Volume 9 Number 24 1981 Nucleic Acids Research

Rifampicin inhibition of RNA synthesis by destabilisation of DNA-RNA polymerase-oligonucleotide-complexes

Wolfgang Schulz and Wolfram Zillig

Max-Planck-Institut für Biochemie, 8033 Martinsried bei München, GFR

Received 18 August 1981

ABSTRACT

Although the antibiotic rifampicin inhibits the transcription of poly[d(A-T)] by E.coli RNA polymerase, a series of short oligonucleotides is produced. It is claimed that the overall inhibition of RNA synthesis by rifampicin is caused by a destabilising effect on the binding of the intermediate oligonucleotides to the active enzyme-DNA complex.

Rifampicin itself can only interact specifically with RNA polymerase if the enzyme is free or in a binary complex with DNA. However, the enzyme is not susceptible in a ternary complex, even if the "RNA" is as short as a trinucleotide.

INTRODUCTION

The antibiotic rifampicin is a potent inhibitor of eubacterial RNA polymerases and much work has been done to elucidate the mechanism of its action. Sippel and Hartmann showed in 1968 (1) that chain elongation in the transcription reaction is not affected by the antibiotic, but a step or steps in initiation are inhibited. On the other hand, several investigators (2,3) found that the specific binding of the enzyme to promoter sites is not influenced by rifampicin. It was generally accepted that rifampicin blocks the formation of the first phosphodiester bond, until Johnston and Mc Clure showed (4) that dinucleoside tetraphosphate is synthesised abortively from the two starting nucleoside triphosphates in the presence of rifampicin.Recently,Mc Clure and Cech (5) presented a model about the mechanism of rifampicin action.They claimed that rifampicin blocks translocation and thus elongation by a complete steric interference.

In this paper experimental data are presented which support a different model. We conclude that rifampicin destabilises the binding of short oligonucleotides to the RNA polymerase-DNA com-

© IRL Press Limited, 1 Falconberg Court, London W1V 5FG, U.K.

plex, and thus reduces the possibility that they can be elongated.

MATERIALS AND METHODS

Highly purified nucleoside triphosphates were purchased from ICN, Cleveland.

$\alpha[^{32}P]$ labelled ribonucleotides were obtained from Amersham Buchler, Braunschweig. $[^{32}P]$-pyrophosphate was from NEN. Charcoal (0.3-0.5 mm in diameter) and polyethyleneimine cellulose thin layer sheets with fluorescent indicator (PEI TLC) were obtained from Merck, Darmstadt. Rifampicin was from Sigma.

RNA polymerase holoenzyme was purified from E.coli B according to Zillig et al (6) with subsequent chromatography on T_4 DNA cellulose. Saturation with σ was tested according to Hansen and Mc Clure (7).

Poly[d(A-T)] was synthesised with T_4 DNA polymerase as described by Aposhian and Kornberg (8).

Preparation of pppApU: 10 ml reaction mixtures containing 0.1 mg/ml T7 DNA, 200 µg/ml RNA polymerase, 20 µg/ml rifampicin, ATP and UTP (both 1mM) were incubated at 30°C for about 24 hours. The mixture was loaded onto a 1.5 x 70 cm DEAE cellulose column (equilibrated with 0.3M triethyl ammonium bicarbonate (TEAB), pH 8). The column was run isocratically. Fractions were analysed by PEI thin layer chromatography. Peak fractions were pooled and lyophylised. The product was resolved in 50% ethanol and lyophylised repeatedly and finally resolved in water. Its concentration was determined spectrophotometrically at 260 nm, assuming that the extinction (1mM = 25 OD_{260}) for the adenosine or uridine moity in the compound is the same as in free ATP or UTP respectively.

pppApUpA was prepared enzymatically from pppApU and ATP using RNA polymerase and as a template poly[d(A-T)].Purification and determination of the concentration was done as for pppApU (1mM = 39 OD_{260}).

pppApUpApU was prepared from pppApUpA and UTP in a way analogous to that applied for the trinucleotide.Purification was performed by PEI thin layer chromatography.The respective band was removed from the thin layer plate,washed with water and the pro-

duct was eluted with 1M TEAB.

For thin layer chromatography (TLC),PEI plates and potassium phosphate buffer,pH 8 with 10% ethanol were used.For separation of di- and trinucleotides from the nucleoside triphosphates the buffer was 0.2M (R_F values relative to UTP are: UTP:1,ATP:0.53, pppApU:0.43 and pppApUpA:0.24).For separation of the oligonucleotides from each other,it was 0.3M.After application of the samples all TLCs were pre-run in a 5mM phosphate buffer for 15 min. All compounds mentioned remained at the base line at this ionic strength.For quantitative determination of radioactive products, the bands were excised and their Cerenkov radiation was determined and corrected according to the total radioactivity of the TLC track.

Electrophoresis was carried out on 20% acrylamide gels 200x 400x0.5 mm) at room temperature according to Maxam and Gilbert (9). The gels were pre-run at 1200 V before sample application.

Sample preparation:5 µl of a "reaction-stop-mixture" containing 9M urea,0.05M EDTA and 0.03% of the dyes orange G,bromphenol-blue and xylene cyanol FF was pipetted onto a Teflon sheet and evaporated to dryness.5 µl of the samples was pipetted onto the dried "stop-mixture" and mixed.3-5 µl amounts of this mixture were sucked into small polyethylene tubes and applied to the gel.

Quantitative analysis of the products : The total radioactivity applied to the gel was determined by counting the radioactivity in the polyethylene tubes before and after application of the samples.The radioactivity of the product bands was determined after they had been cut out from the gel and was corrected according to the total radioactivity.

Elution of oligonucleotides from acrylamide gels: the bands were excised from the gel and the gel pieces were chopped and mixed with water. After standing for some hours at room temperature the polyacrylamide was removed by centrifugation. The supernatant was put onto a small DEAE cellulose column. After extensive washing with water the oligonucleotides were eluted with 1M TEAB and lyophylised.

General reaction conditions: If not otherwise stated, all reactions were performed at 22°C in low salt buffer (LS-buffer:

30mM Tris pH 7.9, 6mM $MgCl_2$, 60mM NH_4Cl, 0.1 mg/ml gelatine). The concentrations of the reaction components were: poly[d(A-T)]: 0.1 mg/ml, RNA polymerase: 0.1 mg/ml, rifampicin: 20 µg/ml, $\alpha[^{32}P]$ UTP and $\alpha[^{32}P]$ ATP : 5µM (specific radioactivity about 100 Ci/mMol).

Gel filtration: Sepharose 4B columns (300x6mm and 250x2.5mm) were equilibrated with LS buffer. All columns were run at 4^oC. The fraction size was 8 or 4 drops respectively.

Routine preparation of ternary complex containing trinucleotide: Poly[d(A-T)], RNA polymerase, pppApU (50 µM) and $\alpha[^{32}P]$ATP (5 µM) were incubated for 20 minutes at 22^oC. The complex was isolated by chromatography on Sepharose 4B. It appears in the void volume of the column.

The pyrophosphate exchange was assayed by determining the amount of ^{32}PP (specific radioactivity of PP_i was about 100 Ci/mol) incorporated in the nucleoside triphosphates. 100 µl samples were mixed with 1 ml suspension of granulated activated charcoal in 10ŏ trichloroacetic acid containing 0.2 M $Na_4P_2O_7$. The suspension was filtered through a cotton filter in a large Eppendorf tip and washed with about 20 ml 5% trichloroacetic acid and in addition with about 5 ml water. The nucleoside triphosphates were eluted with 10 ml eluting buffer (48% water, 50% ethanol, 1% ammonia). Cerenkov radiation of the eluate was determined.

RESULTS

It has been previously reported by Mc Clure and Cech (5) that E.coli RNA polymerase synthesises dinucleoside tetraphosphate in the presence of rifampicin, but that no detectable amount of tri-or tetranucleotide is produced, with the only exception of pppApApC using the $\lambda P_{R'}$ promoter as a template. We could not confirm this finding. Further incubation of a preincubated mixture of poly [d(A-T)], RNA polymerase and rifampicin with ATP and $\alpha[^{32}P]$ UTP for 4 hours leads to the formation of pppApU, but several other reaction products are formed in addition, as shown by autoradiography of a polyacrylamide-urea gel (Fig.1). In the upper part of the gel there is some high molecular weight RNA. However, most of the reaction products are of low molecular

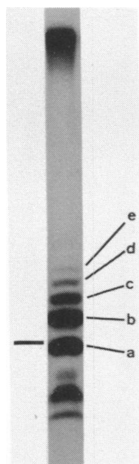


Fig.1: Products of the poly[d(A-T)]dependent synthesis in the presence of rifampicin. A mixture of 10 µl containing poly[d(A-T)] RNA polymerase and rifampicin was preincubated at 22°C for 10 minutes. Incubation was continued for 4 hours after addition of ATP and α[^{32}P] UTP (50 µM or 5 µM respectively). The bar indicates the position of the marker dye orange G.

weight.For identification,bands a, b, c, d and e were cut out from the gel,isolated as described in Materials and Methods, and subjected to PEI thin-layer chromatography.By comparison with references,bands a, b and c were identified as pppApU, pppApUpA and pppApUpApU respectively.Bands d and e have the mobilities expected for the penta- and hexa-nucleotides (Fig.2). In spite of the high molar ratio of rifampicin to RNA polymerase one could argue thatpppApU is only elongated by enzyme molecules from which rifampicin is transiently dissociated. In In this case, one would expect that very high concentrations of rifampicin would suppress or at least decrease the formation of tri-,tetra-,and longer oligonucleotides.The experiment shown in Fig.3 rules out this possibility: There is no further change

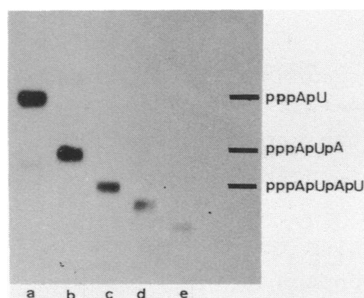


Fig.2: Thin layer analysis of the oligonucleotides recovered from the acrylamide gel.The positions of pppApU,pppApUpA and pppApUpApU are indicated at the margin.

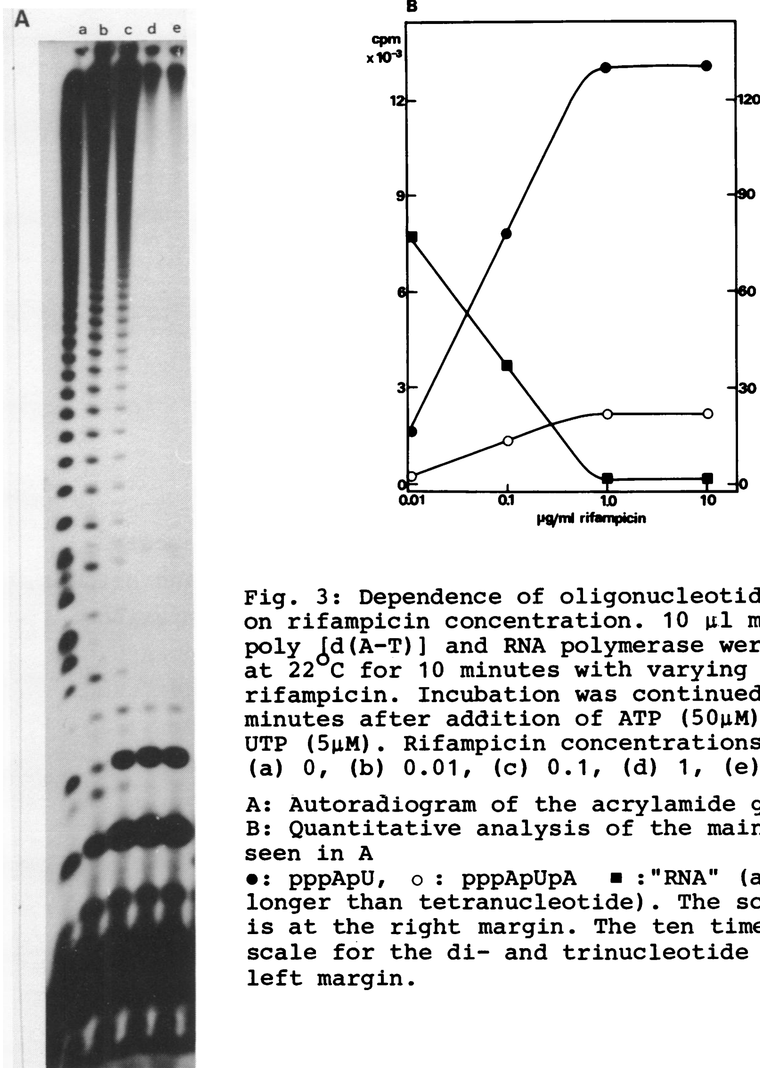


Fig. 3: Dependence of oligonucleotide synthesis on rifampicin concentration. 10 µl mixtures of poly [d(A-T)] and RNA polymerase were incubated at 22°C for 10 minutes with varying amounts of rifampicin. Incubation was continued for 15 minutes after addition of ATP (50µM) and $\alpha[^{32}P]$ UTP (5µM). Rifampicin concentrations were: (a) 0, (b) 0.01, (c) 0.1, (d) 1, (e) 10 µg/ml.

A: Autoradiogram of the acrylamide gel.
B: Quantitative analysis of the main products seen in A
●: pppApU, ○ : pppApUpA ■ :"RNA" (all products longer than tetranucleotide). The scale for "RNA" is at the right margin. The ten times larger scale for the di- and trinucleotide is at the left margin.

in the product pattern once the rifampicin concentration exceeds the point of equivalence with RNA polymerase.Fig.3A shows an autoradiogram of an acrylamide gel with the separated reaction products,while Fig.3B presents the corresponding quantitative analysis.

There are two mechanistical possibilities for the formation of oligonucleotides.The primary product pppApU may be elongated

either directly without a preceding dissociation or may alternatively first dissociate and accumulate before elongation. Both possibilities seem to be realised.First evidence comes from the time course experiment,shown in Fig. 4.
During the initial 30 minutes the synthesis of di-,tri- and tetranucleotide proceeds linearely and no "lag" is observed for tri- or tetranucleotide synthesis.In the late phase of the kinetics,the production of dinucleotide ceases due to consumption of the UTP,whereas trinucleotide production continues.
An isotopic exchange experiment confirms the hypothesis that both of the above mentioned possibilities occur (Fig.5).
A reaction mixture of RNA polymerase, poly[d(A-T)], rifampicin, ATP and α[^{32}P] UTP was incubated together with increasing amounts of unlabelled pppApU. The ratio of radioactive dinucleotide to trinucleotide should remain constant independent of the pppApU concentration, if the dinucleotide is elongated directly. However, this ratio should increase with the pppApU concentration, if the dinucleotide is only elongated after prior dissociation and subsequent re-binding. The horizontal line illustrates the case in which only that fraction of the dinucleotide which re-

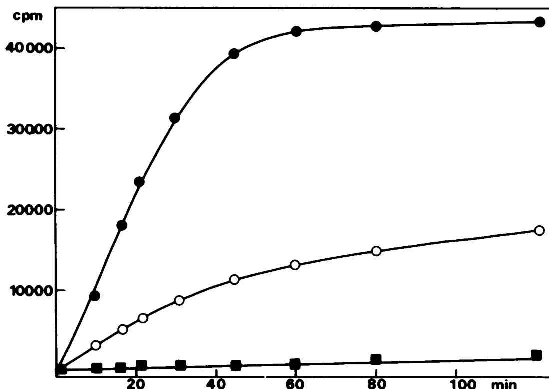


Fig.4: Time course of oligonucleotide synthesis.A preincubated mixture of poly {d(A-T)},RNA polymerase and rifampicin was supplemented at time zero with ATP (50 μM) and α{^{32}P} UTP (5 μM).Aliquots of 5 μl were removed at the indicated times and samples were loaded onto an acrylamide gel.The quantitative amounts of di-,tri- and tetranucleotides were determined.
•: dinucleotide, ○: trinucleotide, ▪: tetranucleotide

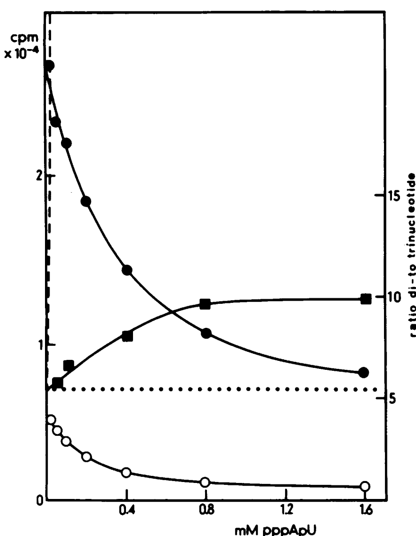


Fig.5: Isotopic exchange between the pppApU synthesised during reaction and exogenous dinucleoside tetraphosphate.ATP (50 μM) and α{^{32}P}UTP were added to a 10 μl mixture containing poly {d(A-T)},RNA polymerase,rifampicin and increasing amounts of pppApU and incubated for 15 minutes.Products were separated by acrylamide gel electrophoresis and determined quantitatively.
--- : theoretical line for immediate and complete exchange,
..... : theoretical line for no exchange
●: dinucleotide product, ○: trinucleotide product
■ : ratio of di- to trinucleotide

mains bound to the enzyme-DNA complex may be elongated, while the broken line has been calculated for the case in which all the pppApU synthesised from ATP and labelled UTP exchanges immediately with the unlabelled dinucleotide. The other curves demonstrate the actual experimental findings. The dinucleotide competes with the ATP in the initiation reaction and thus reduces the formation of labelled di-, and trinucleotide. The ratio of di- to trinucleotide becomes at first higher with increasing concentrations of pppApU, indicating that free pppApU can be bound and elongated.The plateau of the curve indicates that only a limited proportion of the synthesised dinucleotide can exchange with the unlabelled dinucleotide before it is elongated.

Fig. 6 shows the time course of trinucleotide formation from free pppApU and ATP in the presence and absence of rifampicin.

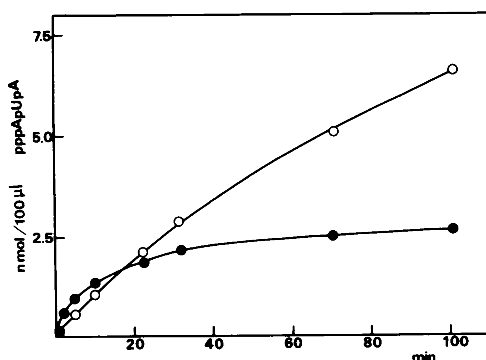


Fig. 6: Time courses of trinucleotide synthesis. Reaction was started by addition of pppApU and α[^{32}P] ATP (final concentrations 50 µM and 5 µM respectively) to two pre-incubation mixtures both containing poly [d(A-T)], RNA polymerase and one also rifampicin. 5 µl aliquots were removed at the indicated times and samples were loaded onto an acrylamide gel. o: with rifampicin. ● :without rifampicin.

In its absence,the rate of trinucleotide production is initially high,but declines to a low value when trinucleotide concentration becomes stoichiometric to that of RNA polymerase. In the presence of rifampicin,the reaction rate is initially lower,but remains almost constant,even if the concentration of the synthesised trinucleotide far exceeds the enzyme concentration.This suggests that the rate limiting step in the absence of rifampicin is the release of the trinucleotide product, and that rifampicin enables the enzyme to release the product faster,rendering the enzyme free to bind new substrates.

To test directly the influence of rifampicin on complex stability,a ternary complex was prepared from poly {d(A-T)} ,RNA polymerase,pppApU and α{^{32}P} ATP and isolated as described in Materials and Methods.The decay kinetcs at 22°C of this isolated complex was analysed in the presence and absence of rifampicin.Surprisingly,the same stability was found whether rifampicin was present or not: In both cases the time course of the decay of active ternary complex was the same as measured by its ability to elongate the bound trinucleotide to tetranucleotide (data not shown) or poly {r(A-U)} respectively (Fig.7A)

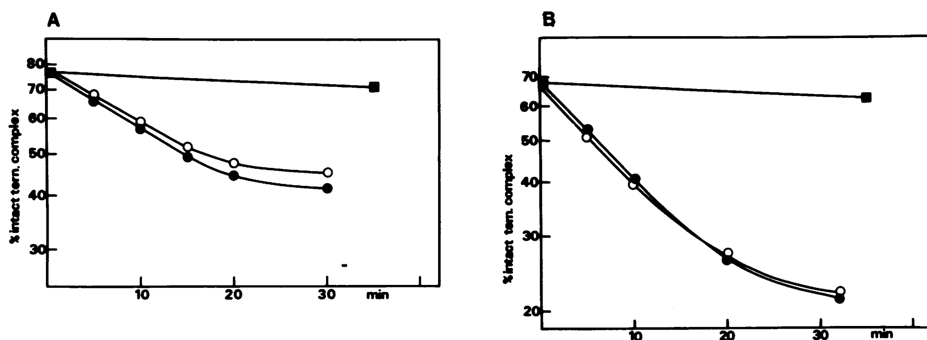


Fig.7: Decay kinetics of ternary complex. Isolated complex was incubated at 22°C or 0°C in the presence or absence of rifampicin. 5 µl aliquots were removed at the indicated times and either supplemented with ATP and UTP (Fig. 7A) or rechromatographed on Sepharose 4B columns (Fig. 7B).

A)After incubation for 4 minutes with ATP and UTP,the samples were applied to a PEI thin layer.The proportion of active ternary complex was calculated from the amount of radioactivity at the base line.

o: with rifampicin at 22°C, •: whithout rifampicin at 22°C,
▪: without rifampicin at 0°C.

B)Samples were put onto small Sepharose 4B columns.The proportion of intact ternary complex was calculated from the ratio of radioactivity in the void volume to radioactivity in the "salt volume".

o:with rifampicin,22°C •:without rifampicin,22°C,
▪:without rifampicin,0°C.

A direct measurement of the decay of ternary complex has been obtained by rechromatography of aliquots taken at different incubation times (Fig. 7B).From the linear part of the decay curves a half life of about 20 minutes at 22°C was estimated. At 0°C however,it was more than ten times higher.
The experiments show clearly that rifampicin does not inhibit transcription by interactions with the ternary complex,even if the "RNA" therein is as short as a trinucleotide.Therefore, the difference between the time courses with and without rifampicin still needs an explanation.In the time course experiment the rifampicin was added to the polymerase <u>before</u> ternary complex formation could occur.In the tests for complex stability, however,the antibiotic was added <u>after</u> complex formation.

While trinucleotide is synthesised from dinucleotide and

ATP in the presence of rifampicin,it is not possible to isolate the corresponding ternary complex.The ternary complex could only be isolated in the absence of the antibiotic.The evidence is given in Fig.8A which shows the elution profiles of reaction mixtures with and without rifampicin from Sepharose 4B columns. Gel analysis (Fig. 8B) of the fractions shows that trinucleotide appears only in the ternary complex peak if the reaction was performed without rifampicin.

The same result is obtained in trying to isolate ternary complex directly formed from its components poly {d(A-T)},RNA polymerase and α{^{32}P}-labelled pppApUpA,in the presence or absence of rifampicin.Fig.9 shows the elution curves from Sepharose 4B columns.A stable ternary complex could be recovered only in the absence of rifampicin.It showed the same stability as the ternary complex produced by elongation of pppApU.The bound trinucleotide could also be elongated.

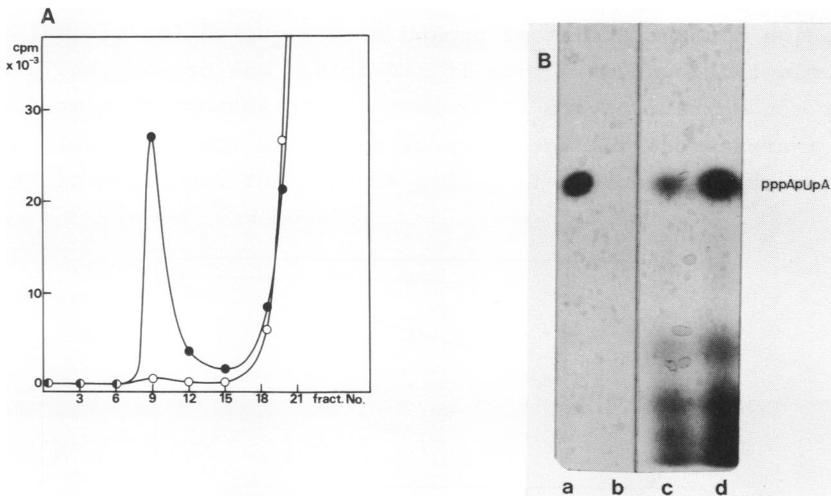


Fig.8, A)Elution profiles of reaction mixtures with and without rifampicin.15µl mixtures of poly {d(A-T)},RNA polymerase, pppApU and α{^{32}P} ATP were incubated at 22oC for 20 minutes in the presence or absence of rifampicin and separated on Sepharose 4B. o: with rifampicin, •:without rifampicin.

B)Analysis of the column fractios by acrylamide gel electrophoresis: a: fraction 9,without rifampicin, b: fraction 9,with rifampicin, c: fraction 21,without rifampicin, d: fraction 21,with rifampicin

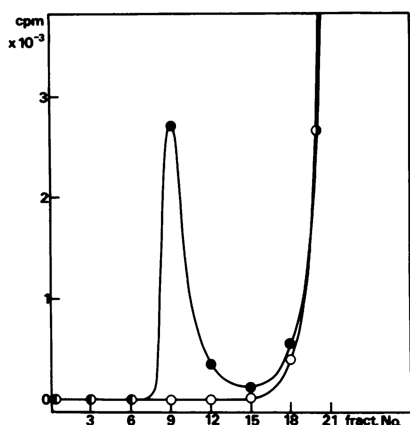


Fig.9: Preparation of ternary complex from its components. 5 µl mixtures of poly[d(A-T)], RNA polymerase and [^{32}P] labelled pppApUpA (0.1 mM, specific activity about 1000 Ci/mol) were incubated with or without rifampicin for 10 minutes at 22°C, chilled to 0°C and separated on Sepharose 4B columns.
o :with rifampicin
• :without rifampicin

If rifampicin inhibits transcription by reducing the stability of the binding of short oligonucleotides to the enzyme DNA complex and thus decreases the probability of elongation,its effect should be suppressed by increasing the oligonucleotide concentration.The experiment shown in Fig.10 gave the expected result.High concentrations of pppApU were required for maximal trinucleotide synthesis rate if rifampicin was present,while much lower concentrations are sufficient in its absence.When we further increase the concentration of pppApU we found not only no further stimulation,but in fact a significant reduction of tri-

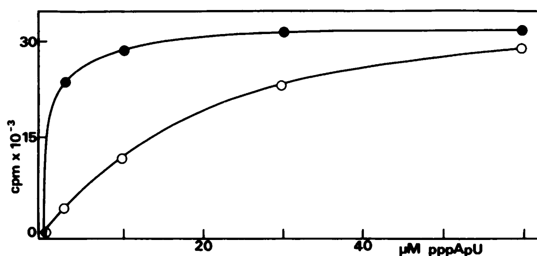


Fig.10 : Suppression of rifampicin inhibition by high concentrations of pppApU. α[^{32}P] ATP and increasing amounts of pppApU were added to two sets of 18µl preincubation mixtures containing poly [d(A-T)] and RNA polymerase; one set contained in addition rifampicin. Incubation was continued for 10 minutes. Quantitative determination of trinucleotide product was done after acrylamide gel electrophoresis.
o: with rifampicin, •: without rifampicin.

nucleotidesynthesis,both in the presence and absence of rifampicin(data not shown).It is not yet clear if this effect is meaningful or due only to some unknown contaminants in our pppApU preparation.

The destabilising effect of rifampicin should be seen not only in the elongation of di- to trinucleotide.but also in the pyrophosphorolosis of the dinucleoside tetraphosphate to nucleosidetriphosphates.Fig.11 shows the strong influence of rifampicin on this reaction.We monitored the back reaction of pppApU formation by measuring the pyrophosphate exchange according to:

pppApU + $\overset{*}{P}P_i$ = ATP + UT$\overset{*}{P}$. T7 D111 DNA (deletion mutant D111) was used as a template.At the only strong promoter (A_1) the reaction of ATP with UTP is limited to the formation of dinucleoside tetraphosphate.Under the chosen conditions the reaction rate is not significantly different whether rifampicin is present or not.

All experiments described so far,except that just discussed, were performed with poly [d(A-T)] as template.As preliminary evidence that our hypothesis about the destabilising effect of rifampicin holds also for natural DNA,we have shown that the synthesis of trinucleotides and not only dinucleotides occurs at the A_1 promoter of whole T7 D111 DNA.Fig. 12 shows that the trinucleotide pppApUpC (marked by an arrow) is in fact synthesised in the presence of rifampicin from the nucleoside triphosphates and also by elongation of pppApU.The arrowed band is almost

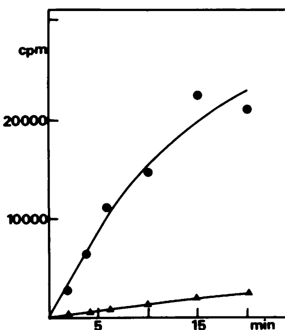


Fig.11: Kinetics of pyrophosphate exchange in the presence and absence of rifampicin. ATP, UTP and {^{32}P} - pyrophosphate (final concentrations: 1 mM, 0.5 mM and 0.2 mM) were added at zero time to two 1 ml mixtures preincubated at 30^{0}C, one containing 0.1 mg/ml T7 D111 DNA, 20 µg/ml RNA polymerase and the other containing in addition, 20µg/ml rifampicin. 100 µl aliquots were removed at the indicated times and the extent of exchanged pyrophosphate was determined.
▲: with rifampicin,
●: without rifampicin.

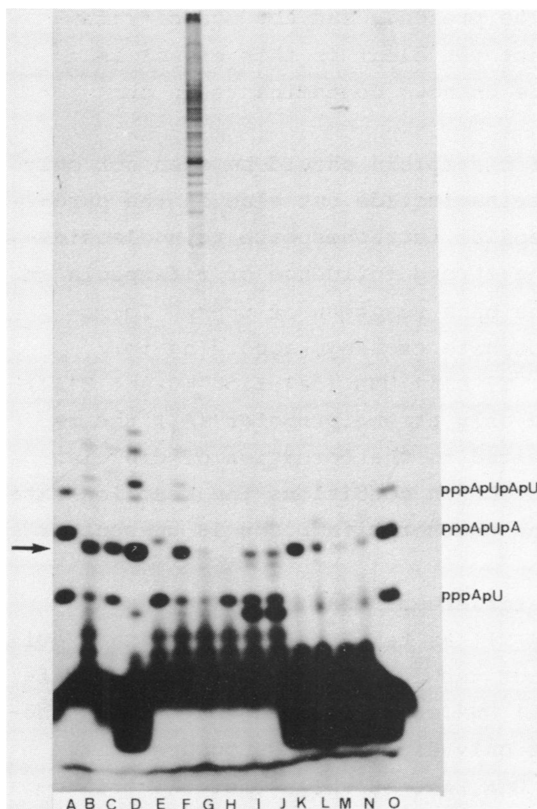
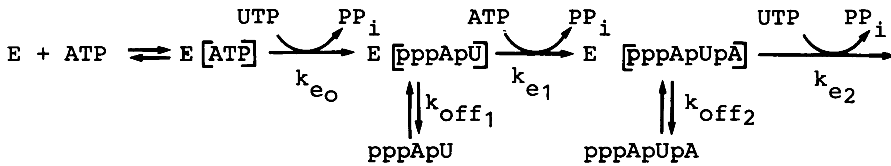


Fig. 12 : Products of the D 111 DNA dependent oligonucleotide synthesis in the presence and absence of rifampicin. Different combinations of nucleotides were added to preincubated mixtures of T7 D111 DNA (0.1 mg/ml), Eσ (20 μg/ml) with and without rifampicin (20 μg/ml). Incubation was continued for 30 min. at 22°C. The reaction was stopped and samples were loaded onto an acrylamide gel as described in Materials and Methods. The final volume was 10 μl and the final concentrations were : pppApU: 30μM; ATP: 25μM; UTP: 5μM; CTP: 5μM; GTP: 5μM. A and O: markers produced at poly[d(A-T)] ; B-D: ATP, UTP and CTP without rifampicin, ATP is labelled in lane B, UTP in C and CTP in D; E-G: without rifampicin, ATP is labelled, E: ATP and UTP, F: ATP, UTP and CTP; G: ATP, UTP, CTP and GTP; H-J: The same as E-G, but with rifampicin; K: pppApU and α[^{32}P] CTP without rifampicin; L: pppApU and α[^{32}P] CTP with rifampicin; M: pppApU, α[^{32}P] CTP and GTP without- and N: pppApU, α[^{32}P] CTP and GTP with rifampicin.

certainly pppApUpC because it always appears whether ATP, UTP or CTP carries the label. In addition to pppApU and pppApUpC two other products are synthesised overstoichiometrically in the presence of rifampicin (lane I and J). We assume these to be pppApC and pppApCpA, synthesised at the C promoter (initiation sequence: ACAG··· (10)). They only appear when either ATP or CTP is labelled.

DISCUSSION

The first steps in poly [r(A-U)] chain initiation and elongation can be outlined by the following simplified scheme:



The DNA-bound enzyme E first binds ATP, then UTP in an ordered reaction (11,12).After formation of the first phosphodiester-bond the product pppApU either dissociates (abortive initiation) or is elongated. The relationship between abortive product formation and elongation in each elongation step is determined by the ratio of the "off-constant" $k_{off\,i}$ to the "elongation constant" $K_{e\,i}$ for a given concentration of the elongating nucleotide.

We conclude from our data that the reaction scheme remains essentially the same when rifampicin is bound to the enzyme. Not only dinucleotide but also higher oligonucleotides are formed.However,the relative amounts of the products decrease very strongly with increasing lengths, if the reaction is run only for a short time where mainly direct elongation of the nucleotides occurs. Therefore, RNA synthesis ceases after a few steps. A very simple explanation for this finding is that rifampicin destabilises the binding of the oligonucleotide to the enzyme-DNA complex, or - in terms of our scheme - increases the k_{off}. This reduces the probability that an oligonucleotide is elongated.

We had demonstrated the destabilising effect of rifampicin directly only for the complex with the pppApUpA and could thereby confirm a previous finding of Sylvester and Cashel (13), who used however ApU instead of pppApU.Further strong indirect evidence for our hypothesis that rifampicin generally destabilises the binding of oligonucleotides comes from the results of the pyrophosphate exchange experiment at the A_1 promoter of T7 D111 and from the experiment in which we could counteract the effect of rifampicin on trinucleotide synthesis by high pppApU concentration.Both experiments illustrate that the antibiotic acts in reducing the stationary concentration of the dinucleotide.

Our view of the mechanism of rifampicin inhibition is diffe-

rent from that of Mc Clure and Cech (5).These authors stated that rifampicin causes a complete steric block which does not allow translocation.This hypothesis was deduced mainly from their failure to detect trinucleoside tetraphosphate in the presence of rifampicin.However,they found one exception: at the promoter λP_R, the trinucleotide pppApApC was synthesised in detectable amounts from ATP and CTP.A similar observation was reported by Carpousis and Gralla (14) who found that besides the dinucleotide pppApA the trinucleotide pppApApU is also produced in the presence of rifampicin using the lac UV5 promoter as a template.These findings do not fit Mc Clure's model well but are easily explained by ours,if we assume that the binding of the intermediate pppApA to the complex is tight (low dissociation rate) compared with the binding stability of purine-pyrimidine dinucleotides.It has in fact been reported by several authors (13,15) that a growing oligonucleotide produces a much stabler ternary complex if its 3' end is a purine rather than a pyrimidine nucleotide. Generally we would predict that the synthesis of trinucleotides in the presence of rifampicin depends on the basic binding stability of the dinucleotide intermediate.A comparison of the ratio of the yields of di-to trinucleotide in the poly[d(A-T)] system with that in the A_1-D111 system shows however,that this stability appears to be not solely determined by the initiation sequence.In our model,rifampicin does not affect the reaction mechanism principally but exerts its inhibitory effect in a quantitative manner by changing dissociation constants.In contrast,however.the binding of rifampicin,or-to be more cautious- its specific interaction with RNA polymerase is not reduced gradually,but is eliminated completely by the transition binary → ternary complex,even if the "RNA" of the latter is not longer than a trinucleotide.As a consequence, rifampicin cannot re-bind to an RNA polymerase in a ternary complex, once it has been dissociated. This gives an intriguing explanation why there is always the formation of a small but significant amount of high molecular weight RNA in the presence of rifampicin because the proportion of all those individual ternary complexes that have got rid of the antibiotic

is able to accomplish transcription in a normal manner.

Although not directly concerned with the mechanism of rifampicin inhibition,one finding requires clarification.Sylvester and Cashel (12) emphazised that the formation of a phosphodiesterbond is an absolute prerequisite to obtain a stable ternary complex.Our data do not support this view.We were able to reconstitute directly from its components a ternary complex which is indistinguishable from an indirectly prepared complex,both in its resistance to rifampicin and in its ability to elongate the trinucleotide.Sylvester and Cashel's failure to obtain a stable ternary complex simply by mixing the components ApUpA,RNA polymerase and poly [d(A-T)] may be due to one of two reasons: a) They used ApUpA istead of pppApUpA,which binds more tightly,b) Their trinucleotide concentration was far to low,considering that in this special preparation procedure the equilibrium constant determines the amount of bound trinucleotide,whereas the kinetic stability of the complex is important for its succesful isolation if the ternary complex is created by elongation of dinucleotide.

ACKNOWLEDGEMENTS

We wish to thank Dr. Don Dennis for stimulating discussions and Heidi Lindemann for excellent technical assistance.

REFERENCES

1 Sippel A. and Hartmann G. (1968), Biochem.Biophys.Acta 157, 218-219
2 Kassavetis G.A.,Kaya K.M.,Chamberlin M.J. (1978),Biochemistry 17, 5798-5804
3 Bordier C. (1974) FEBS LETT. 45, 259-262
4 Johnston D.,Mc Clure W.(1976) in RNA Polymerase (Losick Chamberlin eds.) Cold Spring Harbor Press.Cold Spring Harbor,N.Y. 413-428
5 Mc Clure W.R.,Cech C.L. (1978), J.Biol.Chem.253,8949-8956
Zillig W.,Zechel K.and Halbwachs H. (1970) Hoppe Seyler's Z.Physiol.Chem.351,221-224
7 Hansen U.,Mc Clure W. (1979) J.Biol.Chem. 254,5713-5717
8 Aposhian H.V.,and Kornberg A. (1962) J.Biol.Chem.237, 519-525
9 Maxam A.M., and Gilbert W. (1980) in Methods in Enzymology, Vol. 65,495-560
10 Mc Connell D.J. (1979),Nucleic Acids Res. 6,3491-3506
11 Smagowicz W.J.,Scheit K.H. (1978) Nucleic Acids Res.5, 1919-1932

12 Mc Clure W.R.,Cech C.L.,and Johnston D.E. (1978) J.Biol. Chem. 235, 8941-8948
13 Carpousis A.J.,Gralla J.D. (1980),Biochemistry 19,3245-3253
14 Sylvester J.E.and Cashel M.(1980) Biochemistry 19, 1069-1074
15 So A.G.,and Downey K.M. (1970) Biochemistry 9,4788-4793