The mechanism of rifampicin inhibition of Escherichia coli RNA polymerase was studied with a newly developed steady state assay for RNA chain initiation and by analysis of the products formed with several 5'-terminal nucleotides. The major effect of rifampicin was found to be a total block of the translocation step that would ordinarily follow formation of the first phosphodiester bond. These effects were incorporated into a steric model for rifampicin inhibition. Additional minor effects of the enzyme bound inhibitor were to increase slightly the lifetime of RNA polymerase on the λPR promoter and to increase by two the apparent Michaelis constants of the initiating triphosphates. The products formed by RNA polymerase in the presence of rifampicin belong nearly exclusively to the class ppPupN. No evidence for the accumulation of such molecules was obtained in vivo.

The rifamycin class of antibiotics have been intensively studied ever since the observation of Sippel and Hartmann that rifampicin inhibited the initiation of RNA synthesis in Escherichia coli (1). Several comprehensive reviews on many aspects of the structure, RNA polymerase binding properties, and in vitro or in vitro mode of action of the inhibitor are available (2-4). A precise description of the mechanism of action of the rifamycins would be intrinsically interesting, but, in addition, such a description would allow a more precise dissection of the steps that occur during the process of RNA chain initiation.

Physical studies on the binding of rifampicin (a semisynthetic derivative of naturally produced rifamycin SV) to RNA polymerase have led to two proposed models. In the first, Baehr et al. (5) favor the notion that RNA polymerase exists in two conformations to which rifampicin binds in a simple bimolecular fashion. On the other hand, Yarbrough et al. (6) have proposed, on the basis of essentially the same type of rapid kinetic fluorescence data, that the interaction occurs in a sequential two-step process:

\[ E + R \rightleftharpoons E\,R \rightleftharpoons E\,R' \]

These authors also reasoned that the unimolecular step, \( k_3 \), is responsible for the inhibition observed in RNA chain initiation.

The physical detail of this interaction was not matched by

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† Postdoctoral fellow of the Jane Coffin Childs Memorial Fund.

‡ Present address, Chemistry Department, University of Colorado, Boulder, Colo. 80309.

The published sequence for the λPR (also termed, “6 S” promoter transcript begins ppApCpG (10). These authors point out, however, that the possibility of another Ap or Cp between the first two bases could not rigorously be ruled out. We have found that the principle product from our reactions in the presence of ATP and CTP to be ppApApC. This was determined as follows. The product as resolved on WASP chromatography was eluted and desalted on a small DEAE-Sephadex column (A-25). The product was treated with bacterial alkaline phosphatase to remove the 5'-triphosphate (100 µg/ml Worthington BAPC for 90 min at 65°C). The dephosphorylated product was deproteinized with phenol and run on polyethyleneimine TLC plates with authentic ApC as marker. Development in 0.5 M LiCl or 1.5 M LiCl followed by autoradiography showed that the product migrated slower than ApC (Rf = 0.50 and 0.69, respectively). Similarly, chromatography on Whatman No. 3 MM paper (in 1 M NH4OAc-ethanol = 1:1) indicated that the product was a trimethylsilyl derivative rather than ApC (Rf = 0.38 and 0.59, respectively). We
have also found that when the full length RNA transcript of the P74 promoter labeled with [α-32P]CTP was eluted from a gel, no detectable label was found in pppAp following limited base hydrolysis. Finally, Sklar and Weissman have sequenced the DNA in the region of the start of P74 transcription and found that the pppApApC product we find is indeed coded by the DNA template strand. The same conclusion was to the P74 start sequence was reached by Calva and Burgess by different means. Although this transcript can start with only a single A, as shown by normal base of production of pApC, we have not varied the ATP concentration over a wide enough range to determine whether it ever does start with a single A.

Polyacrylamide gel electrophoresis in 7 M urea was carried out essentially as described by Maniatis et al. (11): 15% acrylamide, 0.4% bisacrylamide in 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3. Samples for electrophoresis were brought to 6.5 M urea with the addition of solid urea equal to one-half the sample mass. Autoradiography followed by excision of product bands and Cerenkov counting was used to quantitate the species present.

The experiment performed to determine whether rifampicin treatment could result in an in vivo accumulation of dinucleotides employed E. coli B/r growing at 37°C in a Tris (0.12 M)/sals medium, pH 7.5, 0.2% glucose, 1 mM KP, (specific activity, 1100 cpm/pmol). The experiment performed to determine whether rifampicin treatment could result in an in vivo accumulation of dinucleotides employed E. coli B/r growing at 37°C in a Tris (0.12 M)/sals medium, pH 7.5, 0.2% glucose, 1 mM KP, (specific activity, 1100 cpm/pmol). When the turbidity had reached an A595 = 0.905 (viable count assay a yield of 4.6 × 10⁹/ml), half of the culture was brought to 200 μg/ml of rifampicin and to 50 μg/ml of rifampicin. After 30 min additional incubation, the two cultures were brought to 1.15 M HCOOH. Soluble nucleotides were extracted, neutralized, diluted, and applied to a DEAE-Sephadex column. A linear gradient of urea was carried out with the use of a gradient eluter. The result was a linear gradient of urea was carried out with the use of a gradient eluter.

The results of an experiment in which ATP was varied in the presence and absence of rifampicin are shown in Fig. 1. (A similar set of data for UTP is shown in Fig. 2.) Only the slopes of the double reciprocal plots were affected by the inhibitor. Although the abortive initiation assay is a steady state reaction, the results in Figs. 1 and 2 can not be analyzed in terms of simple competitive inhibition. The reason for this complexity is 2-fold: rifampicin binds to RNA polymerase with very high affinity, Kd = 10⁻¹⁰ M (12). Second, the lifetime of the rifampicin-RNA polymerase complex is about 60 min (6). Thus, under the conditions employed here, we can consider the RNA polymerase to be entirely complexed by inhibitor and to remain in such complexes during many turnovers of substrate into product. In other words, rifampicin is a partial inhibitor of the formation of the first phosphodiester bond in RNA synthesis having an effect only on the binding of the first two triphosphates and having no effect whatever on the maximal velocity of their conversion into a dinucleoside tetraphosphate. We have defined an inhibition constant, Kᵢ, for rifampicin, but in using it to modify the rate equation for abortive initiation introduced in the accompanying paper (8), we have not included the rifampicin concentration dependence. The rate equation for this reaction in the presence of rifampicin becomes:

\[
\frac{V}{K_r} = \frac{V(A)}{K_r + K_r(A)} + \frac{V(A)}{K_r + K_r(A)}
\]

This equation is consistent with the observation that rifampicin only alters the slope of the reciprocal plot. The dimensionless constant, Kᵢ, is thus a direct measure of the extent to which the slope is increased. Kᵢ was evaluated by taking the ratio of the two slopes in Fig. 1 and found to be 2.2 under our standard conditions. The ratio of slopes in Fig. 2 is more complicated because it includes the degree of saturation of ATP i.e. Kᵢ/KA.

In principle, we could use steady state kinetic methods to determine a dissociation constant for rifampicin. At concentrations of inhibitor approaching Kᵢ, the slopes of a reciprocal plot would depend on the free rifampicin concentration. The analysis of such data is simple enough (13), but the very high affinity of rifampicin (Kᵢ is comparable to the enzyme concentration employed here) would require extensive corrections for the amount of free and bound rifampicin in solution. Direct methods for determining the true Kᵢ are far more reliable (6, 12). We have, therefore, focused our attention only upon the effect of the tightly bound rifampicin. This effect is seen to be simple interference with triphosphate binding, corresponding to about 50% inhibition when standard assay conditions are employed.

The effect of rifampicin on dinucleotide synthesis was also determined for several ATP analogs to determine whether Kᵢ varied as a function of the 5'-terminal nucleotide. The results are shown in Table 1. In all cases, we observe comparable extents of inhibition with the possible exceptions of AMPP,P and AMPβP,β where the inhibition was found to be 85% and 78%, respectively. Because we have not determined Kᵢ values directly for each analog, the differences in the observed inhibition could easily be accounted for by differences in their respective Kᵢ values.

The rate (5%) that observed with ATP observed with AMP,PP in the absence of rifampicin is interesting because it illustrates the importance of the bridging oxygen between the α- and β-phosphates. Although transcription is impossible with this analog, it appears that binding of this analog even to initiate transcription is also greatly disfavored. Substitution of the β-y bridging oxygen with CH₂ or NH is seen to have less of an effect. Indeed, we have found that if the adenine base is intact, RNA polymerase can form a phosphodiester bond between many unlikely molecules and UTP. For example, diphosphoCoA and NADH can substitute for ATP at 2%
and 3% of the control rate. Systematic experiments on the specificity and fidelity of initiation will be reported elsewhere.

In Fig. 3, we show the results of rifampicin inhibition of abortive initiation on three different DNA templates as a function of temperature. In each case, we observed about 50% inhibition at 37°C; we, therefore, assume that the simple explanation of decreased triphosphate binding obtains for the X promoters Pa and Ps, however (Fig. 3, A and B). The control rates show a steep temperature dependence within a particular region that is a characteristic of the initiation reaction of RNA polymerase. The striking result is that for RNA polymerase bound to each of these promoters, rifampicin is not an inhibitor of the abortive initiation reaction below 30°C. Indeed, for the Pa promoter, a small reproducible increase in rate was observed at 25°C and below. Rifampicin binds tightly to free and to template-bound RNA polymerase at all temperatures in this range (12). Thus, the tight binding and long lifetimes of the complex that lead to an inhibition of triphosphate binding at 37°C have very little effect below 30°C on natural promoter-polymerase complexes.

Although the above experiments have allowed us to characterize some of the subtle effects that rifampicin exerts on the binding of the first two triphosphates in initiation, we were more interested in discovering an explanation for the major effect of this inhibitor: near total inhibition of long chain RNA synthesis. We approached the problem by analyzing the products formed when the third triphosphate in a sequence was added to a reaction synthesizing dinucleotides. The Pa promoter codes for an initiating sequence, AUGUA. We have previously shown that the addition of GTP to a reaction synthesizing pppApU on this promoter resulted in a marked inhibition of dinucleotide synthesis (7). This occurred because the longer chains synthesized at this site resulted in a stably initiated complex. The results in Table II show that in the presence of rifampicin there was no inhibition of pppApU synthesis when GTP was added. Furthermore, product corresponding to pppApUpG or longer species did not appear on the chromatograms used for the product analysis (data not shown). Similarly, the template-specific dinucleoside phosphate, Cpa, was elongated to CpaP in the presence of rifampicin but longer products were not found, nor was significant inhibition of CpaP synthesis observed.

In contrast to the results found with ATP and Cpa, we found that reactions in which ADP or AMP were used as the 5'-terminal nucleotides were significantly inhibited by the addition of GTP. In these two cases, rifampicin did not prevent the formation of second phosphodiester bond. The inhibition caused by GTP with AMP present was not as dramatic as that observed with ADP, so we investigated the reaction products of the AMP reaction more carefully. The two prod-

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Relative rate</th>
<th>Inhibition by rifampicin</th>
<th>Transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>11</td>
<td>66</td>
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</tr>
<tr>
<td>AMP</td>
<td>23</td>
<td>55</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Adenosine tetraphosphate</td>
<td>119</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>AMP-C-PP</td>
<td>5</td>
<td>63</td>
<td>-7</td>
</tr>
<tr>
<td>AMP-P-C-P</td>
<td>21</td>
<td>85</td>
<td>66</td>
</tr>
<tr>
<td>AMPpP-P</td>
<td>13</td>
<td>78</td>
<td>51</td>
</tr>
</tbody>
</table>

**Fig. 3.** The effect of rifampicin on abortive initiation from three DNA templates as a function of temperature. The velocities of abortive initiation under standard conditions is plotted versus temperature of the incubation medium. The templates in the three panels were: A and B, A2 DNA (the product in A, pppApU, corresponds to the Pa and Ps promoters; the product in B, pppApApC, is from the Pa promoter). The C panel corresponds to poly[d(A-T)].poly[d(A-T)]. To observe the reaction on the latter template, AMP (2 mM) was substituted for ATP at 1 μM. When present was added to 100 μM. [α-32P]UTP was present at 50 μM.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Product synthesized in 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without GTP</td>
</tr>
<tr>
<td></td>
<td>μM</td>
</tr>
<tr>
<td>Cpa</td>
<td>0.81</td>
</tr>
<tr>
<td>ATP</td>
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</tr>
<tr>
<td>ADP</td>
<td>0.07</td>
</tr>
<tr>
<td>AMP</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**Table III**

**Effect of rifampicin on synthesis of pApU or pApUpG on λP_n promoter**

The standard assay system containing 1 mM AMP and 1 mM λP_n restriction fragment was employed. Rifampicin (50 μg/ml) and GTP (200 μM) were added as indicated.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM/min</td>
<td>nM/min</td>
</tr>
<tr>
<td>None</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>9.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Rifampicin + GTP</td>
<td>3.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>
inhibition of pApU synthesis as expected from the nonsaturating concentration of AMP employed. Addition of GTP alone resulted in a comparable reduction of total UTP incorporation, but the products were about equally distributed between pApU and pApUpG. The addition of rifampicin and GTP together still allowed both products to be synthesized although at reduced rates (45% inhibition of total UTP incorporation relative to the reaction in which GTP alone was added).

A similar set of experiments was performed on the \( \lambda_R \) promoter where the starting sequence is AUC (data not shown). Again, we found that rifampicin allowed formation of the second phosphodiester bond when the \( 5' \) initiating nucleotide was AMP, but not when ATP was employed. In this case, we used labeled CTP to identify the labeled trinucleotide because the WASP mobilities of the dinucleotide and trinucleotide were overlapping. The dinucleoside phosphate, CpA, which is predicted to be an initiator for \( \lambda_R \) was not as specific as it was on \( \lambda_R \). In fact, all NpA's tested on \( \lambda_R \) were equally good substrates for elongation to NpApU, whereas on \( \lambda_R \), CpA was at least 10 times better than any of the other NpA's. In addition, we have had difficulty in resolving the \( \lambda_R \) DNA fragment from a Hae III digest after separation on acrylamide gels. Because of these two ambiguities, we have not done all of the same experiments on \( \lambda_R \) as were done on \( \lambda_R \).

These product analysis experiments and others on the T7 promoters suggest that there may be steric interference of the first translocation step when rifampicin is bound to RNA polymerase. For the normal ATP substrate, the block is complete after the formation of the first phosphodiester bond. For initiating nucleotides with smaller \( 5' \) groups (e.g. AMP, ADP), the inhibition does not become complete until after the formation of the second phosphodiester bond. This simple model and more complex possibilities will be discussed below.

Are there any promoters which break the rule stated above?

The four promoters in the early region of T7, (A1, A2, A3, and D) yielded only dinucleoside tetraphosphates in the presence of rifampicin. Three \( \lambda \) promoters (\( \lambda_R \), \( \lambda_R \), and \( \lambda_D \)) also yield similar results. The \( \lambda_R \) promoter, as discussed under "Methods," begins with the sequence AAC. However, it can also template the synthesis of pApC when AMP is used in place of ATP. In the presence of rifampicin, pppApApC can be formed as shown in Fig. 3B. It is, thus, the only exception we have found to the simplest model of rifampicin inhibition.

In part, because of this exception, we studied rifampicin inhibition of total RNA synthesis from the \( \lambda_R \) promoter. This transcript has been sequenced (10). RNA polymerase terminates synthesis after 199 bases in the absence of additional factors. In vivo, this transcript is probably not terminated in the lytic phase of phage \( \lambda \) growth, thus allowing late gene transcription to occur. We preincubated RNA polymerase with the \( \lambda_R \)-containing DNA fragment and initiated 30 min of synthesis at 37°C with the addition of NTP's and [\( ^{32}\)P]CTP. The reaction products were resolved on a 12% acrylamide, 7 mM urea gel. This allowed quantitation of all RNA chains greater than about 20 bases long. The products were also run on the standard WASP chromatography system. The reaction containing rifampicin was also preincubated for 10 min prior to initiation with triphosphates. The control reaction resulted in the synthesis of 7.8 RNA chains and 16.3 pppApApC molecules per molecule of template DNA. In the presence of rifampicin, 0.3 RNA chain and 289 pppApApC molecules per molecule of template were determined.

**Figure 4.** Effect of rifampicin on RNA synthesis on poly[d(A-T)]•poly[d(A-T)]. The radioactivity in products is plotted versus mobility in the WASP chromatography system. Long chain RNA remained at the origin. Oligonucleotides migrated from 3 to 10 cm as discussed in the text. Labeled substrate (L[\( ^{32}\)P]UTP) ran between 13 and 15 cm and is not shown in this figure. Standard assay conditions were employed for the control (A) without inhibitor and for the reaction in which rifampicin (50 \( \mu \)g/ml) was added (B). In each case the DNA was 100 \( \mu \)M.
The RNA priming of \( \Phi X174 \) DNA synthesis (14, 15). The inhibition by rifampicin of total RNA synthesis on this template was 94%. Nevertheless, 17 UTP per genome were incorporated into long (≥5) product during the 30-min incubation. The reaction was also dependent on a subunit. Only 10% of the long chain product was produced by core enzyme. The rate of dinucleotide synthesis (pppApU and pppGpU) was about 10 times higher than that observed on the \( \lambda H \) promoter when all four triphosphates were present (8). When rifampicin was added, however, very little increase in dinucleotide production was observed. The number and nature of initiation sites on this template is not well understood. A general conclusion is that the balance between abortive and productive was added, however, very little increase in dinucleotide phosphate binding to RNA polymerase: ATP, \( K_d = 2 \times 10^{-5} \) M; and even triphosphate binding to RNA polymerase is predicted. The inhibitor in close proximity to the product RNA binding region of RNA polymerase (i.e., steric inhibition); more complex schemes in which rifampicin binding results in a change in RNA polymerase conformation such that the same blockage occurs (i.e., allosteric inhibition) cannot be ruled out at this time.

**DISCUSSION**

A summary of the in vitro effects of rifampicin that have been observed is shown in Table V. Small changes in the observed lifetime of rifampicin-bound polymerase to the \( \lambda H \) promoter are not considered mechanistically significant. There is certainly no effect of rifampicin on the selectivity with which RNA polymerase ordinarily binds to bacteriophage promoters. With these minor reservations, we conclude that the effect of rifampicin on promoter binding can be described as small. When rifampicin is bound to promoter-associated RNA polymerase, the binding affinity of triphosphates is decreased by a factor of two under standard in vitro conditions. The direct effect may be on only one of the two initiating triphosphates, however. This is because in an equilibrium-ordered mechanism a competitive inhibitor of either triphosphate is predicted to affect the slope of a reciprocal plot in which the other triphosphate is varied. This prediction is confirmed by the data in Figs. 1 and 2. Indeed, independent binding evidence obtained by Wu and Goldthwait (17, 18) indicates that rifampicin may only interfere with binding of triphosphates to the low affinity purine-specific site on RNA polymerase. The binding studies were done in the absence of template, but, when these results are taken together with our kinetic data on promoter-bound RNA polymerase, it seems likely that rifampicin might interact with ATP or its binding site to increase the dissociation constant. Then, because of the close interaction between ATP and UTP discussed in the preceding paper (8), an inhibitory effect is seen on UTP binding as well.

### Table IV

<table>
<thead>
<tr>
<th>DNA Template</th>
<th>C</th>
<th>G</th>
<th>T</th>
<th>A</th>
<th>C</th>
<th>A</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>F</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Initiating Triphosphates</th>
<th>U</th>
<th>G</th>
<th>U</th>
</tr>
</thead>
<tbody>
<tr>
<td>pppApU</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pppApU</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pppApU</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pppApU</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 5. Steric model for rifampicin inhibition of RNA initiation on \( \Phi X174 \) DNA. This highly schematic interpretation, we view rifampicin as acting in simple steric fashion to prevent translocation of the larger 5'-terminated dinucleotides while allowing translocation of those with only a 5'-mono- or diphosphate. Additional effects of rifampicin and the generality of this model are discussed in the text.

In summary, we propose a simple steric model for the early inhibition of in vitro RNA synthesis by rifampicin that explains most in vivo effects as well. Fig. 5 depicts the main idea of this model superimposed on the initiation sequence of the \( \lambda H \) promoter. The accompanying table also summarizes our product analysis data qualitatively. In this view, the primary result of rifampicin binding to RNA polymerase is to prevent the translocation of an initiated dinucleoside tetraphosphate. In its simplest form, the model would have some portion of the inhibitor in close proximity to the product RNA binding region of RNA polymerase (i.e., steric inhibition); more complex schemes in which rifampicin binding results in a change in RNA polymerase conformation such that the same blockage occurs (i.e., allosteric inhibition) cannot be ruled out at this time.
phosphodiester bond has been formed. The product analysis done on λPr and λPt promoters unambiguously supports this conclusion. In addition, these data also show that rifampicin does not block the intrinsic ability of RNA polymerase to translocate. The elongation of pApU to pApUpG in the presence of inhibitor suggests that a simpler (probably steric) interference occurs.

The results obtained from the λPr promoter in which pppApApC was observed in the presence of rifampicin seem to challenge the simplest of the steric models we propose. However, this transcript can initiate at the second purine site normally as well, i.e. pApG is synthesized at normal rates. The ambiguity of an exact starting site is normally a property of only a few promoters. The lac operon transcript can begin with 5'-GTP or -ATP (19). At low triphosphate concentrations, other promoters can be "forced" to initiate synthesis at sites upstream from the normal starting sites (20). In fact, of the promoters we have studied, and whose DNA sequences are known, only λPr and lac have DNA sequences that could lead to a purine initiated chain with ambiguity in starting sequence. All of the other λ promoters and T7 promoters we have studied have purines in the template strand immediately upstream from the normal starting template base. Thus, the preference of RNA polymerase to begin chains with 5'-purine triphosphates is guaranteed by this property of the DNA sequences. If this is a common property of promoters, the strict steric model for rifampicin inhibition may only apply to this class of promoters and not to lac or λPt. Further experiments will be required to resolve this discrepancy. We emphasize, however, that no products longer than pppApApC were observed when rifampicin was present on the RNA polymerase-Pr-Pt promoter complex.

We concluded that the inhibition of RNA synthesis beyond a dinucleotide or in some cases, trinucleotide, was total. However, on long incubation in the presence of rifampicin full length transcripts were observed from the λPr promoter. The amount of product was about 5% that of the control without rifampicin. The amount of long chain product on poly[d(A-T)] and ΦX single-stranded DNA was also 4 to 6% of the control without added rifampicin. If we consider again the mechanism of rifampicin binding to RNA polymerase and assume that the two-step scheme proposed by Yarbrough et al. obtains, an explanation for the 5% residual activity can be hypothesized as follows. The number of chains that were synthesized in the presence of rifampicin at the Pr promoter corresponded to 0.3 molecule/promoter/30 min. We know that while rifampicin is bound to the enzyme, the complex is rapidly turning over pppApApC. If we assume that RNA polymerase can elongate the trinucleotide to full length product each time an inhibitor molecule dissociates and calculate from the above data a frequency of initiation under these conditions of 0.01 min⁻¹, the t½ for rifampicin would be 68 min. The reported value for rifampicin bound to RNA polymerase on T7 promoters under comparable solution conditions is 44 min (12).

If the binding of rifampicin to RNA polymerase were a simple bimolecular association step, the above explanation would not be possible because new complexes would form in kₘₐₓ × [rifampicin] seconds. Because both forward and reverse steps are limited in their overall rate by a unimolecular process, there is a short time after inhibitor dissociation when RNA polymerase is refractory to the presence of greater than saturating amounts of rifampicin. This explanation of our results identifies the second step in the scheme of Yarbrough et al. as the one with functional consequences. A strong prediction of this idea is that the same number of RNA chains should be produced per unit time independent of the concentration of rifampicin in the range well above Kd. A variety of data suggest that this is the case, but a systematic study has not been done. The residual synthesis of RNA in the presence of rifampicin would then be a function only of the polymerase-inhibitor complex lifetime.

The binding mechanism proposed by Yarbrough et al. is also substantiated by our kinetic data. If binding to rifampicin were to produce two different states of RNA polymerase as suggested by Baehr et al., we might expect to find evidence for such a mixture in our kinetic experiments. There was no indication of two enzyme forms when saturation with either ATP or UTP was determined. We would also conclude that the two species had the same temperature dependence of abortive initiation on the three templates investigated. Finally, of course, we could not explain the low residual RNA synthesis in the presence of rifampicin without the function only of the polymerase-inhibitor complex lifetime.

The interaction of rifampicin with RNA polymerase is likely to be more complex than a simple two-step binding process, however. Indeed, the differences in the temperature dependence of velocity on the three promoters suggest important steps taking place that may not be observable with spectroscopic techniques. We have left the steep temperature dependence of the control rates observed in Fig. 3 uninterpreted. There is already ample evidence in the literature that a major change in DNA and therefore enzyme conformation occurs at temperatures in the range of 20-30°C (21). Such data, usually in the form of "transition curves," are generally analyzed with a two-state formalism to yield various thermodynamic quantities. For the data reported here, that type of analysis seems singularly inappropriate because, in addition to having to make unwarranted assumptions about two or more states, the normal Arrhenius dependence of the enzyme catalyzed reaction is superimposed upon any conformational transitions. We note simply that rifampicin loses its triphosphate binding inhibitory effect on both promoters at about the midpoint of the steepest portion of the velocity versus temperature curve and that for poly[d(A-T)]-poly[d(A-T)] such a midpoint seems not to exist. Further, as postulated in the previous paper (8), product release in abortive initiation is not likely to be rate-determining unless lower temperature is viewed as having a substantial stabilizing effect on weakly initiated complexes.

The low temperature stimulation of abortive initiation by rifampicin has also been observed on T4 templates (22). The extent of stimulation was greater than what we report here on the λ promoters and may reflect a difference in the two templates. However, apparent stimulation can also be caused by using triphosphates with traces of cross-contamination.
The control reaction rate would then be lower than if pure triphosphates were used because limited chain elongation would result in stably initiated complexes that no longer synthesized dinucleotides. The addition of rifampicin would, in our model, prevent any elongation and in turn result in a larger fraction of total enzyme available to synthesize dinucleotides.

The above explanation for the presence of long chain RNA synthesis in the presence of rifampicin applies not only to promoters such as $\lambda$P$_R$, but also to $\Phi X$174 single strands and to poly[d(A-T)]-poly[d(A-T)]. In the absence of rifampicin, the results in Fig. 4 show that a small amount of pppApU is formed, and that longer oligomer products, pppApUpA and pppApUpApU, also accumulate. In the rifampicin-containing reaction, long RNA chain synthesis (origin radioactivity) is reduced about 20-fold and pppApU is the only small product in evidence. The degree of inhibition of long chain synthesis is not as complete as that observed on the $\lambda$P$_R$ promoter. This could be due in part to the direct covalent elongation of the 3'-OH ends of the template as described by Nath and Hurwitz (23). In our model, rifampicin is predicted to have no significant effect upon this aberrant reaction because the RNA product groove that we picture as being occluded by rifampicin would already be occupied by the 3'-OH end of the DNA primer strand. We have not as yet investigated this template system further. Therefore, we emphasize only that, on a wide variety of templates, the several effects of rifampicin can be seen as deriving from its principal effect on RNA polymerase, to wit that, when bound on this enzyme, absolute blockage of chain elongation occurs, usually after the formation of the first phosphodiester bond.

As a practical matter, the use of rifampicin in vivo or in crude extracts to block RNA priming of DNA synthesis appears justified on the basis of our experiments. Although short chains were made in the presence of rifampicin on $\Phi X$174 single-stranded DNA, long incubation times were required to observe significant quantities. From our observations in vivo, we predict that any short chains that were not immediately incorporated into longer product would very quickly be degraded to mononucleotides. The rate of stable RNA synthesis in E. coli after rifampicin treatment has been measured (24), and corresponds to about 90 to 95% inhibition of initiation. This drug is clearly an effective RNA initiation inhibitor, but the block is by no means absolute. In fact, if our interpretation of the residual RNA synthesis in the presence of rifampicin is correct, then the in vitro and in vivo synthesis results suggest approximately equal lifetimes for RNA polymerase-rifampicin complexes under both conditions.

Rifampicin has been used as a probe of the individual steps occurring in the RNA polymerase initiation reaction (25-27). The results reported in the preceding paper showed that there are multiple pathways available to an initiated RNA polymerase complex. A general conclusion was that an elongating RNA polymerase was not stably initiated until a variable number of nucleotides had been incorporated into the nascent RNA chain. We have shown here that the principal block imposed by rifampicin usually occurs after the formation of the first phosphodiester bond. The rifampicin challenge technique referred to above assumed a bimolecular competition between triphosphates and rifampicin to form initiated complexes or inhibited complexes, respectively. From an analysis of their data, $k^*$, the rate constant for initiation, was defined and evaluated under a variety of conditions. Although many of the conclusions resulting from this work have been shown to be generally applicable to the initiation mechanism, certain aspects of the interpretation should now be reevaluated. In particular, the observation that ATP did not display saturation behavior and that $k^*$ was only slightly dependent on temperature are both counter intuitive based upon how enzymes usually react. Further, in a subsequent detailed analysis, Rhodes and Chamberlin used the same approach to determine an order of addition of UTP and ATP (28) in the initiation reaction that was reversed from that determined by the direct methods we have reported in the preceding paper (8). Finally, $k^*$ was strikingly insensitive to the DNA template or individual promoter investigated.

We will not present a detailed kinetic analysis of how $k^*$ in the rifampicin challenge assay relates to the steps we have identified both in the initiation reaction and in the rifampicin inhibition scheme. But we indicate in the following how one simple idea might rationalize at least part of the complexity. First, we now know from physical evidence that the rifampicin-RNA polymerase binding process is composed of two steps: one bimolecular step followed by (at least) one unimolecular conformational change. We have argued above that the block in RNA synthesis occurs after the unimolecular step. In other words, although rifampicin binding to RNA polymerase is usually kinetically second order, the relevant step for inhibition is likely to be the first order conformational change of the complex. Second, the sequence of steps leading to a stably initiated RNA chain is composed of at least three bimolecular triphosphate binding steps, two chemical reaction steps and a unimolecular translocation event. As a very minimum, the $k^*$ determined from the rifampicin challenge technique would of necessity include all six steps listed above in the initiation reaction weighted in proportion to their contribution to the overall rate of achieving a stably initiated complex. A simpler possibility would be that $k^*$ is in fact related to the rate at which initially bound rifampicin induces the inhibitory conformational change in RNA polymerase. In other words, if the essential competition in the rifampicin challenge experiment is between the unimolecular conformational change caused by bound inhibitor and the pseudo-first order steps leading to stable initiation, the $k^*$ for “initiation” will really be a measure of the rate at which the rifampicin induced conformational change occurs. A quantitative derivation of the above view would be hopelessly complex and lead to little new understanding. Indeed, the essential point we emphasize here is that both rifampicin binding and the initiation process (i.e., series of many discreet steps) can no longer be viewed as simple competing steps. The added complexity of the initiation reaction portrayed here is simultaneously a basis for more detailed experiments on both initiation and its control and on rifampicin inhibition.

Although most rifamycin analogs are efficacious in direct proportion to their RNA polymerase binding constant (2), the work presented here suggests that there may be a class of tight binding analogs that merely slow down the translocation process rather than block it completely. On the other hand, some of the weakly bound inhibitors might still block translocation when present, but by virtue of their lowered affinity allow more detailed kinetics to be applied to the dissociable interaction. Streptolydigin, while not a rifamycin may belong to the second class described above.

In conclusion, we point out that the steric model we have proposed is based on kinetic and product analysis data and is, therefore, inadequate to determine structural relationships in the enzyme active site. The pseudo-structural aspects of this proposal are intended only to reduce our findings to a single simple idea.

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