clear. Obviously, to compare these effects on RNA polymerase properly, it would first be necessary to establish whether or not these complexes inhibit RNA polymerase by the same mechanism and whether or not differences in the binding constants between DNA and the various phenanthridinium derivatives can account for the variation in their effectiveness as RNA polymerase inhibitors.

Mechanism of inhibition of RNA polymerase by 3,8-diaminophenanthridinium derivatives. Ethidium bromide is known to inhibit RNA synthesis by interfering with the template activity of DNA rather than by acting directly on the enzyme (29, 30). A similar conclusion can be reached about the other 3,8-diaminophenanthridinium derivatives, as indicated by the double-reciprocal plots of reaction velocity against template concentration constructed for the DNA-DMEB and DNA-DEMB complexes (Fig. 2). These plots suggest that inhibition of the enzyme is related in a competitive manner to the amount of template present. Apparently, then, the 3,8-diaminophenanthridinium derivatives inhibit RNA polymerase by a common indirect mechanism, i.e., by preventing the template from directing RNA synthesis effectively.

Dependence of RNA polymerase activity on concentration of DNA-bound phenanthridinium. Template inhibition implicates the template-bound phenanthridinium derivatives, rather than the free compounds, as inhibitors of the RNA polymerase function. This suggests that a comparison between the inhibitory effects

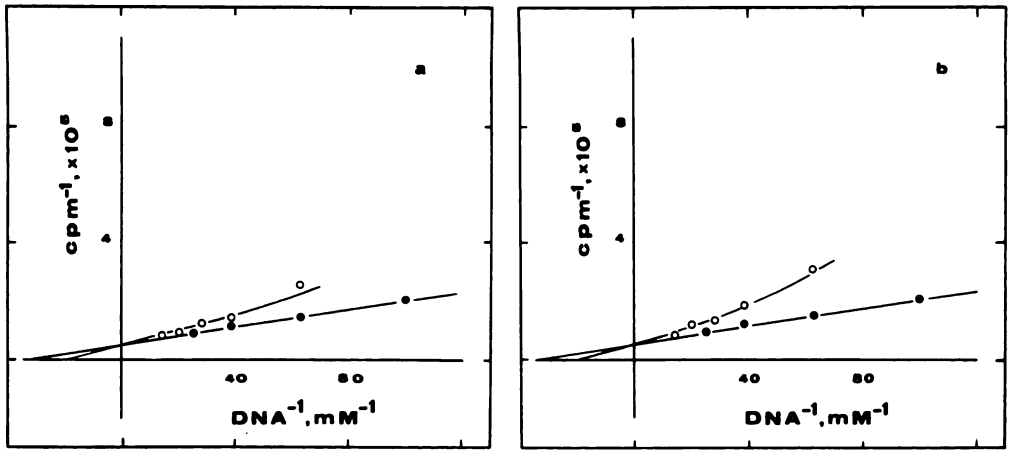

FIG. 2. *Effect of template concentration on rate of RNA synthesis*

The rate of RNA synthesis was calculated from the incorporation of [8-^{3}H]UTP (specific activity, 0.25 μCi/nmole) catalyzed by 4 μg of RNA polymerase for 7 min at 37° in the absence (●——●) and presence (○——○) of 11.0 μM DMEB (a) and 15 μM DEMB (b).

of the various phenanthridinium derivatives must take into account the concentrations of DNA-bound inhibitors rather than total inhibitor concentrations.

The association constants for the binding between DNA and EB, DMEB, and DEMB obtained from the Scatchard plots shown in Fig. 3, under conditions similar to those used for the polymerase assays, are 0.53×10^5 M^{-1}, 0.31×10^5 M^{-1}, and 0.26×10^5 M^{-1}, respectively. Reliable binding data for DMNC are difficult to obtain, because of the limited solubility of this phenanthridinium derivative in aqueous media.

Using these association constants, the data shown in Fig. 1 can be replotted as rate of RNA synthesis against molar ratio (r) of bound inhibitor to DNA phosphate (Fig. 4). It is apparent that the phenanthridinium derivatives compared in Fig. 4 inhibit RNA polymerase quite effectively. The inhibition curves exhibit a sigmoid character, in agreement with the inhibition data previously reported for EB (31). At a ratio (r) of 0.08, RNA synthesis is almost completely inhibited by each of the compounds used. Below certain concentrations of bound inhibitor, inhibition is much less effective than it is at intermediate concentrations. A striking difference in the inhibitory effectiveness of EB compared with DMEB and DEMB is also apparent. At a ratio of 0.02, for instance, the latter phenanthridinium derivatives have very little, if any, effect on the reaction while EB slows the rate of RNA synthesis by almost 40%.

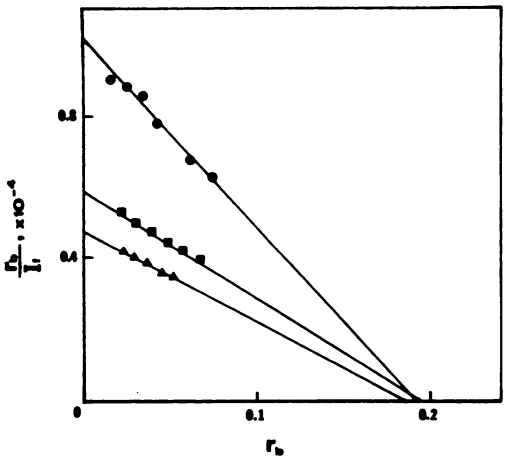

FIG. 3. *Scatchard plots for EB* (●——●), *DMEB* (■——■), *and DEMB* (▲——▲)

Spectrophotometric measurements were carried out as described under MATERIALS AND METHODS.

Effect of phenanthridinium inhibitors on initiation of RNA synthesis. In attempting to explain the above differences in the inhibitory effectiveness of phenanthridinium derivatives it is not sufficient simply to determine that these derivatives inhibit the over-all process of RNA synthe-

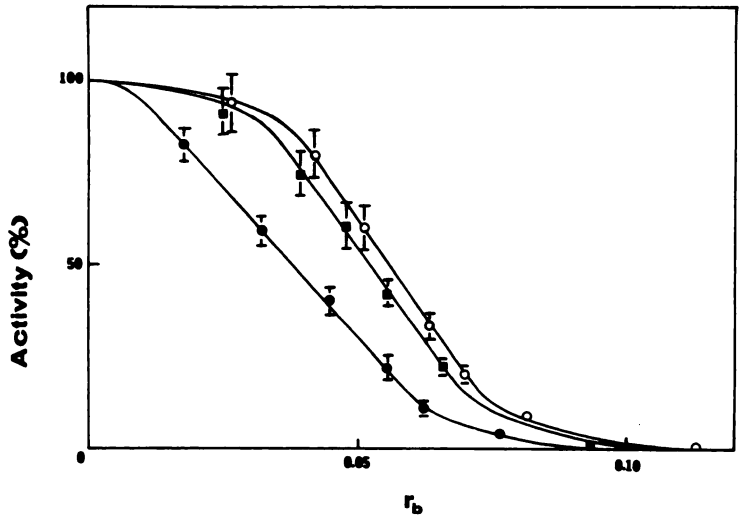

FIG. 4. *Dependence of rate of RNA synthesis molar ratio of bound inhibitor to DNA nucleotide* ●——●, *EB;* ○——○, *DMEB;* ■——■, *DEMB.*

sis. It is also necessary to establish whether the same or different steps of the polymerase catalyzed-reaction are inhibited.

RNA polymerase is believed to bind initially to the DNA template in a nonspecific manner prior to the formation of a stable binary complex (I) in which the enzyme binds at the promoter region on the outside of the DNA helix. Complex I can then be transformed, in a temperature-dependent, rate-limiting step, to the so-called readily starting (RS) complex, from which RNA chains are rapidly initiated. This transition apparently involves localized separation of DNA strands, during which the enzyme is repositioned around or within the strand opening. Before RNA chain elongation can take place, the RS complex must proceed to form the initiation complex, in which the first phosphodiester bond is established (32).

Ethidium bromide has been reported to interfere with template function primarily by inhibiting an early step in RNA synthesis. In contrast, the process of RNA chain elongation appears to be unaffected by the inhibitor, at least when PM2 DNA is used as the template (30). With such a template, initiation of RNA chains can be inhibited at relatively low concentrations of EB and, at these concentrations, the inhibition has no apparent effect on elongation.

To determine whether the structural analogues of ethidium bromide used in these studies would exhibit similar properties, the simultaneous incorporation of [^{32}P]ATP-[^{3}H]UTP and [^{32}P]GTP-[^{3}H]UTP into RNA (33) in the presence of EB and DEMB was measured. The results (Fig. 5), which were obtained using a linear T2 coliphage DNA as template, indicate that the decrease in rate of RNA formation in the presence of increasing amounts of inhibitor closely parallels the reduction in the number of RNA chains initiated with either ATP or GTP.

Since ATP and GTP are the major nucleotides which can provide the first base for an RNA chain (34), this behavior indicates that the 3,8-diaminophenanthridinium derivatives inhibit RNA synthesis primarily by limiting the number of chains which can be initiated, rather than the rate of growth of chains which have already been initiated. The extent, if any, to which each inhibitor interferes with chain elongation may be estimated from the insets of Fig. 5, in which the ratio of ^{3}H to ^{32}P (which is a measure of the degree of polymerization of the average RNA chain) is plotted against inhibitor concentration. The zero slopes obtained for both DEMB

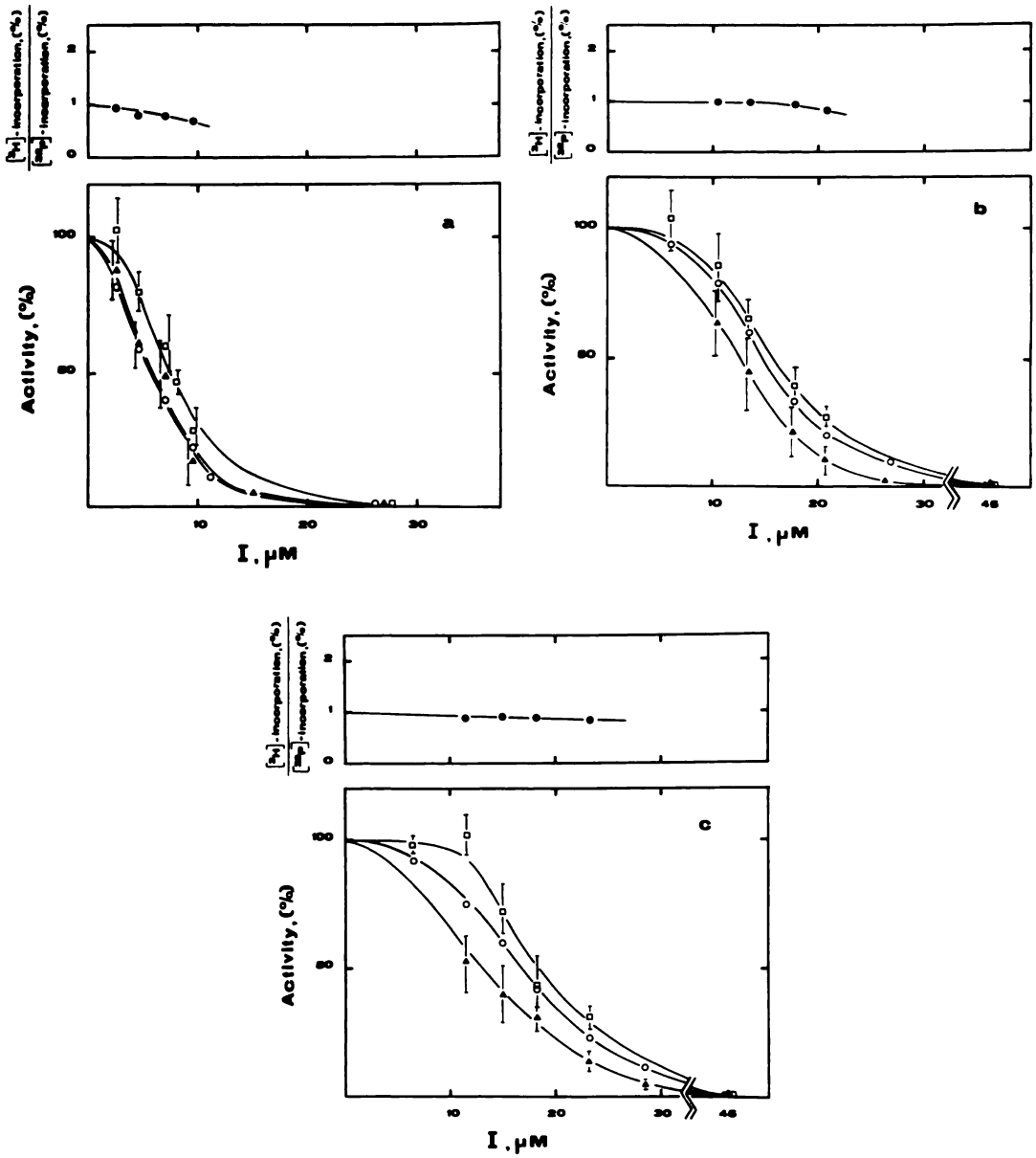

FIG. 5. *Inhibition of RNA synthesis and RNA chain initiation by EB (a), DMEB (b), and DEMB (c)*

○——○, [^{3}H]UMP incorporation; □——□, [γ-^{32}P]ATP incorporation; ▲——▲, [γ-^{32}P]GTP incorporation. Since, in the absence of inhibitor, 4 molecules of RNA are initiated with ATP for every molecule initiated with GTP (as determined by the molar ratio of A starters to G starters) under conditions of identical UMP incorporation, the ratio of percentage ^{3}H to percentage ^{32}P incorporation (●——●) was calculated using the formula

$$\frac{\%\ ^3\mathrm{H}}{\%\ ^{32}\mathrm{P}} = \frac{5 \times (\%\ [^3\mathrm{H}]\mathrm{UMP})}{4 \times (\%\ [\gamma\text{-}^{32}\mathrm{P}]\mathrm{ATP} + \%\ [\gamma\text{-}^{32}\mathrm{P}]\mathrm{GTP})}$$

In the absence of inhibitor, 2.5×10^4 cpm of [^{3}H]UMP, 5×10^3 cpm of [γ-^{32}P]ATP, and 2×10^3 cpm of [γ-^{32}P]-GTP were incorporated. Each bracket denotes the standard deviation from the mean of six determinations. The DNA concentration was 30 μM, and the enzyme concentration was 40 μg/ml.

and DMEB indicate that these inhibitors interfere primarily with RNA chain initiation and do not exert any appreciable effect on the process of chain elongation. Ethidium bromide, however, exhibits a low negative slope, which might indicate that, when a T2 coliphage template is used, EB has a minor effect on the rate of RNA chain elongation—a finding analogous to that previously reported for T5 coliphage (30). In any event, the primary effect of EB on RNA synthesis is the same as that of DEMB and DMEB, i.e., inhibition of a step prior to the formation of the initiation complex.

Effects of phenanthridinium inhibitors on substeps of initiation. Since the initiation of RNA synthesis proceeds through a number of discrete steps, a systematic comparison of the inhibitory activities of different 3,8-diaminophenanthridinium derivatives would require further knowledge of the specific initiation step or steps which these derivatives inhibit. Ethidium bromide has been reported to inhibit RNA synthesis by interfering with the formation of stable complexes between RNA polymerase and initiation sites on the DNA (30). More recent studies have suggested further that this inhibitor has no effect either on the rate of transformation of the I complex to the RS complex or on the rate of transformation of the RS complex to the initiation complex, but that it acts by limiting the number of enzyme molecules which form I complexes (35).

By analogy, DEMB and DMEB may also be expected to act by limiting the number of RNA polymerase molecules that can participate in the formation of I complexes. Whether or not this is the case can be ascertained by examining the effects of these phenanthridines on the rate of RNA synthesis in the presence of rifampicin. Such measurements can yield useful information because of the unique properties of this antibiotic. It exerts a pronounced effect on the over-all synthesis of RNA but has no appreciable effect on this reaction once the initiation complex has been formed. Because of these properties it can be shown that the rate constant for the transformation of the RS complex to the initiation complex can be estimated from a plot of enzymatic activity, expressed as V/V', against rifampicin concentration (Fig. 6), where V stands for the rate of RNA synthesis in the absence of rifampicin, and V' for the rate in its presence. The slope obtained is a measure of the rate constant of rifampicin attack on the binary RNA polymerase-DNA complex over the apparent first-order rate constant for RNA chain initiation. In addition, the intercept on the V/V' axis is a measure of the number of RS complexes which are transformed to the rifampicin-resistant ternary complex (24). Assuming that the rate of rifampicin attack on the complex is not altered in the presence of the phenanthridinium derivatives, the near identity of the slopes for the inhibited and uninhibited reactions indicates that the rate constant for enzyme molecules which proceed from their respective RS complexes to the ternary complex is not appreciably affected by the presence of the inhibitors. However, the V/V' intercept values for EB at $r = 0.04$ and for DEMB at $r = 0.065$, which are 47% and 36% of the values for the uninhibited reaction, respectively, indicate that both these inhibitors substantially limit the number

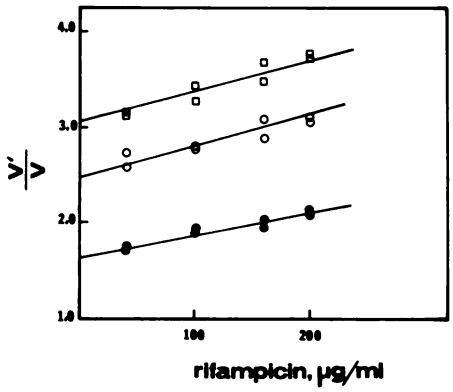

FIG. 6. *Dependence of rate of RNA synthesis on rifampicin concentration*

The rate of incorporation of radioactivity into product in the absence of inhibitor and rifampicin (V') was 8.4×10^3 cpm/90 sec at 30°. V is the rate of the reaction in the presence of rifampicin only (●——●), rifampicin and ethidium bromide at r_b = 0.035 (□——□), and rifampicin and DEMB at r_b = 0.050 (○——○). The DNA concentration was 50 μM, and the enzyme concentration was 40 μg/ml.

of enzyme molecules which participate in this step. This decrease in the number of enzyme molecules which can initiate RNA chains may result from interference by the inhibitors with either the formation of the RS complex or possibly the formation of a precursor of the RS complex, the I complex.

Effect of phenanthridinium derivatives on formation of I complex. In order to distinguish between the two possibilities listed above, the rate and number of RNA polymerase molecules which are involved in the transformation of the I complex to the RS complex were examined. This can be done by determining enzymatic activity as a function of the time of preincubation at 30° of binary complexes formed between the polymerase and DNA at 0°, i.e., I complexes, which are subsequently initiated at 30° with a mixture of nucleotides either with or without rifampicin (Fig. 7). The slope of the curve is a measure of the rate constant of the transformation of the I to the RS complex (32). The effect of DEMB at $r = 0.05$ on the dependence of activity on preincubation time is also shown in Fig. 7. The similarities noted in the dependence of RNA synthesis on preincubation time in the presence and absence of DEMB suggest that the rate of transformation of the I complex to the RS complex is not affected by the presence of the inhibitor.

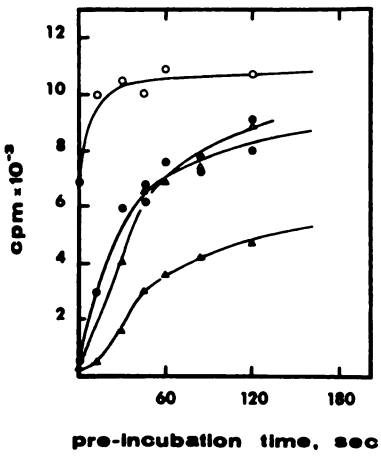

FIG. 7. *Rate of RNA synthesis as a function of preincubation time*

O——O, in the absence of rifampicin; ●——●, in the presence of rifampicin (30 μg/ml) only; ▲——▲, in the presence of both rifampicin and DEMB at $r = 0.050$; △——△, in the presence of DEMB only.

Because the kinetics of transformation of the I complex to the RS complex is first-order, the apparent half-life of the complex can be calculated from a plot of the logarithm of $P_\infty - P_t$ against preincubation time (Fig. 8).

DISCUSSION

Ethidium bromide and its structural analogue DEMB appear to inhibit the initiation of RNA chains by interfering with an early step in the synthesis of RNA, namely, by limiting the number of enzyme molecules which interact with specific initiation sites on DNA to form the so-called I complex.

Ethidium bromide and DEMB, as well as the other 3,8-diaminophenanthridinium compounds used in these studies, form complexes with DNA which exhibit physical similarities to one another. Further-

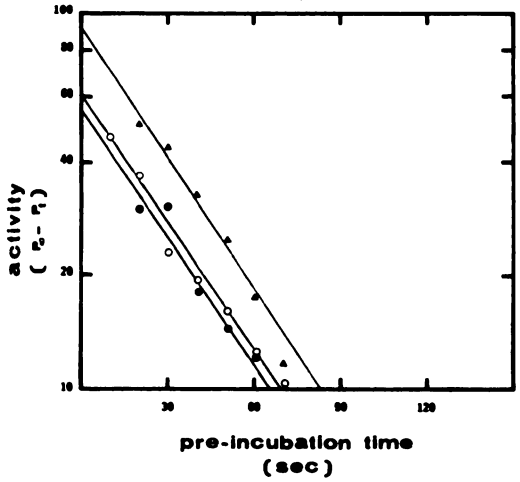

FIG. 8. *Dependence of logarithm of enzymatic activity (per cent) at infinite preincubation time, minus enzymatic activity (per cent) at time t, vs. preincubation time (t)*

Activity is the percentage of counts per minute incorporated in the presence of rifampicin relative to the counts incorporated in its absence either without inhibitor (O——O) or with EB (▲——▲) or DEMB (●——●). The activity levels corresponding to 160 sec of preincubation time are taken as the activity levels at infinite time. DNA and enzyme concentrations were 90 μM and 20 μg/ml, respectively. All other conditions were the same as described under MATERIALS AND METHODS.

more, the inhibition of RNA polymerase by these compounds may be attributed to binding of the inhibitor to the DNA template. Under conditions which produce effective inhibition, an intercalation complex may be formed almost exclusively. Specifically, the Scatchard plots obtained at the relatively high ionic strength used for the RNA polymerase assay indicate limited secondary interactions between DNA and these derivatives. A similar conclusion was drawn previously regarding the complex formed between DNA and ethidium bromide (20).

In spite of the above similarities in the mode of interaction between DNA and phenanthridinium derivatives, some striking differences are apparent in the effectiveness of these compounds as inhibitors of RNA polymerase. Both EB and DMNC, derivatives which carry a phenyl or a nonyl group, respectively, at position 6 of the ring, appear to inhibit the enzyme at lower concentrations than the phenanthridinium derivatives with either an ethyl or a methyl substituent at position 6 of the ring, DEMB and DMEB.

These differences in inhibitory effectiveness between the two groups of 3,8-diaminophenanthridinium compounds are evident even though EB and DEMB, and quite likely DMEB and DMNC as well, inhibit RNA polymerase by interfering with the same early step of RNA synthesis, formation of the I complex. Thus these differences must be related in some way to the nature of the particular substituent present at position 6 of the ring.

In the intercalation complexes formed between DNA and 3,8-diaminophenanthridinium derivatives, the phenanthridinium ring is inserted between base pairs in an orientation which permits the formation of hydrogen bonds between the amino groups of the ring with the phosphate residues of the DNA backbone. In this orientation a large substituent at position 6 of the ring is projected outside the intercalating site, as substantiated in the case of the DNA–ethidium bromide complex (13).

Intercalation, of course, produces a distortion in the conformation of the template which apparently interferes with the function of RNA polymerase by limiting the ability of the enzyme to recognize and bind productively to sites at which phenanthridinium molecules are intercalated. Under these conditions the interaction of the enzyme with appropriate DNA sites, leading to the formation of the specific I complex, may be severely limited. Furthermore, it appears that the precise geometry of the binding site for the RNA polymerase is also of importance in determining the formation of the I complex.

The present of a phenyl substituent at position 6 of the ring may be expected to produce a greater distortion in the geometry of the intercalation site than that resulting from the methyl substituent present in DEMB. This modification of the binding site may be both a direct result of the presence of a bulkier group within the site and an indirect outcome of the presence of such a group. In the latter instance a bulky group may influence the geometry of the binding site by modifying the relative orientation of the other atoms which form the binding site. Intercalation of ethidium, dimidium, and propidium, all 3,8-diaminophenanthridinium derivatives with a phenyl substituent in position 6 of the ring, brings about an apparent unwinding for the double helix of 12°, 11.5° ± 2.2°, and 12° ± 3.4°, respectively.[2] In contrast, the intercalation of 3,8-diamino-5-methylphenanthridinium chloride, which does not carry a substituent at position 6, is calculated to result in an angle of unwinding of 8.3° ± 0.7° (28).

In view of these conformational differences it should be recalled that although DEMB produces substantial inhibition of RNA polymerase, this inhibition is significantly lower than that produced by the 3,8-phenanthridinium derivatives which carry a phenyl group at position 6. Thus it appears that, above and beyond the effect of intercalation of the phenanthridinium ring on the rate of formation of the I complex, the formation of this complex is fur-

[2] These angles are calculated relative to an assumed angle of unwinding for ethidium bromide of 12°. More recent reports, however, suggest a value of 26° for ethidium bromide (37, 38).

ther inhibited by the presence of bulky substituents at position 6 of the intercalated ring. This enhanced interference may be related directly to the more pronounced distortion of the sites of intercalation, which may render such sites less recognizable to the enzyme, or it may result from some other, indirect effect on the process of formation of the I complex.

One such effect could arise if, for instance, ethidium bromide were characterized by a lower rate of dissociation from DNA than the other derivatives examined. This could obviously account for a lower inhibitory effectiveness for easily dissociating inhibitors, which would give the DNA template greater opportunity to form the I complex. It has been suggested that the differences in inhibitory effectiveness toward RNA polymerase between actinomycin and many of its structural analogues reside in differences in the rates of dissociation among these derivatives (38). Although actinomycin inhibits RNA polymerase by a different mechanism than ethidium bromide, i.e., by interfering with the process of RNA chain elongation, and other differences are present that make the two systems not strictly comparable, the influence of possible differences in dissociation rate constants among phenanthridinium derivatives on their effects on RNA polymerase cannot be discounted.

In any event it is clear that the presence of a phenyl group has a substantial effect on the inhibitory effectiveness of the phenanthridinium ring, and this effect may have some significance in relation to certain previously established structure–biological activity relationships among phenanthridinium derivatives. Specifically, phenathridinium derivatives which carry bulky groups at position 6 have been found to be the most effective trypanocides (39), and although the mechanism which produces the trypanocidal effects of phenanthridinium compounds is not well understood, it is most probably related to drug-template interactions which result in modification of template properties.

The present findings therefore suggest that a more systematic examination of the effects of a varied group of phenanthridinium derivatives on RNA polymerase activity may reveal a relationship between inhibitory effectiveness and trypanocidal activity. In turn, the effectiveness of phenanthridinium derivatives as inhibitors of RNA polymerase appears to be related to the geometry of the intercalation site as it is influenced by the precise structure of the intercalated molecule.

ACKNOWLEDGMENTS

We are indebted to Dr. T. I. Watkins for his kind cooperation and his generous gift of a number of phenanthridinium derivatives.

REFERENCES

1. Brownlee, G., Goss, M. D., Goodwin, L. G., Woodbine, M. & Walls, L. P. (1950) *Br. J. Pharmacol. Chemother.*, 5, 261–276.
2. Seaman, A. & Woodbine, M. (1954) *Br. J. Pharmacol. Chemother.*, 9, 265–270.
3. Dickinson, L., Chantrill, B. H., Inkley, G. W. & Thompson, M. J. (1953) *Br. J. Pharmacol. Chemother.*, 8, 139–142.
4. Zylber, E. & Penman, S. (1969) *J. Mol. Biol.*, 46, 201–211.
5. Nagley, P., Gingold, E. B., Lukins, H. B. & Linnane, A. W. (1973) *J. Mol. Biol.*, 78, 335–350.
6. Guntaka, R. V., Mahy, B. W. J., Bishop, J. M. & Varmus, H. E. (1975) *Nature*, 253, 507–511.
7. Waring, M. J. (1964) *Biochim. Biophys. Acta.*, 87, 358–364.
8. Eron, L. J. & McAuslan, B. R. (1966) *Biochim. Biophys. Acta*, 114, 633–636.
9. Müller, W. E. G., Zahn, R. K. & Seidel, H. J. (1971) *Nature*, 232, 143–145.
10. Elliott, W. H. (1963) *Biochem. J.*, 86, 562–567.
11. Meyer, R. B. & Simpson, M. V. (1969) *Biochem. Biophys. Res. Commun.*, 34, 238–244.
12. Aktipis, S. & Kindelis, A. (1974) *FEBS Lett.*, 49, 149–151.
13. Fuller, W. & Waring, M. J. (1964) *Ber. Bunsenges. Phys. Chem.*, 68, 805–808.
14. Douthard, R. J., Burnett, J. P., Beasley, F. W. & Frank, B. H. (1973) *Biochemistry*, 12, 214–220.
15. Freifelder, D. (1971) *J. Mol. Biol.*, 60, 401–403.
16. Waring, M. J. (1965) *J. Mol. Biol.*, 13, 269–282.
17. Aktipis, S., Martz, W. W. & Kindelis, A. (1975) *Biochemistry*, 14, 326–331.
18. Aktipis, S. & Martz, W. W. (1970) *Biochem. Biophys. Res. Commun.*, 39, 307–313.
19. Dalgleish, D. G., Peacocke, A. R., Fey, G. & Harvey, C. (1971) *Biopolymers*, 10, 1853–1863.
20. Aktipis, S. & Kindelis, A. (1973) *Biochemistry*,

12, 1213-1221.
21. LePecq, J. B. & Paolletti, C. (1967) *J. Mol. Biol.*, 27, 87-106.
22. Thomas, C. A. & Abelson, J. (1966) *Procedures Nucleic Acid Res.*, 1, 553-561.
23. Rubenstein, I., Thomas, C. A. & Hershey, A. D. (1961) *Proc. Natl. Acad. Sci. U. S. A.*, 47, 1113-1124.
24. Burgess, R. R., Travers, A. A., Dunn, J. J. & Bautz, E. K. F. (1969) *Nature*, 221, 43-47.
25. Mangel, W. F. & Chamberlin, M. J. (1974) *J. Biol. Chem.*, 249, 2995-3001.
26. Mahler, H. R., Kline, B. & Mehrotra, B. D. (1964) *J. Mol. Biol.*, 9, 801-811.
27. Li, H. J. & Crothers, D. M. (1969) *J. Mol. Biol.*, 39, 461-477.
28. Wakelin, L. P. G. & Waring, M. J. (1974) *Mol. Pharmacol.*, 10, 544-561.
29. Waring, M. J. (1965) *Mol. Pharmacol.*, 1, 1-13.
30. Richardson, J. P. (1973) *J. Mol. Biol.*, 78, 708-714.
31. Richardson, J. P. & Parker, S. R. (1973) *J. Mol. Biol.*, 78, 715-720.
32. Mangel, W. F. & Chamberlin, M. J. (1974) *J. Biol. Chem.*, 249, 3007-3013.
33. Maitra, U., Nakata, Y. & Hurwitz, J. (1967) *J. Biol. Chem.*, 242, 4908-4918.
34. Maitra, U. & Hurwitz, J. (1965) *Proc. Natl. Acad. Sci. U. S. A.*, 54, 815-822.
35. Aktipis, S. & Panayotatos, N. (1976) *Biochem. Biophys. Res. Commun.*, 68, 465-470.
36. Wang, J. C. (1974) *J. Mol. Biol.*, 87, 797-816.
37. Tsai, C.-C., Jain, S. C. & Sobell, H. M. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, 72, 628-632.
38. Müller, W. & Crothers, D. M. (1968) *J. Mol. Biol.*, 35, 251-290.
39. Woolfe, G. (1956) *Br. J. Pharmacol. Chemother.*, 11, 330-333, 334-338.