Micro-analysis of Pure Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase from *Escherichia coli*

ACTION OF HEPARIN AND RIFAMPICIN ON STRUCTURE AND FUNCTION

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By using micro disc electrophoresis and micro-diffusion techniques, the interaction of pure DNA-dependent RNA polymerase (EC 2.7.7.6) from *Escherichia coli* with the template, the substrates and the inhibitors heparin and rifampicin was investigated. The following findings were obtained: (1) heparin converts the 24S and 18S particles of the polymerase into the 13S form; (2) heparin inhibits RNA synthesis by dissociating the enzyme–template complex; (3) rifampicin does not affect the attachment of heparin to the enzyme; (4) the substrates ATP and UTP are bound by enzyme loaded with rifampicin; (5) rifampicin is bound by an enzyme–template complex to the same extent as by an RNA-synthesizing enzyme–template complex. From this it is concluded that the mechanism of the inhibition of RNA synthesis by rifampicin is radically different from that by heparin. As a working hypothesis to explain the inhibitory mechanism of rifampicin, it is assumed that it becomes very firmly attached to a position close to the synthesizing site and only blocks this when no synthesis is in progress.

In previous publications (Neuhoff, Schill & Sternbach, 1968*a,b*, 1969*a,b,c*) it has been shown that pure DNA-dependent RNA polymerase from *Escherichia coli* (K12 Hfr) in aqueous solution consists of differing combinations of subunits, which behave as 24S, 18S and 13S particles of the polymerase. The particles are interconvertible and even then preserve their enzymic activity (Neuhoff *et al.* 1969*c*). The *E. coli* polymerase-specific inhibitor rifampicin (Hartmann, Honikel, Knüsel & Nüesch, 1967; Wehrli, Nüesch, Knüsel & Staehelin, 1968*b*; Hartmann, Behr, Beissner, Honikel & Sippel, 1968; Sippel & Hartmann, 1968; Sippel, 1968) does not affect the protein fractionation pattern in 8% polyacrylamide gels and does not prevent the formation of a complex between enzyme and template (Neuhoff *et al.* 1969*b*). From this it could be deduced that the polymerase has different sites for attachment to the template and for RNA synthesis. In this paper results of experiments with ³H-labelled rifampicin are reported. These results support the previous deductions and, further, demonstrate that the influence of rifampicin on the structure and function of the polymerase is completely different from that of heparin, which is another potent inhibitor of RNA synthesis by DNA-dependent RNA polymerase (Doerfler, Zillig, Fuchs & Albers, 1962; Walter, Zillig, Palm &

Fuchs, 1967). Additionally, it is demonstrated that the enzyme possesses different binding sites for the substrates ATP and UTP.

METHODS

RNA polymerase. The pure RNA polymerase used in these investigations was the same preparation as that described previously (Neuhoff *et al.* 1969*c*). It was stored in small portions, either in 50% (v/v) glycerol at –20°C or without glycerol at –70°C, and thawed at 0°C for analysis and then kept at 0°C.

To check the results obtained with micro methods for the binding of rifampicin to the polymerase, [³H]-rifampicin (specific radioactivity 0.26 Ci/g; rifampicin from CIBA A.-G., Basle, Switzerland, tritiated at The Radiochemical Centre, Amersham, Bucks., U.K.) was added to normal RNA-synthesis media (Zillig, Fuchs & Milette, 1966) at different times. After termination of the synthesis by cooling to 0°C, the solution was applied to Whatman 3MM paper. Excess of [³H]rifampicin was removed by repeated alternate rinsing in ethanol and 10% (w/v) trichloroacetic acid. After drying, chromatography was performed (Heller, 1967; Matthaei *et al.* 1966) with 1M-ammonium acetate (pH 5.5)–ethanol (5:2, v/v). The dried chromatograms were cut into small strips and their radioactivities measured in the scintillation counter.

Micro disc electrophoresis. This was performed in 8% polyacrylamide gels in 5μl capillaries with elution of single protein components from the gel after fractionation, and was carried out as previously described (Neuhoff &

Schill, 1968; Neuhoﬀ *et al.* 1968*a,b*, 1969*a,b,c*). Gels used in measurement of radioactivity of protein particles treated with [^{35}S]heparin (specific radioactivity 0.91 mCi/g; The Radiochemical Centre) or [^3H]rifampicin contained ethylenebisacrylate in the fractioning gels instead of *NN'*-methylenebisacrylamide as cross-linking agent (Choules & Zimm, 1965) but at the same concentration. The fractionations obtained with these gels were identical with those obtained on normal gels. After fractionation and staining with Amido Black 10B, the gel segments whose radioactivity was to be counted were removed (Neuhoﬀ & Schill, 1968) and completely dissolved in 200–300 μl of aq. ammonia (sp.gr. 0.91) in the counting vials (Spear & Roizman, 1968). After evaporation of the ammonia, 1 ml of ethanol and 7 ml of scintillation solution [2,5-diphenyloxazole (4g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.1g/l) in toluene (Packard Instruments Inc., La Grange, Ill., U.S.A.)] were added and the radioactivity was measured in a Tri-Carb model 3380 liquid-scintillation counter (Packard Instruments Inc.).

For assay of enzyme activity after fractionation of the polymerase the gels were stained with 0.1% Bromophenol Blue at 0°C and the sections of the gels corresponding to the protein bands were cut out under a stereomicroscope and transferred to the usual incubation mixture (Zillig *et al.* 1966).

Determination of R_f values. To determine the migration rate of individual protein bands, for comparisons between different fractionations, it is necessary to have a reference point that is dependent on the conditions of the electrophoresis. Reference proteins are unsuitable (Neuhoﬀ, 1968; Neuhoﬀ *et al.* 1969*c*). By using macro-scale disc electrophoresis, one can use the distance between the fluorescein band, which marks the buffer front, and the top of the gel as reference, since these are clearly visible before staining. This procedure cannot be applied with sufficient accuracy in the present method because of the small diameter of the microgels (450 μm) and the short distance over which fractionation occurs (approx.

4–5 mm). Therefore the micro gels are first stained in 1% pyronine in 7.5% (v/v) acetic acid and differentiated in acetic acid. This produces a complex, insoluble in acid, between the fluorescein, which has migrated with the buffer front, and the pyronine. Subsequently the usual Amido Black staining of the proteins is carried out. The migration rate may then be determined by analysing the microdensitometer records, because the fluorescein-pyronine complex is also registered as a peak.

Two-dimensional micro-diffusion technique. In the experiments on the dissolution of the enzyme-template complex by heparin (Heparin-Na, pyrogen-free, lot no. 24590; Serva, Heidelberg, Germany) or pancreatic deoxyribonuclease (lot D8KH; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) the complexes were formed in 0.75% agarose plates (Neuhoﬀ *et al.* 1969*b*) in TMA buffer (10 mm-tris-acetate, pH 7.5, containing 22 mm- NH_4Cl , 10 mm-magnesium acetate and 1 mm-dithiothreitol) through two-dimensional micro-diffusion of the enzyme against poly d(A-T) or calf thymus DNA (lot no. 15130 RDAA; C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). For quantitative analysis, maximal curves from unstained and unfixed complexes were first obtained on a Joyce-Loebl double-beam microdensitometer by means of suitable filter combinations. Subsequently all holes on the agarose plate were charged with heparin or deoxyribonuclease and after fixed time-intervals the same part of the complex was repeatedly measured without altering the microdensitometer settings. In experiments of longer duration drying of the agarose and the formation of artifacts was prevented by placing a cover slip on the preparation. Fig. 1 shows the microdensitometer curves from such an experiment, in which the time-course of the dissolution of an enzyme-DNA complex by deoxyribonuclease may be clearly observed.

Radioautography. To obtain radioautograms of enzyme-template complexes that had been treated with [^{35}S]heparin or [^3H]rifampicin, the complexes in the agarose were fixed in 7.5% acetic acid, and unbound radioactivity

Fig. 1. Original microdensitometer curves of an unfixed and unstained complex of polymerase with calf thymus DNA in 0.75% agarose in TMA buffer. After registration of the first curve, 1 μl of deoxyribonuclease solution (0.25 mg of protein/ml) was added to each hole in the agarose plate and further curves were drawn at the stated intervals, without alteration of the filter and slit setting of the microdensitometer. The decrease of the curve amplitude clearly indicates the lysis of the complex.

was removed by repeated washing in further fresh acetic acid. After drying of the agarose in air, highly sensitive Du Pont dosage-meter film (no. 044/544) from personal radiation-exposure tags was placed on the plate, covered with a 3 cm × 3 cm glass plate, fixed with Sellotape and exposed for 4–8 days. Plate 2 confirms the findings from scintillation counting, from which it was concluded that radioactivity is only found in the regions of the agarose where there is also protein (Neuhoff *et al.* 1969b). Thus there is no unspecific attachment to the agarose.

Radial micro-diffusion technique. The radial micro-diffusion test is a simple procedure for the qualitative and quantitative investigation of attachment of [³H]-rifampicin, [³⁵S]heparin or labelled substrates to the polymerase. For this, a layer (approx. 1 mm thick) of 0.75% agarose in TMA buffer was applied to a 3 cm-square glass plate, as for a two-dimensional micro-diffusion test. After storage in a moist chamber at 0°C for 12–15 h, holes (diam. 0.5 mm) were punched in the layer, by using suction through a 10 μ l Drummond Microcap. Polymerase was then introduced into the holes with a suitable micro-pipette under the stereomicroscope. For quantitative analysis a 1 μ l or 2 μ l Drummond Microcap was first filled with enzyme solution and completely transferred to the holes with a micro-pipette. After complete diffusion of the enzyme, the holes were regularly loaded two or three times with TMA buffer, after which the test solution was applied and then the holes were again rinsed with TMA buffer. Then the plate was fixed in 7.5% acetic acid and all radioactivity not bound to the enzyme was rinsed out with several changes of acetic acid. The protein was then easily seen, even without staining, as a milky white clouding around the hole in the agarose. Finally, the protein-loaded agarose was removed with a fine scalpel, dehydrated

in ethanol and dissolved in toluene scintillation solution directly in the counting vials.

RESULTS

Plate 1 shows the effect of heparin on the fractionation of the RNA polymerase. All nine gels were each loaded with 0.3 μ l of the enzyme solution (0.45 μ g of protein). For mol.wt. 720 000 this corresponds to 0.63 pmol of enzyme. All gels were first loaded with 0.9 μ l of the appropriate solution and the enzyme was added on top of this, so that the polymerase had to run through 0.9 μ l of water in the control gel and 0.9 μ l of heparin, in increasing concentration, in all the other gels before being fractionated in the gel. The same result was obtained if the enzyme was first mixed with heparin and then fractionated. The amount (mol) of heparin contained in 0.9 μ l of solution (assuming median mol.wt. 17 500) and the heparin/enzyme molar ratio are also given in Plate 1. With a molar ratio of 0.3 (gel 3) there was already a distinct decrease in the 24S fraction. With an excess of heparin, molar ratios 1.6 and 3.4 (gels 5 and 6), a clearly faster 'heparin band' appeared, which accounted for 30% of the total protein in both cases. These concentrations of heparin produced 30% inhibition in activity tests with unfractionated polymerase. Further increase of the heparin concentration to molar ratios 7.7, 15.5 and 31 led to a progressive

Table 1. *Demonstration of the attachment of [³⁵S]heparin to the protein components of the polymerase, the enzymic activity and reconversion of the 'heparin band' into 24S, 18S and 13S particles*

(1) Initial fractionation of [³⁵S]heparin-RNA polymerase complex

(a) ³⁵S radioactivity from nine 0.9 μ l portions of [³⁵S]heparin (1.36 mg/ml) + 0.45 μ l of polymerase (4 mg/ml). Unbound heparin migrates in front of the protein during electrophoresis. (b) Polymerase activity test on various fractions carried out for 90 min at 37°C with corresponding slices from eight gels after fractionation of 0.9 μ l of heparin (0.38 mg/ml) + 0.6 μ l of polymerase (4 mg/ml).

(2) Refractionation of the 'heparin band'

The medium contained 5 μ l of polymerase (5 mg/ml) + 20 μ l of [³⁵S]heparin (36 mg/ml). Of this mixture ten 1.8 μ l portions were fractionated and then the 'heparin band' from each was eluted. These were combined and refractionated.

strengthening of this 'heparin band' to 54, 64 and 70% of the total protein respectively. Still further increase in the heparin concentration produced no further changes in the fractionation pattern.

Determination of migration rates in six gels produced R_F values ($\pm$ S.D.) 0.098 ($\pm$ 0.0075), 0.13 ($\pm$ 0.012) and 0.207 ($\pm$ 0.016) for bands 1, 2 and 3 respectively. The R_F of the 'heparin band' was 0.270 ($\pm$ 0.024), this thus being faster than a 13S RNA polymerase particle. In contradiction to this seems to be a finding by Walter *et al.* (1967), which we have confirmed, that an RNA polymerase solution, saturated with heparin, sediments at 15S in a linear sucrose gradient [7–25% (w/v) sucrose in TMA buffer, pH 7.4, containing glycerol (10%, v/v)].

To demonstrate the attachment of heparin to the enzyme various experiments were carried out with [35 S]heparin. Table 1 presents the results of an experimental series in which the polymerase was fractionated with [35 S]heparin, and the 'heparin band' eluted and refractionated. Heparin not bound to protein ran mainly with the buffer front. A control experiment, in which [35 S]heparin alone was subjected to electrophoresis, showed that no heparin remained in the portion of the gel in which the protein bands were found, and thus the 35 S radioactivity bound to the protein could not have originated from free heparin. If the 'heparin band' was eluted the eluate also showed 35 S radioactivity. After refractionation of this band the usual 24S, 18S and 13S particles were observed, with only a minor content of the 24S component. This fractionation pattern was similar to that found on refractionation of 13S particles (cf. Figs. 3 and 4 in Neuhoft *et al.* 1969c). After refractionation these protein particles exhibit only very low 35 S radioactivity. The heparin, which in contrast with rifampicin is only loosely bound to the protein, mostly became detached and appeared as free heparin in the gel ahead of the protein. The 'heparin band' was no longer to be found; it had been completely reconverted into normal protein. These results explain the fact that the 'heparin band' exhibited enzymic activity when tested in the normal manner (see Table 1).

The observation that heparin is only so loosely attached to the enzyme that it becomes detached during electrophoresis makes it impossible to perform stoichiometrical calculations on the binding of heparin to the enzyme on the basis of these experiments. The analysis of a radial diffusion test, in which 1.5 μ l of a polymerase solution containing 5 mg of protein/ml, i.e. 21 pmol (mol.wt. 360 000), had freely diffused in an agarose plate and was subsequently treated with excess of [35 S]heparin (5 μ l containing 6.8 μ g and 11 600 c.p.m.) produced 1525 c.p.m. (0.89 μ g) of protein-bound heparin. With an estimated molecular weight of 15 000–

Fig. 2. Analysis of microdensitometer curves from experiments of the type shown in Fig. 1. Complexes of RNA polymerase and calf thymus DNA were subjected to lysis with 1 μ l of heparin solution of different concentrations. Heparin concentrations: 1 ($\circ$), 600 μ g/ml; 2 ($\bullet$), 400 μ g/ml; 3 (Δ), 200 μ g/ml; 4 ($\blacktriangle$), 100 μ g/ml; 5 ($\square$), 20 μ g/ml. The dependence of the rate of lysis on the concentration of heparin used should be noted. The same results are obtained with enzyme-poly d(A-T) complexes.

Fig. 3. Analysis of microdensitometer curves from experiments of the type shown in Fig. 1. RNA polymerase-poly d(A-T) complexes were treated: 1 ($\bullet$), with 1 μ l of heparin solution (600 μ g/ml); 2 ($\circ$), first with an excess of 1 mM-ATP+1 mM-UTP solution, then with heparin as for 1; 3 (Δ), the complex was first saturated with excess of rifampicin, rinsed with TMA buffer and then treated with the same heparin solution as for 1. After treatment with rifampicin, a 50% lysis of the complex is only to be seen after double the time taken with an untreated complex.

20 000 for heparin this corresponds to 59–45 pmol of heparin, i.e. 2–3 mol of heparin/mol of enzyme.

That a protein particle loaded with heparin should migrate quicker under electrophoretic conditions and slower in a density gradient than a 13S particle is easily explained by the demonstrated attachment of the polyanion, heparin, which thus increases the charge and molecular weight of the protein.

The inhibition of the enzymic activity of the polymerase by heparin may be attributed to the dissociation of the enzyme-template complex by heparin, as indicated in Figs. 2 and 3. Fig. 2 shows the relationship between the concentration of heparin used and the time for dissociation of the complex. The same behaviour could be obtained by treating the complex with pancreatic deoxyribonuclease (cf. Fig. 1). Fig. 3 shows that the dissolution took place more

Gel:	1	2	3	4	5	6	7	8	9
Heparin (pmol)	—	0.1	0.2	0.5	1.0	2.4	4.8	9.7	19.3
Heparin/enzyme molar ratio	—	0.16	0.3	0.8	1.6	3.4	7.7	15.5	30.9
Percentage of total protein content contributed by the 'heparin band'	—	—	—	—	30	28	54	64	70

EXPLANATION OF PLATE I

Fractionation of RNA polymerase in 8% polyacrylamide gels: photograph of gels after staining with Amido Black (magnification $\times 18$). Gel 1, control ($+0.9\mu\text{l}$ of water); gels 2–9, results for the same quantity [$0.3\mu\text{l}$, i.e. $0.45\mu\text{g}$, or 0.63 pmol (mol.wt. 720000)] of enzyme passing through the same volume ($0.9\mu\text{l}$) of heparin solution (of increasing concentrations) before fractionation. The molecular weight of heparin is approx. 17500. HB, 'heparin band'.

EXPLANATION OF PLATE 2

Radioautogram, on the Du Pont film of a radiation-dosage tag, of complexes of RNA polymerase and poly d(A-T) that had been loaded with $[^3\text{H}]$ rifampicin (a) and $[^{35}\text{S}]$ heparin (b) (magnification approx. $\times 10$). The exposure time was 6 days. The experiment with $[^{35}\text{S}]$ heparin was terminated a short time after the addition of heparin, before lysis was completed, by fixation in acetic acid. The lysis, demonstrated by the diffuse blackening, and the binding of the heparin to the protein also shown by this should be noted. No radioactivity is demonstrable in the surrounding agarose.

Table 2. Analysis of a two-dimensional micro-diffusion test

A complex consisting of polymerase and poly d(A-T) was first loaded with [^3H]rifampicin, then with TMA buffer and finally with excess of [^{35}S]heparin. Afterwards it was fixed and rinsed with 7.5% acetic acid. An identical complex was loaded with excess of ATP and UTP (each 1 mM), TMA buffer and the same heparin solution, and rinsed and fixed in acetic acid. Background radioactivities were obtained from areas of agarose of the same size and in close proximity to the complex.

Treatment	^3H radioactivity (c.p.m.) (background 137)	^{35}S radioactivity (c.p.m.) (background 110)
[^3H]Rifampicin, then [^{35}S]heparin	4485	1442
ATP + UTP, then [^{35}S]heparin	—	916

slowly in the presence of substrates (ATP and UTP), i.e. under conditions of synthesis (Neuhoff *et al.* 1969b), than when no substrates were present. Table 2 shows that at the same time less heparin was bound by the protein. Pretreatment with rifampicin clearly slowed down the dissolution of the rifampicin-enzyme-template complex by heparin or deoxyribonuclease. That both rifampicin and heparin may become attached to the complex is demonstrated by the radioautograms in Plate 2, which show the results of adding [^3H]rifampicin or [^{35}S]heparin to all the holes in an agarose plate containing an already formed enzyme-template complex. In the radioautogram of the heparin experiment the advanced lysis of the complex is also visible. In agreement with these observations is the finding that an enzyme-template complex is not even formed when heparin-loaded enzyme is diffused against the template.

Just as lysis of the enzyme-template complex proceeds according to the nature of the template and the concentration of heparin, so there is no abrupt termination of RNA synthesis under normal conditions of synthesis on the addition of heparin (see Fig. 4). Synthesis is terminated with a certain latency, which depends on the template and conforms to the dissociation time found in the two-dimensional micro-diffusion tests. With calf thymus DNA in its native state as template, synthesis is terminated less rapidly than with poly d(A-T). Addition of heparin to a preparation with denatured calf thymus DNA terminates RNA synthesis within 4 min. This finding is in agreement with those reported by Walter *et al.* (1967).

If an enzyme-template complex was dissolved with heparin and then fractionated by micro disc electrophoresis, the same pattern was observed as

Fig. 4. Dependence of inhibition of RNA synthesis by heparin on the template (the arrow indicates the time of addition; final concn. $20\text{ }\mu\text{g}$ of heparin/ml). With calf thymus DNA ($1.75\text{ }E_{260}$ units/ml) as template (b) the synthesis is inhibited more slowly than with poly d(A-T) ($0.5\text{ }E_{260}$ unit/ml) as template (a). The incubation mixture contained: $7.2\text{ }\mu\text{g}$ of protein/ml, 30 mM -tris-acetate buffer, pH 7.9, 0.13 M - NH_4Cl , 30 mM -magnesium acetate and 1 mM -[^{14}C]-ATP, -CTP, -GTP and -UTP. $\circ$, Control; $\bullet$, + heparin.

when polymerase alone had been incubated with heparin. The same result, including the appearance of a 'heparin band', was obtained after a rifampicin-enzyme-template complex has been lysed with heparin. Experiments with enzyme loaded with [^3H]rifampicin revealed that the ^3H radioactivity of the rifampicin could also be demonstrated in the 'heparin band', thus demonstrating that heparin does not disrupt the binding of rifampicin to the enzyme. As shown in Table 2, [^{35}S]heparin is taken up by enzyme loaded with [^3H]rifampicin. This indicates that rifampicin and heparin are attached to different sites on the polymerase. Lill, Lill, Sippel & Hartmann (1969a), using the gel-filtration method, come to the same conclusion. Even after a [^3H]rifampicin-enzyme-template complex had been dissolved with deoxyribonuclease, [^3H]rifampicin radioactivity could be demonstrated in the protein bands obtained after electrophoretic fractionation.

In contrast with heparin, rifampicin is firmly

bound to the polymerase; [^3H]rifampicin can be demonstrated even after the three ^3H -labelled protein fractions have been eluted and refractionated. The 24S, 18S and 13S particles exhibit the same characteristics in the presence of rifampicin as has been described for normal unloaded enzyme (Neuhoff *et al.* 1969b,c); each single fraction can be reconverted into the other particles, and these newly formed particles still retain the ^3H radioactivity of the rifampicin. Microdensitometric analysis of the gels, stained with Amido Black, and measurement of the radioactivity of the individual fractions allow one to determine the amount of rifampicin taken up by the individual particles (Table 3). Further, once bound to RNA polymerase, rifampicin cannot be removed by treatment with 1% (w/v) sodium dodecyl sulphate solution and subsequent fractionation in the presence of 0.1%

sodium dodecyl sulphate. Further, rifampicin cannot be removed from the enzyme by micro-dialysis (Neuhoff & Kiehl, 1969) against TMA buffer at 0°C for 3 days. Only by treatment with 6M-guanidine hydrochloride can the RNA polymerase-rifampicin complex be dissociated (Lill *et al.* 1969a).

As shown in Table 4, RNA polymerase has binding sites for ATP and UTP, in addition to those for rifampicin and heparin, or the template, which could be demonstrated by application of the two-dimensional micro-diffusion technique. In addition to ^3H radioactivity of rifampicin, it is possible to demonstrate [^{14}C]ATP- and also [^{14}C]UTP-binding activity. That these substrates are bound as triphosphates could be deduced from the identical result with [$\gamma\text{-}^{32}\text{P}$]ATP. In further experiments enzyme-template complexes, with and without unlabelled rifampicin, were loaded with [^3H]UTP and also with [^{14}C]ATP and their radioactivities measured as described. Here, too, comparable labelling with ^3H and ^{14}C could be demonstrated. The simultaneous attachment of [^3H]rifampicin and [^{14}C]ATP to the polymerase can be confirmed with a radically different technique (see Table 5, lines 14 and 15). From this it may be deduced that pure RNA polymerase from *E. coli*, in addition to a specific binding site for the template (or heparin), has further binding sites for the substrates ATP and UTP, which in turn are not identical with the binding site for rifampicin. These results are in good agreement with those from Lill, Santo, Sippel & Hartmann (1969b).

It is known that rifampicin only totally inhibits RNA synthesis by polymerase if it is added to the medium before the commencement of synthesis. Synthesis already in progress is inhibited little or not at all, depending on the time of addition. This finding is only true if the course of synthesis is only followed for 5–10 min after the addition of rifampicin.

Table 3. *Analysis of the results of microdensitometer curves, after staining protein fractions on six gels with Amido Black and determining the ^3H radioactivity after fractionation of a mixture of RNA polymerase with excess of [^3H]rifampicin*

For the calculations the total area under the curves or the total radioactivity of all three components was taken as 100. Rifampicin not attached to protein is characterized by its yellow colour and migrates behind the buffer front but ahead of the fastest protein. A control electrophoresis with [^3H]rifampicin alone confirmed that there is no rifampicin in the part of the gel normally occupied by protein. Results are expressed as means $\pm$ s.d.

Protein fraction	Rifampicin (%)	Protein (%)	Rifampicin/protein ratio
24S	9.0 $\pm$ 2.1	8.3 $\pm$ 1.2	1.1
18S	22.7 $\pm$ 1.9	43.2 $\pm$ 1.9	0.5
13S	68.3 $\pm$ 3.5	48.5 $\pm$ 2.5	1.4

Table 4. *Two-dimensional micro-diffusion of RNA polymerase against poly d(A-T) or calf thymus DNA in 0.75% agarose plates in TMA buffer*

The complexes were either loaded only with excess of [^3H]rifampicin, or, after rinsing with TMA buffer, either [^{14}C]ATP (1 mM) or [^{14}C]UTP (1 mM) was added. After diffusion they were fixed and rinsed in acetic acid. The complexes and control segments of agarose, of equal area and from the vicinity of the complexes, were removed under a stereomicroscope, dehydrated in ethanol and subsequently dissolved in the toluene scintillation fluid.

Complex	[^3H]Rifampicin		[^3H]Rifampicin, then [^{14}C]ATP		[^3H]Rifampicin, then [^{14}C]UTP	
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
	radioactivity (c.p.m.)	radioactivity (c.p.m.)	radioactivity (c.p.m.)	radioactivity (c.p.m.)	radioactivity (c.p.m.)	radioactivity (c.p.m.)
Enzyme-poly d(A-T) (background)	1963 (35)	34 (10)	2030 (59)	981 (30)	1876 (341)	1663 (158)
Enzyme-DNA (background)	2225 (67)	26 (14)	2040 (235)	837 (57)	1009 (92)	1093 (63)

Table 5. *Enzymic-activity tests of RNA polymerase by using the method of Zillig et al. (1966) and the binding of [³H]rifampicin to the enzyme-template complex on addition to the synthesizing medium at different times*

³H-labelled or unlabelled rifampicin was added to the different media at concentrations approx. 100 times that of the normal inhibitory concentration. The reaction mixture contains 30 mM-tris-acetate buffer, pH 7.9, 130 mM-NH₄Cl, 30 mM-magnesium acetate, 1 mM each of ATP, GTP, UTP and CTP, and 50 µg of calf thymus DNA, or 0.25 E₂₆₀ unit of poly d(A-T) or poly dA, as appropriate.

	Radioactivity	
	¹⁴ C (c.p.m.)	³ H (c.p.m.)
1. Enzyme + DNA + [¹⁴ C]ATP, CTP, GTP, UTP: synthesis 0–8 min	5950	—
2. Enzyme + DNA + [¹⁴ C]ATP, CTP, GTP, UTP: synthesis 0–12 min	7960	—
3. Enzyme + rifampicin + DNA + [¹⁴ C]ATP, CTP, GTP, UTP: synthesis 0–12 min	70	—
4. Enzyme + DNA + [¹⁴ C]ATP, CTP, GTP, UTP, then at 8 min rifampicin: synthesis 0–12 min	7800	—
5. Enzyme + [³ H]rifampicin + DNA + ATP, CTP, GTP, UTP: synthesis 0–12 min	—	1406
6. Enzyme + DNA + ATP, CTP, GTP, UTP, then at 8 min [³ H]rifampicin: synthesis 0–12 min	—	1460
7. Enzyme + [³ H]rifampicin + poly d(A-T) + ATP, UTP: synthesis 0–12 min	—	2170
8. Enzyme + poly d(A-T) + ATP, UTP, then at 8 min [³ H]rifampicin: synthesis 0–12 min	—	2140
9. Enzyme + [³ H]rifampicin + poly dA + UTP: synthesis 0–12 min	—	1750
10. Enzyme + poly dA + UTP, then at 8 min [³ H]rifampicin: synthesis 0–12 min	—	1759
11. [³ H]Rifampicin alone	—	54
12. [³ H]Rifampicin + DNA	—	47
13. [³ H]Rifampicin + poly d(A-T)	—	86
14. Enzyme + [³ H]rifampicin + DNA	—	2085
15. Enzyme + [³ H]rifampicin + DNA + [¹⁴ C]ATP + UTP	445	2068

Fig. 5. Dependence of inhibition of RNA synthesis by rifampicin on the time at which rifampicin is added (↓). When rifampicin is added at a later point, the synthesis continues unaltered for some time. The incubation mixture was the same as that described in Fig. 4. The template was calf thymus DNA. The final rifampicin concentration was 0.4 µg/ml in each case. (a) ○, Normal synthesis at 25°C; ●, synthesis at 37°C after addition of rifampicin. (b) and (c) ○, Normal synthesis at 25°C; ●, synthesis at 25°C after addition of rifampicin.

Fig. 6. Model of mechanism of inhibition of RNA synthesis by rifampicin.

However, if the course of synthesis is followed for a longer period (Fig. 5) inhibition is found to occur (Sternbach, NeuhoFF & Schill, 1969; Lill *et al.* 1969a). Table 5 shows that the same amount of [^3H]rifampicin is bound to the enzyme irrespective of the time at which it is added to the enzyme, even if this is at a time when synthesis is not inhibited by the rifampicin (lines 1–4). Hartmann *et al.* (1969) independently have presented analogous findings. This effect is independent of the template used (lines 5–10).

DISCUSSION

Heparin and rifampicin are the most effective inhibitors of DNA-dependent RNA polymerase from *E. coli* so far known. The inhibitory mechanisms of the two substances differ radically. Heparin interrupts RNA synthesis because the enzyme-template complex is totally disrupted by the polyanion, as shown by formation of the complexes in agarose plates and their lysis with heparin. If added before the start of the synthesis, heparin prevents the formation of the enzyme-template complex.

In contrast, rifampicin has no effect on the formation of the polymerase-template complex (NeuhoFF *et al.* 1969b) and completely inhibits RNA synthesis only if it has been added before its commencement (Hartmann *et al.* 1967, 1968, 1969; Sippel & Hartmann, 1968; Sippel, 1968; Wehrli *et al.* 1968b; di Mauro *et al.* 1969). Since rifampicin contrasts with heparin in not affecting the complex and both inhibitors can be simultaneously attached to the enzyme, they must be attached to different sites on the polymerase. The ability of heparin to disrupt already formed enzyme-template complexes,

even under conditions of synthesis, indicates that heparin displaces the template from its attachment to the enzyme. Therefore it is likely that this polyanion binds to the same site as the template. In the presence of substrates or rifampicin the lysis time is prolonged, which supports the hypothesis that the configuration of the enzyme is modified (Lill *et al.* 1969b) and more stable (Stead & Jones, 1967).

In contrast with that of heparin, the binding of rifampicin to the polymerase is so stable that neither gel filtration, nor centrifugation (Wehrli, Knüsel, Schmid & Staehelin, 1968a), nor electrophoresis, nor long-term dialysis, nor elution and refractionation can detach it from the protein particles. Further, if rifampicin is added during the course of synthesis, the same amount is taken up as that if it had been added before the start. This finding of the equally strong uptake of rifampicin before and during synthesis enables a hypothesis of the mechanism of this inhibition to be proposed (Fig. 6). This is that rifampicin becomes very firmly attached to a specific site on the enzyme, in close proximity to the synthesizing site. If rifampicin is added before the start of the synthesis, it blocks the synthesis site, but does not prevent the attachment of the substrate to the polymerase, i.e. rifampicin does not inhibit the formation of the initiation complex. This conclusion is also presented by Lill *et al.* (1969a). However, if a growing RNA chain is already on the site of synthesis, the rifampicin may still become attached to its own binding site, but does not block the continuing growth of the chain. Only on detachment of the completed chain can rifampicin exhibit its usual inhibition.

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