The Effects of Antimicrobial Agents on Ribonucleic Acid Polymerase

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SUMMARY

A range of antibiotics and other drugs was tested for possible inhibitory activity toward the DNA-dependent RNA polymerase of *Escherichia coli*. Several agents believed to interfere primarily with protein or DNA synthesis in microorganisms were shown to have no significant effect on AMP incorporation into RNA by the enzyme system. Neomycin, Antrycide, and pentamidine precipitated the DNA primer and gave substantial inhibition; suramin, prothidium bromide, proflavin, and ethidium bromide were more powerfully inhibitory, and actinomycin D was the most potent inhibitor found. These results are discussed with reference to the probable modes of action of the drugs *in vivo*.

Inhibition by ethidium bromide was found to be related to the concentration of DNA, as would be expected if the inhibition were due to the drug's forming a complex with the primer. Calculations of the expected level of binding of ethidium to DNA under the assay conditions led to the conclusion that RNA polymerase activity was inhibited in direct proportion to the amount of drug bound to the primer.

INTRODUCTION

Studies on the biosynthesis of RNA in a variety of living organisms have shown that RNA is synthesized from nucleoside triphosphates by DNA-dependent enzyme systems. These enzymes, RNA polymerases (nucleosidetriphosphate:RNA nucleotidyltransferases, E.C. 2.7.7.6), have been discovered in bacteria (1–5), plants (6, 7), and animal tissues (8, 9). Several of the bacterial enzymes have been extensively purified (1–4). Their activity is dependent upon the presence of DNA, which acts as a template for the synthesis of the product so that the base composition and nearest-neighbor nucleotide frequency of the RNA produced are complementary to those of the DNA primer. Moreover, the RNA synthesized in a reaction primed by bacteriophage T2 DNA was shown to form a stable hybrid with denatured T2 DNA, indicating that the base sequences of primer and product must be at least to a large extent complementary (10). RNA synthesized *in vitro* on a template of double-stranded DNA is able to stimulate the incorporation of amino acids into protein by cell-free extracts (4, 11), and the evidence indicates that RNA polymerase catalyzes the DNA-directed synthesis not only of messenger RNA, but also of the other forms of RNA *in vivo* (12).

The ability of actinomycin D to inhibit the activity of DNA-dependent RNA polymerase has been studied in a number of laboratories (5, 13, 14). This inhibition has been shown to be due to complex formation between the antibiotic and DNA and appears to represent the primary mode of action of actinomycin against living cells (13, 15, 16). Because of its specificity of action, actinomycin has proved to be a valuable tool for demonstrating the existence of DNA-directed RNA synthesis in biological systems.

Several workers have also noted inhibition of RNA polymerase by proflavin (12, 14, 17). While actinomycin is known to inhibit RNA synthesis more powerfully than DNA synthesis *in vivo* and *in vitro* (14, 15), proflavin is more inhibitory to DNA polymerase activity (12). Nevertheless, proflavin has been used to measure the rate of decay of messenger RNA *in vivo* (17, 18).

This paper describes a survey of the effects of a range of antimicrobial agents on the DNA-dependent RNA polymerase of *Escherichia coli*. The results indicate inhibition by a number of substances, particularly the trypanocidal drugs Antrycide, pentamidine, ethidium bromide, prothidium bromide, and suramin.

MATERIALS AND METHODS

Materials. DNA from bacteriophage T2 was prepared according to the procedure of Shedlovsky and Brenner (19). The purified phage suspension was shaken with an equal volume of water-saturated redistilled phenol at room temperature for 2 min and centrifuged at 12,000 rpm for 10 min. The viscous upper layer was reextracted with phenol and centrifuged again. The DNA was precipitated with 2 volumes of ice-cold 95% ethanol, collected on a glass rod, and dissolved in 0.01 M Tris-HCl (pH 7.4) containing 0.01 M NaCl. A further phenol extraction was carried out, and the DNA was reprecipitated after the NaCl concentration had been adjusted to 0.1 M. The precipitate was redissolved as above, extracted with ether six times to remove traces of phenol, freed from ether by bubbling air through the solution, and stored at 0–4° in the presence of a few drops of chloroform.

Unlabeled nucleoside triphosphates were obtained from the California Corporation for Biochemical Research. ^{14}C-labeled nucleoside triphosphates were products of Schwarz BioResearch Inc. and were adjusted by addition of the unlabeled compounds to give the following specific activities measured in an end-window counter: ATP 262 cpm/mμmole, CTP 415 cpm/mμmole, GTP 487 cpm/mμmole, and UTP 469 cpm/mμmole. Neomycin sulfate (Mycifradin sulfate) was obtained from Upjohn Ltd., proflavin sulfate from British Drug Houses; actinomycin D was a gift from Dr. H. B. Woodruff of Merck, Sharp & Dohme Ltd.; Antrycide dimethosulfate, pentamidine isethionate, and suramin (Na salt) were kindly provided by Dr. B. A. Newton of this department; ethidium bromide was a gift from Dr. G. Woolfe of Boots Pure Drug Co. Ltd. All solutions used in the course of this work were prepared with glass-distilled water.

Assay of RNA polymerase activity. RNA polymerase was isolated from log-phase cells of *E. coli* B according to the procedure of Chamberlin and Berg (1). Specific activities of the purified preparations (fraction IV) were in the region of 2000 units per milligram protein (1). Reaction mixtures (0.25 ml) contained 10 μmoles Tris-HCl buffer (pH 7.9), 1 μmole $MgCl_2$, 0.1 μmole each of ATP, CTP, GTP, and UTP (one of which was radioactively labeled), 3 μmoles β-mercaptoethanol, T2 DNA as indicated, and enzyme. The amount of enzyme added was chosen such that it catalyzed the incorporation of 4–6 mμmoles of AMP under the conditions used. After incubation at 37° for 10 min the reaction was stopped by heating at 100° for 5 minutes. The mixture was rapidly cooled to 0°, and 200 μg of a crude yeast nucleic acid preparation was added followed by trichloroacetic acid to a final concentration of 5%. The mixture was allowed to stand in ice for 4 minutes and then filtered through a 2-cm membrane filter; the precipitate was washed with 20 ml of 5% trichloroacetic acid followed by 5 ml of 1% acetic acid, and the filter was dried and counted in an end-window counter. The purpose of heating at the end of the incubation period was to convert the T2 DNA to the single-stranded form which permitted more satisfactory precipitation of the reaction mixture. Duplicate determinations were performed; the agreement between the two values was almost invariably within $\pm 4\%$ of the mean. All incorporation results were corrected for a "blank" determined by incubating a complete reaction mixture without enzyme, cooling to 0°, adding the enzyme, heating at 100° for 5 minutes, and assaying

acid-insoluble radioactivity as usual. This blank value lay in the range 13–19 cpm, representing 1% of the incorporation obtained with a complete system. The reaction was completely dependent upon the addition of DNA. When CTP, GTP, or UTP were omitted from the reaction mixture either singly or together, the incorporation of ^{14}C-AMP was reduced to less than 4% of the control incorporation. The incorporation of radioactivity from ^{14}C-CTP, ^{14}C-GTP or ^{14}C-UTP was similarly sensitive to the omission of other triphosphates. In the presence of 100 μg of DNA the reaction proceeded at a constant rate for at least 15 min, and the effect of enzyme concentration was shown to be linear up to a concentration catalyzing the incorporation of 5 mμmoles of AMP in a 10-min incubation.

RESULTS

Before antimicrobial agents were tested on the RNA polymerase system, the effect of DNA concentration on the reaction was investigated. As shown in Fig. 1, variation of the DNA concentration in the range 10–100 μg per 0.25 ml reaction mixture had little effect on the reaction rate; however, when the concentration was reduced below 10 μg the rate of reaction fell rapidly. Data obtained in this way can be treated according to the method of Lineweaver and Burk (20) yielding quantitative information about the association between the enzyme and the DNA template (21) (see Figs. 7–9).

For work with antimicrobial agents the DNA concentration chosen was 5 μg per reaction mixture, equivalent to 16 mμmoles as deoxyribonucleotides. At this level any agent interfering with the ability of the DNA to act as a template for RNA synthesis would be expected to cause considerable inhibition of the reaction rate (Fig. 1). Moreover, the incorporation of 5 mμmoles of AMP corresponds to a total nucleotide incorporation of almost 16 mμmoles, providing conditions approaching "net" synthesis of product with respect to the amount of DNA present.

Effects of Antimicrobial Agents

Table 1 summarizes the effects of a range of antimicrobial agents in the RNA polymerase reaction mixture. Substances other than those listed in Materials were obtained from standard commercial sources. Most of the agents tested showed no significant effect on the incorporation of ^{14}C-AMP

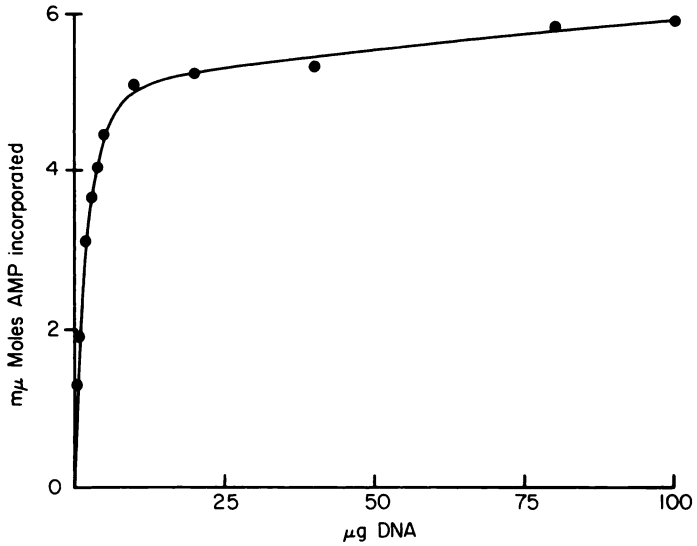

FIG. 1. *Effect of DNA concentration on the RNA polymerase reaction*

Reaction mixtures contained 18 μg enzyme preparation. The values in the abscissa represent the amount of T2 DNA added to each 0.25-ml reaction mixture.

TABLE 1
The effects of antimicrobial agents on RNA polymerase
Standard assay conditions were used with 5 μg of T2 DNA and 22 μg of enzyme preparation.

Substance	Concentration	AMP incorporated (mμmoles)	% of control incorporation
Control	(No addition)	5.11	(100)
Actidione	80 μg/ml	4.95	97
Actinomycin D	14 μg/ml	<0.02	0
Antrycide dimethosulfate	80 μg/ml	1.10[a]	22[a]
Aureomycin	80 μg/ml	4.62	90
Chloramphenicol	80 μg/ml	5.02	98
2,4-Dinitrophenol	4.8×10^{-3} M	5.55	109
Erythromycin	80 μg/ml	4.84	95
Etamycin	80 μg/ml	4.71	92
Ethidium bromide	16 μg/ml	<0.02	0
Kanamycin	80 μg/ml	4.98	98
Mitomycin C	40 μg/ml	5.26	103
Neomycin	80 μg base/ml	0.20[a]	4[a]
Pentamidine isethionate	80 μg/ml	2.03[a]	40[a]
β-Phenethyl alcohol	0.25%	4.17	82
Proflavin sulfate	80 μg/ml	<0.02[a]	0[a]
Prothidium bromide	40 μg/ml	<0.02[a]	0[a]
Puromycin	80 μg/ml	4.95	97
Staphylomycin S	80 μg/ml	5.05	99
Streptogramin	80 μg/ml	4.76	93
Streptomycin	80 μg base/ml	4.45	87
Suramin (Na salt)	80 μg/ml	<0.02	0
Terramycin	80 μg/ml	5.07	99
Tetracycline	80 μg/ml	5.55	109

[a] The primer was precipitated by the added agent.

into RNA; this was particularly true of the antibiotics believed to interfere primarily with protein synthesis in microorganisms, such as chloramphenicol (22), the tetracyclines (including Aureomycin and Terramycin) (22), puromycin (22, 23), streptogramin (24), and the related compounds staphylomycin S and etamycin (25), erythromycin (22, 26), and Actidione (cycloheximide) (27, 28). No effect was found with streptomycin and kanamycin; however, the related antibiotic neomycin precipitated the DNA primer and caused almost complete inhibition. These three aminoglycoside antibiotics are believed to have similar modes of action; they stimulate breakdown of RNA and excretion of nucleotides by *E. coli* (29), but recent evidence suggests that their primary effect is to interfere with the interaction between messenger RNA, soluble RNA, and ribosomes in protein synthesis (30). Hochster and Chang (5) tested a number of inhibitors of protein synthesis on their RNA polymerase from *Agrobacterium tumefaciens* and found no significant inhibitions. The results in Table 1 confirm these authors' findings except for the inhibition by neomycin.

Negative results were also found with mitomycin C, β-phenethyl alcohol, and 2,4-dinitrophenol. Lack of inhibition by mitomycin C has been previously reported (12, 31). β-Phenethyl alcohol at a concentration of 0.25% selectively inhibits DNA synthesis in gram-negative bacteria without affecting RNA synthesis (32); the 18% inhibition by this agent shown in Table 1 is significant, but so low as to be of little interest. Woese *et al.* (18) reported that 5×10^{-3} M 2,4-

dinitrophenol caused a rapid cessation of the incorporation of radioactive uracil into RNA in *E. coli*. A later report (33) showed that at this concentration 2,4-dinitrophenol gave a 10–20% inhibition of *E. coli* RNA polymerase activity primed by calf thymus DNA or *E. coli* DNA *in vitro;* however, no inhibition was observed in the T2 DNA-primed reaction studied here.

The Effect of Concentration of Inhibitory Drugs

The remaining substances included in Table 1 had significant inhibitory effects on the polymerase system and were studied further by investigating the effect of concentration of the inhibitor. Most of the active agents are trypanocidal drugs; the structures of these compounds are reproduced in Fig. 2. Figure 3 shows the inhibition of RNA polymerase produced by increasing concentrations of neomycin, Antrycide, and pentamidine. Because of the uncertainty about the composition of neomycin, the concentrations are expressed in micrograms per milliliter. However, the molecular weights of these substances are all in the region of 600 so that the curves give an approximate indication of their effectiveness on a molar basis. Each substance gave rise to 50% inhibition of AMP incorporation at about 10^{-4} M and caused visible precipitation of the DNA primer. Low concentrations of Antrycide seemed to have a different effect on the enzyme system, giving an apparent 20% stimulation of the reaction.

A second group of substances produced 50% inhibition of AMP incorporation at concentrations in the region of 10^{-5} M (Fig. 4). Neither ethidium bromide nor proflavin caused visible precipitation of the primer, but ethidium was 2–3 times more powerful as an inhibitor than proflavin and completely inhibited the reaction at 2.6×10^{-5} M. Inhibitory concentrations of pro-

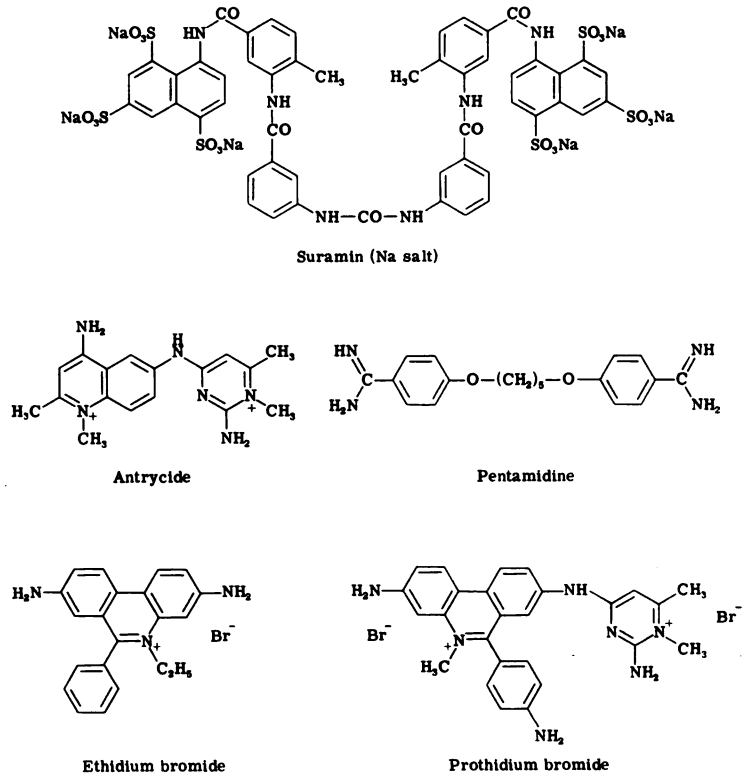

FIG. 2. *Structures of trypanocidal drugs*

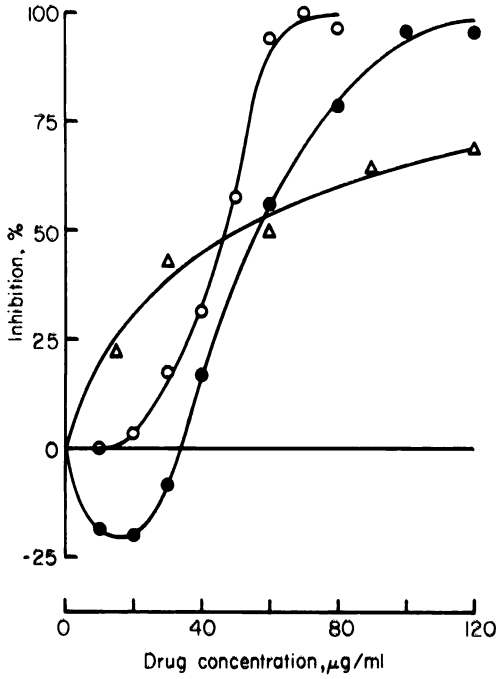

FIG. 3. *Inhibition of RNA polymerase by neomycin, Antrycide, and pentamidine*

Reaction mixtures contained 5 μg T2 DNA and 29 μg enzyme preparation, giving a control incorporation of 3.9 mμmoles of AMP. Drug concentrations are expressed in micrograms per milliliter of neomycin as the free base (○), Antrycide as the dimethosulfate (●), and pentamidine as the isethionate (△).

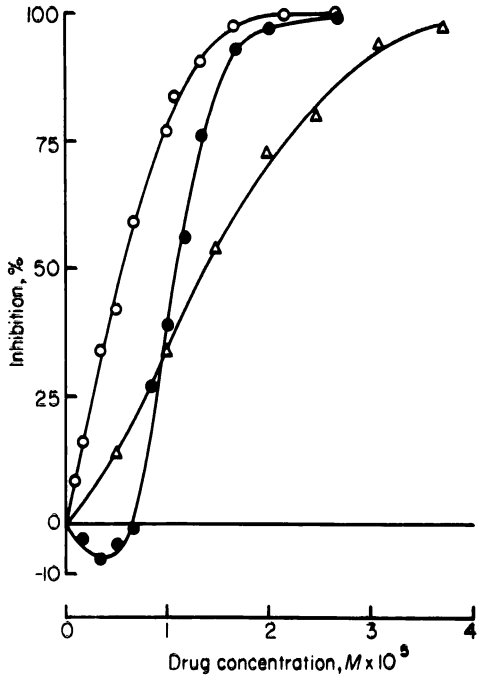

FIG. 4. *Inhibition of RNA polymerase by ethidium bromide, prothidium bromide, and proflavin*

Reaction mixtures contained 5 μg T2 DNA and 36 μg enzyme preparation, giving a control incorporation of 4.5 mμmoles of AMP. ○, ethidium; ●, prothidium; △, proflavin.

thidium bromide led to the appearance of a precipitate, but as in the case of Antrycide low concentrations produced a slight apparent stimulation of AMP incorporation. Another drug with an inhibitory activity of the same order of magnitude was suramin; Fig. 5 shows the effect of suramin concentration, and again a slight stimulatory effect was apparent at low concentrations. Fifty per cent inhibition by suramin occurred at 3.2×10^{-6} M, which is lower than the concentration of ethidium bromide required to produce the same effect; however, the molecular weight of suramin is very high compared with that of ethidium bromide, and on a weight-for-weight basis suramin was 2½ times less effective than the phenanthridinium compound.

The apparent stimulation of the reaction by low concentrations of Antrycide, prothidium, and suramin was reproducibly observed, but further investigation of this effect produced no conclusive results. It was not due to nonspecific precipitation of ^{14}C-ATP in the presence of Antrycide. The possibility that it was due to interaction between the drugs and the reaction product, relieving possible inhibition of further synthesis by the product as it accumulated, was investigated by measuring the time-course of reaction in the presence and absence of 2.5×10^{-5} M Antrycide (14 μg/ml). At all stages of the reaction 10–20% stimulation was apparent, but the limits of accuracy of the measurements did not permit any definite conclusion to be made.

The most potent inhibition of DNA-dependent RNA polymerase activity found was produced by actinomycin D (Fig. 6). This antibiotic gave 50% inhibition at a

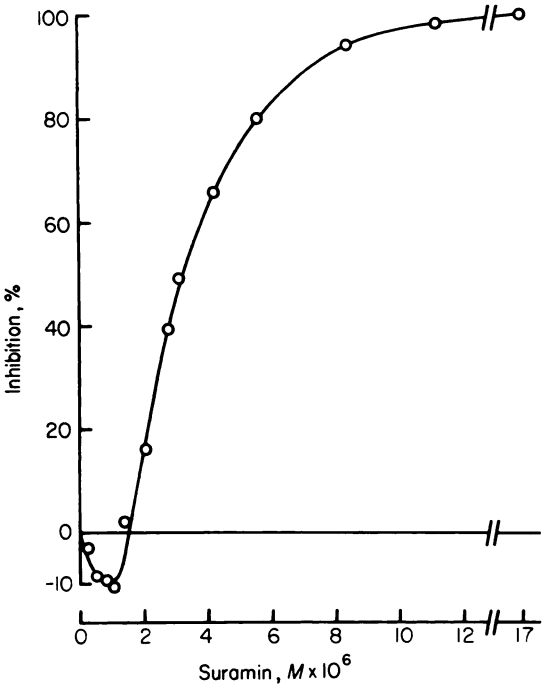

FIG. 5. *Inhibition of RNA polymerase by suramin*

Reaction mixtures contained 5 μg T2 DNA and 26 μg enzyme preparation, giving a control incorporation of 6.6 mμmoles of AMP.

concentration of 8×10^{-8} M, which represents a molar ratio in the reaction mixture of actinomycin:DNA-phosphorus of 0.00125:1. Such a level of inhibition is in close agreement with data presented by Reich (14) which indicate an inhibition of 50% at a molar ratio actinomycin:DNA-P of 0.0012. It can be seen from Fig. 6 that although 75% inhibition occurred with 1.5×10^{-7} M actinomycin the remaining incorporation of AMP was less sensitive to the antibiotic and persisted until the actinomycin concentration was raised to 10^{-5} M. This feature of the inhibition by actinomycin is also apparent in the results of Reich (14) and of Hurwitz *et al.* (12). The two-stage nature of the actinomycin inhibition may be correlated with the existence of two types of site on the double-stranded DNA molecule which are able to bind the antibiotic (34), the initial inhibition arising from binding to the "strong" sites (35), and the slower increase to 100% inhibition reflecting binding to the secondary sites.

The Relationship between Inhibition and DNA Concentration

In the cases of the four drugs which precipitated the DNA primer, it seemed very likely that this precipitation was responsible for the inhibitions observed. The question whether ethidium bromide and suramin also interfered with the priming activity of

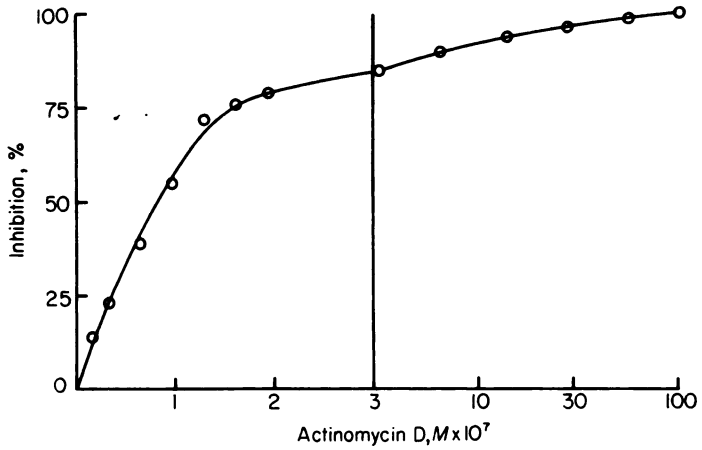

FIG. 6. *Inhibition of RNA polymerase by actinomycin D*

Reaction mixtures contained 5 μg T2 DNA and 36 μg enzyme preparation, giving a control incorporation of 4.2 mμmoles of AMP. The scale of the abscissa is linear up to 3×10^{-7} M and logarithmic from 3×10^{-7} M to 10^{-5} M.

DNA or whether they affected the activity of the enzyme protein itself was investigated by varying the concentration of DNA in the presence and absence of the inhibitor.

Figure 7 shows a double-reciprocal plot of data obtained using a concentration of ethidium bromide which gave 55% inhibition in reaction mixtures primed with 5 μg of T2 DNA. This plot shows that the inhibition was related in a competitive fashion to the amount of DNA present, but the values from the mixtures containing the drug seem to lie on a curve. When these results were replotted in the form $(\text{incorporation})^{-1}$ against $(\mu\text{g DNA})^{-2}$ the points fitted closely to a straight line, suggesting some kind of square-law relationship. The experiment was repeated using 1.56×10^{-6} M ethidium bromide, a concentration which caused 15% inhibition in the presence of 5 μg of T2 DNA. In this case the inhibited reaction mixtures gave values lying on a straight line intersecting with the control line on the ordinate (Fig. 8), showing that ethidium bromide had no effect on the enzyme itself, but rather interfered with the priming of the reaction by DNA. Similar effects were observed when the synthesis of RNA was followed using a different radioactively labeled nucleoside triphosphate (Fig. 9). Again, in both instances the inhibition by ethidium bromide was related in a competitive fashion to the DNA concentration, and the higher drug concentration gave rise to values lying on a curve. Figure 9 also includes results obtained in the presence of suramin; in the case of this drug a straight line was obtained, but on extrapolation the line did not intersect with the control line on either axis, a result suggesting that the inhibition by suramin was partly competitive and partly noncompetitive with respect to DNA. This observation might be explicable in terms of the known structure of suramin (Fig. 2); being a large negatively charged molecule, it might be able to com-

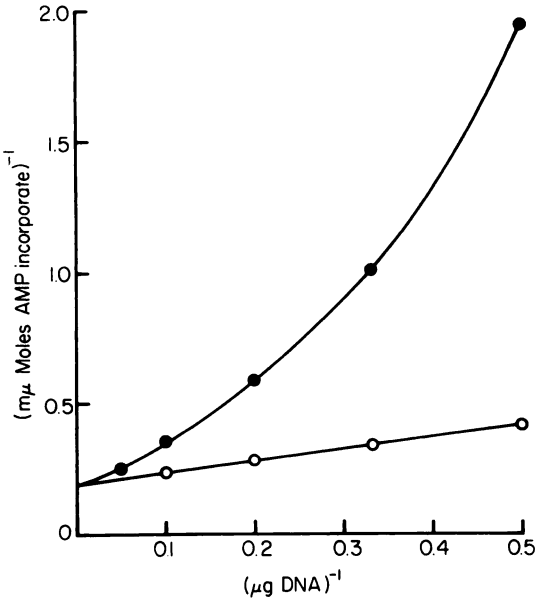

FIG. 7. *Effect of DNA concentration on the inhibition of AMP incorporation by ethidium bromide*

Reaction mixtures contained 22 μg enzyme preparation. The data are plotted according to the method of Lineweaver and Burk (20). ○, Control; ●, 6.25×10^{-6} M ethidium bromide.

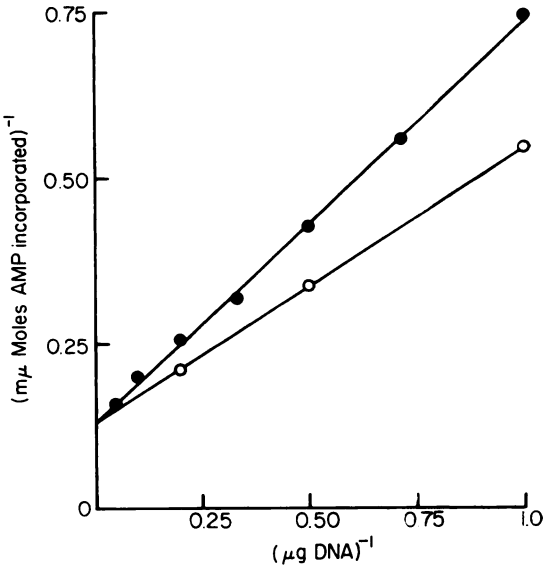

FIG. 8. *Effect of DNA concentration on the inhibition of AMP incorporation by ethidium bromide*

Reaction mixtures contained 22 μg enzyme preparation. The data are plotted according to the method of Lineweaver and Burk (20). ○, Control; ●, 1.56×10^{-6} M ethidium bromide.

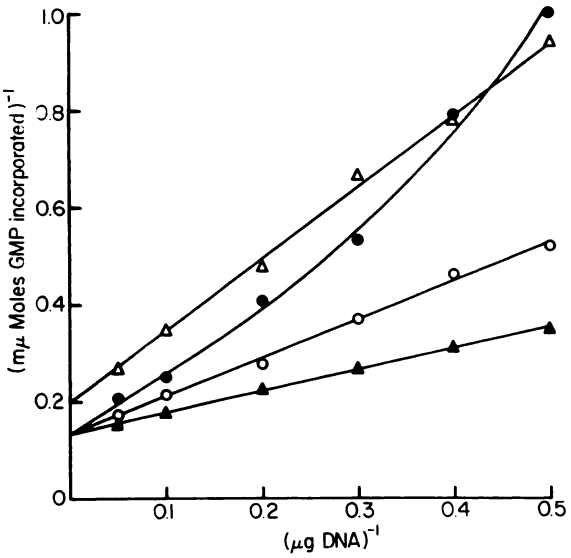

FIG. 9. *Effect of DNA concentration on the inhibition of GMP incorporation by ethidium bromide and suramin*

Reaction mixtures contained 26 μg of enzyme preparation. The data are plotted according to the method of Lineweaver and Burk (20). ▲, Control; △, 3.4×10^{-4} M suramin; ●, 5.0×10^{-6} M ethidium bromide; ○, 2.5×10^{-6} M ethidium bromide.

pete with DNA for attachment to the "active center" of the polymerase and at the same time affect the reaction in some other way—for instance, by interfering with the utilization of the nucleoside triphosphates.

Hurwitz *et al.* (12) have described experiments similar to those reported here using actinomycin D and proflavin. Inhibition of DNA-dependent RNA polymerase by both these agents was shown to be related to the DNA concentration, and in double-reciprocal plots each drug gave rise to a straight line intersecting with the control line on the axis of reciprocal reaction velocity.

Inhibition by Ethidium Bromide

Ethidium bromide forms complexes with DNA, RNA, and synthetic nucleotide homopolymers (36). Physical measurements of the binding of ethidium to these materials showed that the drug was tightly bound up to a maximal level corresponding to 1 molecule per 4–5 nucleotides and that under conditions similar to those used in the assay of RNA polymerase the dissociation constant for the ethidium-DNA complex was 7.4×10^{-6} M, expressing DNA concentration in terms of molarity with respect to deoxyribonucleotides (36). Using these values it was possible to calculate the amount of ethidium bound to the DNA primer in the reaction mixtures employed to determine the data of Figs. 4, 7, and 8, and thus to interpret the results in terms of the amount of drug bound to the primer rather than the total amount added to the system. As previously reported in a preliminary note (37), this approach showed that if the data in Figs. 7 and 8 were expressed in the form of percentage inhibition with respect to the appropriate controls they could be combined with the data in Fig. 4 to yield a plot showing a linear relationship between enzyme inhibition and drug-binding by the DNA template (Fig. 10).

Strictly speaking, Fig. 10 describes only inhibition of AMP incorporation into RNA caused by ethidium bromide, and the ques-

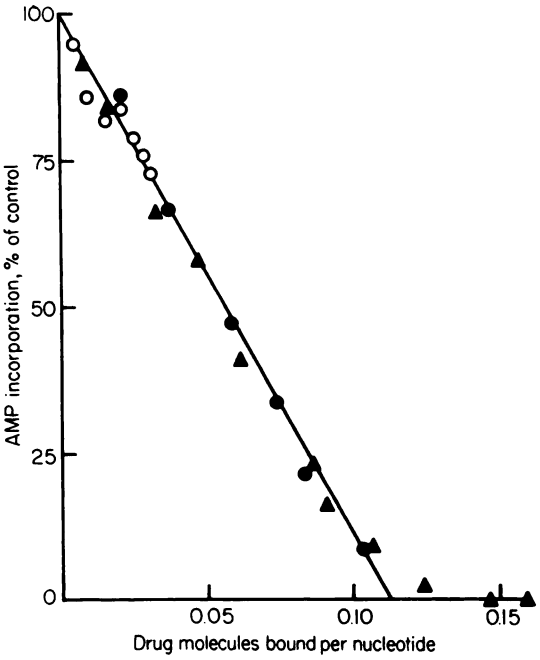

FIG. 10. *Inhibition of RNA polymerase as a function of binding of ethidium to the primer*

▲, Data from Fig. 4; ●, data from Fig. 7; ○, data from Fig. 8.

tion may be asked whether the drug preferentially inhibits the incorporation of some nucleotides into RNA more than the incorporation of others, thus modifying the normal complementary relationship between the base compositions of template and product. This possibility was investigated by studying the effect of ethidium bromide on the incorporation of each nucleotide in parallel experiments (Fig. 11). Clearly the

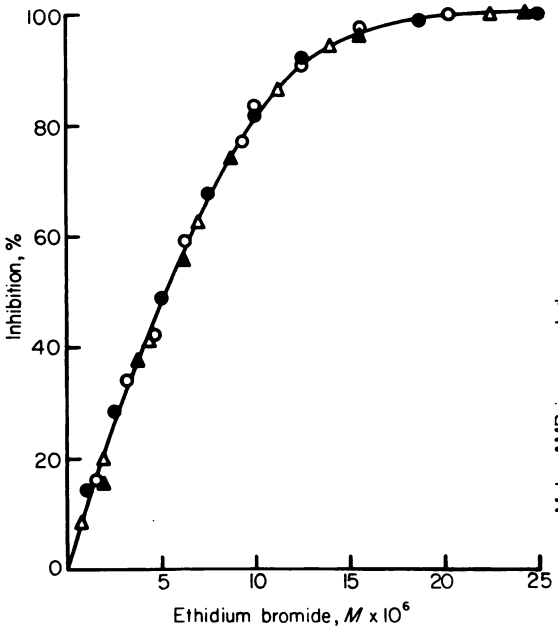

FIG. 11. *Inhibition of incorporation of AMP, CMP, GMP, and UMP by ethidium bromide*

Reaction mixtures contained 5 μg T2 DNA and 26 μg enzyme preparation. Control incorporations were (in mμmoles): AMP 4.5 (○), CMP 4.2 (●), GMP 6.3 (▲), UMP 7.7 (△).

drug had no differential effect on the incorporation of the four nucleotides, but inhibited the incorporation of each to an equivalent degree. It may therefore be concluded that in the presence of ethidium bromide the enzyme still synthesizes a product with base composition determined solely by that of the DNA despite the presence of complexed drug molecules, but that the rate of RNA synthesis is decreased in direct proportion to the amount of bound drug.

Since the ethidium–DNA complex is reversible and the drug is able to form complexes with RNA (36), the kinetics of RNA synthesis by the polymerase system could be complicated by the drug's dissociating from the primer and complexing with the product. If this were the case, the inhibition observed would be expected to decrease as the product accumulated, especially since most of the experiments reported here were performed under conditions giving "net" synthesis during the period of incubation. Time-courses of RNA synthesis in the presence and absence of ethidium bromide are shown in Fig. 12; at any point on the curves

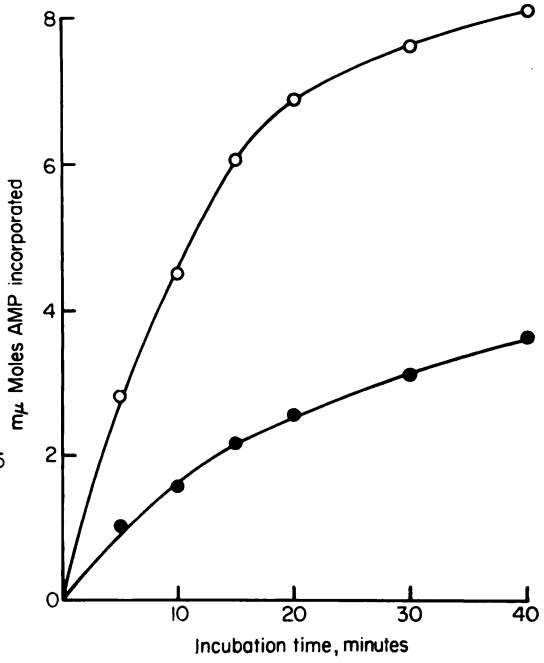

FIG. 12. *Kinetics of RNA synthesis in the presence of ethidium bromide*

Reaction mixtures contained 5 μg T2 DNA and 22 μg enzyme preparation. ○, Control; ●, 8×10^{-6} M ethidium bromide.

the gradient is proportional to the rate of RNA synthesis. It can be seen that over the period of the first 15 min the rate of synthesis in the presence of the drug was considerably depressed. During the first 5, 10, and 15 min the percentage inhibitions were 64, 66, and 64% respectively. However, subsequently the rates of synthesis tended to-

ward equality, and in fact between 30 and 40 min the same amount of RNA was synthesized in each system. This experiment does not prove conclusively that the inhibition was decreasing because of complex formation with the product as it accumulated, but it seems that the present measurements of inhibition after an incubation period of 10 min were not complicated by such an effect.

DISCUSSION

The results on inhibition of RNA polymerase activity *in vitro* by the drugs investigated in this study prompt the question whether these observations have any relevance to the activity of the agents *in vivo*. Three of the agents which precipitated the DNA primer showed this effect only at rather high concentrations, and it does not seem likely that interaction with DNA resulting in inhibition of RNA polymerase activity represents their principal mode of action against living cells. The similarity between the mode of action of neomycin and that of streptomycin (29, 30) suggests that precipitation of nucleic acids is very probably a secondary effect. In the case of Antrycide the position is not quite so clear; it certainly affects RNA synthesis in *Strigomonas oncopelti* (38), and Newton (39) has shown that the drug causes aggregation of ribosomes from the same organism. However, the dual effect of Antrycide on *E. coli* RNA polymerase illustrated in Fig. 3 is difficult to interpret, and the concentrations required to produce substantial inhibition were high. Again, although there is reason to believe that pentamidine is able to interact with nucleic acids in living cells [see the review by Newton (40)], the drug was by no means a potent inhibitor of RNA polymerase *in vitro*.

About the mode of action of prothidium bromide very little is known. The similarity between the structure of this drug and those of Antrycide and ethidium (Fig. 2) suggests that its activity *in vivo* may be related to those of the other quaternary ammonium compounds. Although it produced a substantial inhibition of RNA polymerase, the effect of prothidium was not investigated further because of the difficulty in working with reaction mixtures containing precipitates. By contrast, the powerful inhibition of RNA polymerase seen with suramin is somewhat surprising since previous work on its mode of action has not particularly implicated it in interference with nucleic acid metabolism (40). However, since suramin has been shown to inhibit a number of enzymes concerned in widely different metabolic processes (40), the possibility remains that the inhibition described here may be neither specific nor related to the primary site of action of the drug.

There are good reasons for believing that the observations on inhibition of DNA-dependent RNA polymerase by ethidium bromide are related to the action of this drug against living cells. Ethidium bromide has been shown to inhibit nucleic acid synthesis in bacteria (41), yeasts (27), and protozoa (42). It also inhibits adenine incorporation by tumor cells *in vitro* (43). In each of these systems the effect of the drug on protein synthesis appeared to be minor compared with its interference in nucleic acid synthesis. However, in most instances the drug was more inhibitory to DNA synthesis than to RNA synthesis. Elliott (44) described the inhibition of DNA polymerase *in vitro* by ethidium bromide but produced no evidence that the action of the drug on the enzyme system was related to its ability to interact with DNA. The present studies have shown that ethidium inhibits RNA polymerase activity as a consequence of complex formation with the DNA template. Comparison of the results reported here with the data given by Elliott (44) indicates that DNA polymerase is probably more sensitive to ethidium-binding by the template than is RNA polymerase, correlating with the rather greater effect of the drug on the synthesis of DNA *in vivo* (41, 42). These results provide confirmation at the enzymic level for the suggestion of Seaman and Woodbine (45) that dimidium bromide, the 10-methyl homolog of ethidium bromide, acts on bacteria "by combining with the nucleic acids of the organism so as to disrupt both its metabolism and reproduction."

The fact that the highest concentrations of ethidium bromide tested gave apparently complete inhibition of RNA polymerase activity is of interest. It is possible that this was due to the use of acid-insolubility as a criterion for RNA synthesis and that under conditions of "complete" inhibition acid-soluble oligonucleotides were still formed. Goldberg *et al.* (46) made a similar suggestion to account for the persistence of an exchange reaction between ^{32}P-pyrophosphate and nucleoside triphosphates in the presence of high concentrations of actinomycin.

The most interesting feature of the present results is the discovery that inhibition of the RNA polymerase system by ethidium was directly proportional to the amount of drug complexed to the DNA. In Fig. 10 the inhibition is plotted against molecules of drug bound per nucleotide, but the method of calculation gives no idea of the distribution of ethidium among the population of DNA molecules. If the binding of ethidium to DNA consisted of saturating some molecules completely and leaving others unchanged, rather than binding at independent sites on different DNA molecules throughout the population, then a given concentration of drug would give rise to a "bimodal distribution" of DNA molecules—some completely saturated with the drug and unable to act as primers and others free from drug and able to prime in the normal fashion. Such a mode of interaction would also be expected to give results as in Fig. 10. However, this possibility is unlikely for two main reasons: first, the postulated mode of binding would be expected to show at least some characteristics of a cooperative process, and there was no indication of such characteristics in binding studies (36); secondly, results from analytical ultracentrifugation of complexes with T2 DNA gave no evidence for the existence of a bimodal distribution of molecules (47). It seems highly probable that the interaction between ethidium and DNA up to a binding ratio of 0.2 drug molecule per nucleotide represents the binding of drug molecules to independent sites in a random distribution throughout the population of DNA molecules.

We are left then with the situation that the priming activity of a DNA molecule was decreased in direct proportion to the amount of ethidium complexed to it. Figure 10 suggests that priming activity was lost altogether when one drug molecule was bound for every 9 nucleotides; however, this figure may not be particularly significant since it may well be determined by questions of acid-insolubility. The interpretation of the relationship demonstrated by Fig. 10 depends upon the mechanism of action of RNA polymerase *in vitro*. If the enzyme is able to "transcribe" any section of a DNA molecule without defined starting points or finishing points, the interpretation would seem to be that inhibition by the binding of ethidium consisted of restricting the regions which were free to act as templates to the stretches between successive drug molecules. As the binding ratio increased so the regions available for transcription would be diminished, and as the average distance between drug molecules decreased, a limit would be reached at which the regions were so short that none could act as templates or the oligonucleotides produced were soluble in acid. On the other hand, if the enzyme requires to start at a definite point such as an end and then proceed steadily along the DNA molecule, it would be necessary to postulate that when the enzyme reached a complexed drug molecule its progress was not completely stopped but merely retarded. This postulate would be necessary to account for the 50% remaining activity observed when an average of one drug molecule was bound for every twenty nucleotides.

Other model mechanisms can be constructed to explain the observations. Further studies with inhibited systems similar to that investigated here should throw light on the problem and at the same time yield more detailed information on the mechanism of RNA polymerase action.

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