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Studies of the Topography of the Binding Site of DNA-Dependent RNA Polymerase from *Escherichia coli* for the Antibiotic Rifamycin SV

Walter STENDER and Karl Heinz SCHEIT

Max-Planck-Institut für Biophysikalische Chemie, Abteilung Molekulare Biologie, Göttingen-Nikolausberg

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The synthesis of dimeric derivatives of rifamycin SV differing in the length of spacer, of derivatives of rifamycin SV possessing 2,4-dinitrophenyl groups in varying distances relative to the aromatic part of the antibiotic and a derivative of rifamycin SV carrying a biotinyl residue is described. Rifamycin SV covalently attached to bovine serum albumin was employed to produce antibodies against rifamycin SV in rabbits. Rifamycin SV as well as 2,4-dinitrophenyl-specific antibodies and avidin were used to study the interaction of RNA polymerase with the respective derivatives of rifamycin SV. The results of this investigation can be summarized as follows.

1. Antibodies, which are specific for rifamycin SV do not recognize the rifamycin SV molecule in the enzyme · antibiotic complex.
2. 2,4-Dinitrophenyl-specific antibodies are unable to recognize the 2,4-dinitrophenyl residue in the complex enzyme · 2,4-dinitrophenylaminoethylthio-rifamycin-SV.
3. However, 2,4-dinitrophenyl groups in complexes between enzyme and 2,4-dinitrophenyl derivatives of rifamycin SV are recognized, if those are separated far enough from the rifamycin part.
4. RNA polymerase binds to the rifamycin SV portion in complexes avidin · biotinylaminoethyl-thio-rifamycin-SV.
5. Dimeric rifamycin SV molecules do not form ternary complexes with RNA polymerase.

From those results it is concluded that the binding site of RNA polymerase for rifamycin SV extends 1.40 – 1.90 nm deep into the interior of the enzyme structure and that the ansa chain of the antibiotic extends furthest into the enzyme.

Rifamycin SV and its derivaties are strong inhibitors of DNA-dependent RNA synthesis in bacteria [1 – 5]. They interfere with chain initiation of RNA synthesis when binding to the enzyme, RNA polymerase, or to the complex RNA-polymerase · DNA [6, 7]. Recent investigations yield evidence that the first phosphodiester bond is still formed in the presence of the antibiotic and that the steps of the enzymatic reaction, which lead to the elongation of the phosphodiester bond, are inhibited [8]. Kinetic and thermodynamic studies reveal the high affinity of rifampicin and other rifamycin SV derivatives to enzyme or enzyme · DNA complexes [9 – 12]. Little is known about the topology of the antibiotic binding site of RNA polymerase. An investigation of a rifampicin-insensitive RNA polymerase from *Escherichia coli* and affinity labelling studies with chemically reactive derivatives of rifamycin SV demonstrate that the β subunit seems to carry the antibiotic binding site [13, 14].

The affinity labelling experiments indicate that the bound rifamycin SV is in contact with σ subunit. Structure-activity relations based on chemical modifications of the antibiotic affecting the activity indicate that both the ansa ring, as well as the aromatic part of the antibiotic, are involved in the interaction with the enzyme (Fig. 1). We have attempted to probe the topography of the antibiotic binding site by preparing the following synthetic derivatives of rifamycin SV (Table 1): (a) dimeric derivatives of rifamycin SV differing in the length of spacer, (b) derivatives of rifamycin SV possessing 2,4-dinitrophenyl groups in

Abbreviations. The abbreviations of the various synthetic derivatives of rifamycin SV are given in Table 2; N_2ph = dinitrophenyl.

Enzyme. DNA-dependent RNA polymerase (EC 2.7.7.6).

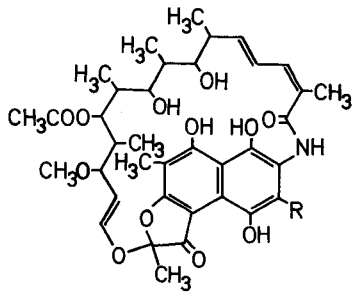

Fig. 1. *Structural formulae of derivatives of rifamycin SV*

Table 1. *Structures and abbreviations for synthetic derivatives of rifamycin SV*
R denotes the substituent in position 3 of the rifamycin SV molecule (see Fig. 1). The 2,4-dinitrophenyl group is abbreviated N_2ph

R	Abbreviation
H	rifamycin SV
$-SCH_2CH_2NH_2$	NH_2EtS-Rif
$-SCH_2CH_2NHCO(CH_2)_4$-	biotinylNHEtS-Rif
$-SCH_2CH_2NHCOCH_3$	AcNHEtS-Rif
$-SCH_2CH_2NHCONHCH_2Br$	BrAcNHEtS-Rif
$-SCH_2CH_2NHCONHCF_3$	F_3AcNHEtS-Rif
$-SCH_2CH_2NHCOCH_2$- (bovine serum albumin)	albumin-CH_2CONHEtS-Rif
$-SCH_2CH_2NHCONHCH_2CH_2S-$	Rif-C_1-Rif
$-SCH_2CH_2NHCOCONHCH_2CH_2S-$	Rif-C_2-Rif
$-SCH_2CH_2NHCO(CH_2)_nCONHCH_2CH_2S-$	
$n = 1$	Rif-C_3-Rif
$n = 2$	Rif-C_4-Rif
$n = 4$	Rif-C_5-Rif
$-SCH_2CH_2NHCNH(CH_2)_6$-$CNHNHCH_2CH_2S-$	Rif-C_8-Rif
$n = 9$	Rif-C_{11}-Rif
$n = 10$	Rif-C_{12}-Rif
$-SCH_2CH_2NHN_2$ph	N_2ph-Rif
$-SCH_2CH_2NHCO(CH_2)_nNHN_2$ph	
$n = 1$	N_2ph-C_2-Rif
$n = 2$	N_2ph-C_3-Rif
$n = 3$	N_2ph-C_4-Rif
$n = 4$	N_2ph-C_5-Rif
$n = 5$	N_2ph-C_6-Rif
$n = 7$	N_2ph-C_8-Rif
$n = 10$	N_2ph-C_{11}-Rif

varying distances relative to the aromatic part of the antibiotic, and (c) a derivative of rifamycin SV, carrying a biotinyl residue.

We employed antibodies to study the interactions of rifamycin SV derivatives with RNA polymerase. The antibodies were either specific for rifamycin SV or for 2,4-dinitrophenyl substituents respectively. These experiments demonstrate whether or not the bound antibiotic is accessible for interaction with the RNA polymerase when the antibiotic is bound to the antibody. With the aid of antibodies which are specific for 2,4-dinitrophenyl groups, we have attempted to show how long the side chain (which protrudes from the C-3 position of the rifamycin SV molecule and is bound to RNA polymerase) must be for the dinitrophenyl group to become accessible for interaction with the antibody. A similar investigation was carried out with avidin, RNA polymerase and a biotinyl derivative of rifamycin SV. The dimensions of the dinitrophenyl binding site on dinitrophenyl antibodies [15–20] and of the biotin binding site on avidin are known [21, 16]. We, therefore, expected the above investigations to yield at least some limiting values for the dimensions of the rifamycin SV binding site on RNA polymerase.

MATERIALS AND METHODS

Enzymes

RNA polymerase core enzyme from *E. coli* has been purified according to Zillig *et al.* through the DEAE-cellulose step [22], and then by phosphocellulose chromatography as described by Burgess [23]. The enzyme had a specific activity of 15000 units/mg assayed with poly[d(A-T)] according to Berg *et al.* [24]. RNA polymerase holo enzyme was prepared according to Arndt-Jovin *et al.* [25], with a specific activity of 6800 units/mg when assayed on T7 DNA, and 16800 units/mg when assayed with poly[d(A-T)].

Chemicals

[^{14}C]ATP and 1-fluoro-2,4-dinitro[^{14}C]benzene were purchased from Amersham-Buchler (Braunschweig). Bovine serum albumin was from Behringwerke AG (Marburg) and poly[d(A-T)] was from Boehringer (Mannheim). Activated charcoal was purchased from Atlas Chemical Industries (Wilmington, U.S.A.) and rifampicin, and rifamycin SV, from Boehringer (Mannheim) and Serva Feinbiochemica (Heidelberg), respectively. T7 DNA was isolated from purified phage by phenol extraction as described by Thomas and Abelson [26].

Synthesis of Rifamycin SV Derivatives

The synthesis of BrAcNHEtS-Rif [13] and [^{3}H]-AcNHEtS-Rif (specific activity 39000 counts min^{-1} nmol^{-1}) [27] has been described elsewhere.

F_3AcNHEtS-Rif. 3-(2-Aminoethyl)-thiorifamycin SV [28] in dimethylformamide was reacted with an excess of *S*-ethyl trifluorothioacetic acid. The desired product was isolated as an amorphous material by silica gel column chromatography with the solvent mixture chloroform/ethanol (2/1, v/v). Ultraviolet absorption (water, pH 7); λ_{max} (ε) = 320 nm (21900 M^{-1} cm^{-1}), 452 nm (12100 M^{-1} cm^{-1}).

N_2ph-NHEtS-Rif. NH_2EtS-Rif (77.3 mg, 0.1 mmol), 1-fluoro-2,4-dinitrobenzene (18.6 mg, 0.1 mmol) and triethylamine (14 µl) were dissolved in 5 ml anhydrous acetonitrile at room temperature with stirring. The reaction was monitored by silica gel thin-layer chromatography using chloroform ethanol (2/1, v/v) as a solvent. The desired product was isolated by silica gel column chromatography employing the same solvent. Ultraviolet absorption (water, pH 7): λ_{max} (ε) 323 nm (23000 M^{-1} cm^{-1}), 379 nm (12400 M^{-1} cm^{-1}), 423 nm (11650 M^{-1} cm^{-1}).

[^{14}C]N_2ph-NHEtS-Rif. The reaction was carried out as described above employing 2.38 µmol of 1-fluoro-2,4-dinitro[^{14}C]benzene (spec. act. 21 Ci/mol). The isolated radioactive compound behaved identically with the nonlabelled authentic material. Specific activity: 26000 counts min^{-1} $nmol^{-1}$.

3-{(2-[ω-(2,4-Dinitrophenyl)-aminoalkylcarbonyl]-amino}-ethylthiorifamycin SV Derivatives. A solution of the ω-amino acid in 5% aqueous sodium carbonate (pH 8–9) was mixed with a solution of an equimolar amount of 1-fluoro-2,4-dinitrobenzene and the resulting reaction mixture was stirred for 5 h at 40–50 °C. The mixture was acidified with concentrated hydrochloric acid. The precipitate formed was collected, washed extensively with 1 M HCl and dried. The products obtained were chromatographically pure. The 2,4-dinitrophenylamino acid (0.1 mmol) dissolved in 5 ml anhydrous tetrahydrofuran was treated with 0.1 mmol 1,1′-carbonyldiimidazole. To this mixture was added a solution of 0.1 mmol NH_2EtS-Rif in 10 ml anhydrous acetonitrile. The reaction was followed by silica gel thin-layer chromatography in chloroform/ethanol (2/1, v/v). The desired product was isolated by preparative silica gel layer chromatography in the same solvent.

All synthesized 2,4-dinitrophenyl derivatives of rifamycin SV gave satisfactory elementary analysis data. The absorption spectroscopic data are summarized in Table 2.

N,N′-*Bis[3-(2-aminoethyl)thiorifamycin SV]urea.* NH_2Ets-Rif (0.1 mmol) and 1,1′-carbonyldiimidazole (0.05 mmol) were dissolved in 10 ml anhydrous acetonitrile and the mixture was stirred at room temperature. The desired product was isolated by preparative silica gel layer chromatography with chloroform/ethanol (2/1, v/v) as solvent.

N,N′-*Bis[3-(2-aminoethylthio)-rifamycin SV] dicarboxylic acid diamides.* The corresponding dicarboxylic acid dichloride (0.06 mmol) and imidazole (0.24 mmol) were dissolved in 5 ml anhydrous acetonitrile. The precipitated imidazole hydrochloride was filtered off under exclusion of moisture. A solution of 0.1 mmol NH_2EtS-Rif in 5 ml anhydrous acetonitrile was added to the filtrate and the resulting mixture kept at room temperature. The reaction was followed by silica gel thin-layer chromatography in chloroform/ethanol (2/1, v/v). The desired product was isolated by preparative silica gel chromatography in the same solvent. All derivatives gave satisfactory elementary analysis data. The absorption spectroscopic data are summarized in Table 2.

Table 2. *Absorption spectroscopic data of 2,4-dinitrophenyl and of dimeric derivatives of rifamycin SV in water at pH 7*

Rifamycin derivative	λ_{max} (ε)	
	nm (M^{-1} cm^{-1})	
N_2ph-C_2-Rif	320 (19600)	437 (10000)
N_2ph-C_3-Rif	323 (19000)	434 (12000)
N_2ph-C_4-Rif	321 (20000)	445 (11600)
N_2ph-C_5-Rif	321 (20000)	442 (11170)
N_2ph-C_6-Rif	321 (17270)	449 (9970)
N_2ph-C_8-Rif	321 (15500)	447 (8480)
N_2ph-C_{11}-Rif	321 (19100)	449 (10780)
Rif-C_1-Rif	321	450
Rif-C_2-Rif	311	435
Rif-C_3-Rif	319	448
Rif-C_4-Rif	320 (31400)	452 (17630)
Rif-C_6-Rif	320 (29700)	449 (18450)
Rif-C_8-Rif	326 (29000)	463 (18200)
Rif-C_9-Rif	321 (31600)	452 (17450)
Rif-C_{10}-Rif	321 (29700)	453 (18400)

N,N′-*Bis[3-(2-aminoethylthio)-rifamycin SV] diimido octanoic diamide.* Diimido dimethyl octanoate dihydrochloride (0.1 mmol), triethylamine (0.2 mmol) and NH_2EtS-Rif (0.2 mmol) were dissolved in 10 ml anhydrous acetonitrile. The reaction mixture was kept at room temperature and then processed as described above.

3-(2-Biotinylamidoethylthio)-rifamycin SV. D(+)-Biotin (0.1 mmol) and 1,1′-carbonyldiimidazole (0.1 mmol) were dissolved in 5 ml anhydrous tetrahydrofuran and the reaction mixture was kept at room temperature for 5 h. To this solution was added 0.1 mmol NH_2EtS-Rif. The reaction went to completion over night. The desired product was isolated as described above. Absorption spectrum (water, pH 7): λ_{max} (ε) 321 nm (20900 M^{-1} cm^{-1}), 452 nm (12300 M^{-1} cm^{-1}).

Other Preparations

Buffer A. 0.04 M *N*-2-Hydroxyethylpiperazine-*N*′-2-ethane sulfonate (pH 8), 0.1 mM EDTA, 0.15 M NaCl and 10 mM $MgCl_2$.

Albumin-CH_2CONHEtS-Rif conjugate. A mixture of 19 mg bovine serum albumin and 7.25 mg BrAcNHEtS-Rif in 2 ml buffer A was incubated for 4 h at 37 °C. The conjugate was separated from excess BrAcNHEtS-Rif by gel filtration on Sephadex G-75 with 0.1 M NaCl as solvent. From the ratio A_{450}/protein concentration the degree of substitution rifamycin/albumin was calculated to be 9.50.

Antisera against AcNHEtS-Rif. Albumin-CH_2CO-NHEtS-Rif (0.5 mg) was dissolved in 5 ml 0.15 M

NaCl, emulsified in an equal volume of Freund's complete adjuvant and injected intradermally into multiple sites on the back of two albino rabbits of about 3 kg each. Booster injections (0.25 mg albumin-$CH_2CONHEtS$-Rif/rabbit) were given after three weeks. Blood was collected by heart puncture before application of the conjugate, three weeks after the first injection and two weeks after the booster injection. After clotting serum was separated by centrifugation at 4 °C and 8000 rev./min for 20 min. The immunoglobulin fraction of the antiserum was purified by ammonium sulfate precipitation followed by DEAE-Sephadex chromatography as described in the literature [29]. The immunoglobulin fraction (17.8 mg/ml) was stored at 4 °C.

2,4-Dinitrophenyl-Specific Antibodies. 2,4-Dinitrophenyl antiserum was purchased from Miles Co. (U.S.A.). The immunoglobulin fraction was purified as described above. A solution of immunoglobulins (21.6 mg/ml) was stored at 4 °C.

Avidin-Sepharose. This was prepared according to literature [30,31] using avidin obtained from Serva (Heidelberg). Sepharose 4B (20 ml) activated with 2 g CNBr was coupled with 4 mg avidin closely following standard procedures. The capacity as measured by binding of [^{14}C]biotin was 2.1 µmol biotin/ml Sepharose. Sepharose Cl-2B (50 ml) was activated employing 0.5 g CNBr and reacted with 10 mg avidin. The capacity was found to 2.4 nmol biotin/ml sepharose.

Binding of Rifamycin SV Derivatives to Rifamycin-SV-Specific Antibodies

[^{3}H]AcNHEtS-Rif and antiserum or immunoglobulin in concentrations as indicated in legends to figures were incubated in buffer A (total volume 50 µl) at 37 °C for 15 min. After cooling to 0 °C, 10 µl of a charcoal suspension [10] were added. The mixture was vigorously agitated, allowed to stand for 15 min at 0 °C and then centrifuged at 16000 × *g* for 20 min. An aliquot of the supernatant was measured for radioactivity in Unisolve. Blank values were obtained without addition of antiserum or immunoglobulin.

Binding of 2,4-Dinitrophenyl Derivatives of Rifamycin SV to 2,4-Dinitrophenyl-Specific Antibodies

Experiments have been performed as described above employing [^{14}C]N_2ph-NHEtS-Rif.

Effects of Immunoglobulins and Avidin on the Inhibition of RNA Polymerase by Derivatives of Rifamycin SV

Poly[d(A-T)] (0.05 mM in nucleotide residues) in buffer A together with RNA polymerase and a rifamycin SV derivative in concentrations as specified in legends to figures were incubated 5 min at 37 °C. Then appropriate amounts of immunoglobulins or avidin were added and incubation continued. At timed intervals 45-µl aliquots were withdrawn from the incubation mixture and added to 5 µl of a solution containing 1.5 mM [^{14}C]ATP (3000 counts min^{-1} · mol^{-1}), 1.5 mM UTP. Incubation was carried out

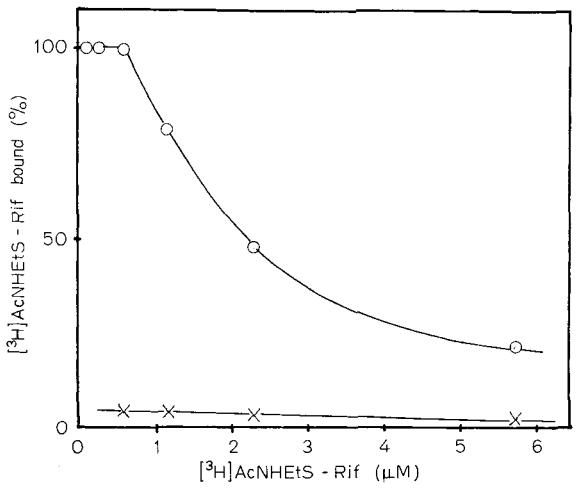

Fig. 2. *Binding of [^{3}H]AcNHEtS-Rif to rabbit antiserum against albumin-$CH_2CONHEtS$-Rif.* Experiments have been carried out as described in Materials and Methods. The incubation mixture contained either 5 µl antiserum against albumin-$CH_2CONHEtS$-Rif (approx. concentration of immunoglobulins 10 mg/ml) (○) or normal rabbit serum (×). The [^{3}H]AcNHEtS-Rif bound is measured as a percentage of the total amount added

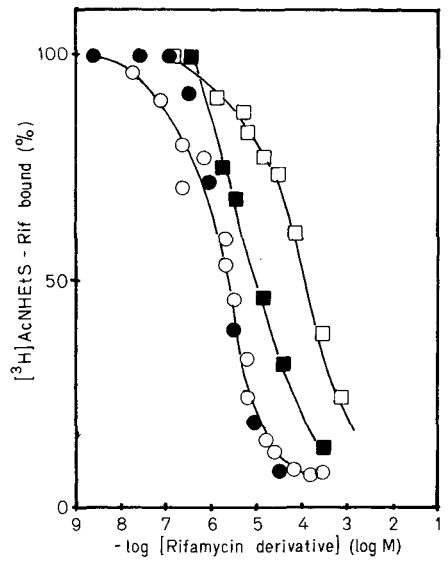

Fig. 3. *The affinity of various derivatives of rifamycin SV to antiserum.* Experimental details as described in Methods. The incubation mixtures contained 10 µl antiserum, 2.3 µM [^{3}H]AcNHEtS-Rif and varying concentrations of the respective rifamycin SV derivative. The percentage [^{3}H]AcNHEtS-Rif remaining bound after addition of rifamycin SV derivative was measured. AcNHEtS-Rif (○); F_3-AcNHEtS-Rif (●); rifamycin SV (■); rifampicin (□)

for 2 min at 37 °C. Synthesized RNA was determined by acid precipitation as described [24]. When necessary the order of addition was changed, rifamycin SV derivative and immunoglobulin or avidin being incubated prior to the addition of enzyme.

Ultracentrifugation Experiments

Sucrose gradient centrifugation has been performed in a Beckman SW-60 Ti swing bucket rotor at 0–5 °C. The sample (0.1 ml) was layered on top of a 4.5-ml gradient (5–20% in 0.04 M Tris-HCl pH 7.9 containing 0.4 M NaCl). The compositions of the applied samples are given in legends to figures. Fractions of the gradient were either measured for radioactivity or absorption at 280 nm. Sedimentation velocity analysis was carried out in a model E analytical centrifuge at 15 °C and 30000 rev./min. The solution contained RNA polymerase core enzyme (0.75 μM) and dimeric rifamycin SV derivative (0.25 μM) in 0.04 M Tris-HCl pH 7.9 containing 0.4 M NaCl.

RESULTS

The Binding of Derivatives of Rifamycin SV to Antibodies against Albumin-CH_2CONHEtS-Rif

Alkylation of bovine serum albumin with BrAcNHEtS-Rif led to an albumin-CH_2CONHEtS-Rif conjugate, which, according to its absorption at 450 nm, contained about 9 residues AcNHEtS-Rif per molecule protein. We assume that alkylation occurred with cysteine and lysine residues.

The conjugate, albumin-CH_2CONHEtS-Rif, was employed to produce antibodies in rabbits against the antibiotic rifamycin SV. The rabbit serum contained proteins with the capacity to bind [^{3}H]AcNHEtS-Rif specifically (Fig. 2). An experiment, in which increasing amounts of [^{3}H]AcNHEtS-Rif are bound to a constant serum protein concentration (see Fig. 2) allows a rough calculation of the content of rifamycin-SV-specific antibodies in the serum. Assuming a molecular weight of 150000 for the antibodies and a total protein immunoglobulin concentration of 10 mg/ml serum, the content of rifamycin-SV-specific antibodies is 0.4–0.5 mg/ml serum. The serum of an untreated rabbit does not contain proteins with comparable affinity for [^{3}H]AcNHEtS-Rif. The specificities of the antibodies formed against various derivatives of rifamycin SV were investigated employing the radioisotope dilution technique. The results are shown in Fig. 3. The ability of the rifamycin derivatives to displace [^{3}H]AcNHEtS-Rif bound to antibodies was measured in these experiments. F_3AcNHEtS-Rif, a close analogue of AcNHEtS-Rif, displayed a similar affinity for the antibodies. Rifamycin SV, lacking substituents in position 3, was four times less effective than bound [^{3}H]AcNHEtS-Rif. The affinity of the antibodies with rifampicin, which has a rather bulky substituent in position 3, is roughly 50 times less than with AcNHEtS-Rif.

Recently, Joniau *et al.* [32] characterized rabbit antibodies which bind rifampicin. These antibodies were induced employing a stable conjugate between bovine serum albumin and 3-formylrifamycin SV. These investigators observed that substituents at C-3 and C-4 of the chromophore nucleus had little effect on the binding of the corresponding rifamycin derivatives to the rabbit antibodies. This finding is not in accord with our results. We observe that the affinity of the rabbit antibodies, which are induced by albumin-CH_2CONHEtS-Rif, for the derivatives of rifamycin SV depends upon the particular substituent at the C-3 position.

The Binding of 2,4-Dinitrophenyl Derivatives of Rifamycin SV to 2,4-Dinitrophenyl-Specific Antibodies

The experiments depicted in Fig. 4 show that [^{14}C]N_2ph-Rif is bound by 2,4-dinitrophenyl-specific immunoglobulins from goat. 3.7 nmol [^{14}C]N_2ph-Rif are bound at a concentration of 1 mg immunoglobulin/ml, indicating that about 20–25% of the immunoglobulins are 2,4-dinitrophenyl specific. We then examined the efficiency of the various derivatives to displace [^{14}C]N_2ph-Rif from the antibodies. The concentrations which are necessary to displace 50% of the bound [^{14}C]N_2ph-Rif from the antibodies are given in Fig. 5. The affinity of the N_2ph derivatives for the antibodies tends to decrease as the distance of the N_2ph moiety from the rifamycin increases.

The Binding of Biotinyl · NHEtS-Rif to Avidin

The affinity of biotinyl · NHEtS-Rif to avidin was determined indirectly. We observed that biotinyl · NHEtS-Rif effectively competed with [^{14}C]biotin in binding to avidin-Sepharose. The data shown in Table 3 reveal a rather similar affinity for avidin of both substances.

The Effect of Antibodies on the Interaction of Derivatives of Rifamycin SV with RNA Polymerase

Do 2,4-dinitrophenyl-specific, or rifamycin-SV-specific antibodies, recognize either N_2ph moieties, or parts of rifamycin SV, in enzyme · N_2ph-Rif or enzyme · rifamycin-SV complexes, respectively? To study this problem we have employed sucrose gradient centrifugation, since a ternary complex would form a distinctly faster-sedimenting species, compared to the binary complex of enzyme · antibiotic. The profiles

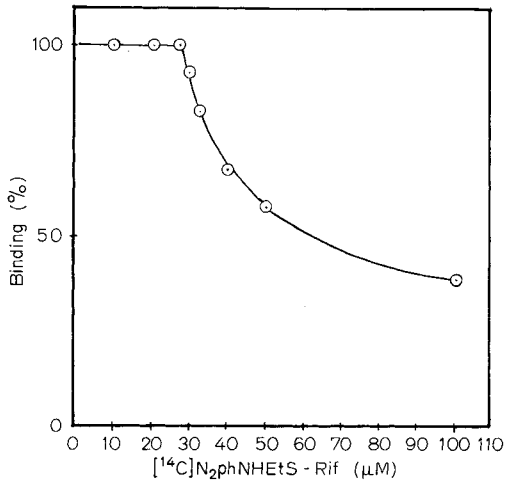

Fig. 4. *The binding of N_2ph-Rif to N_2ph-specific immunoglobulins from goat.* Experimental details as described in Methods. The incubation mixtures contained 35 μg immunoglubulins. The capacity of N_2ph immunoglobulins to bind N_2ph-Rif was determined to 3.9 mmol/mg immunoglobulins

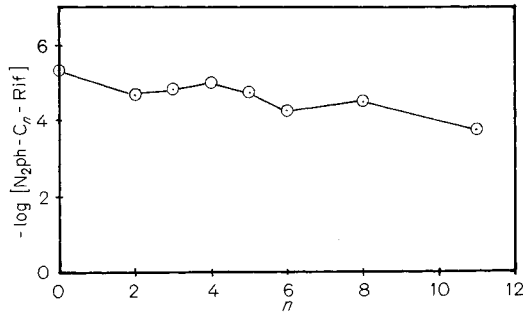

Fig. 5. *The binding of N_2ph derivatives of rifamycin SV to N_2ph-specific immunoglobulins.* Experiments have been carried out in close analogy to those described in legend to Fig. 3. using [^{14}C]-N_2ph-Rif. The concentration of immunoglobulins was 41 μg per assay volume. The concentrations of N_2ph derivatives necessary to displace 50% of the bound [^{14}C]N_2ph-Rif was measured

of the respective sucrose gradients, depicted in Fig. 6 and 7, clearly indicate that no ternary complex of enzyme · antibiotic · antibody is formed in either case. This implies that the dissociation of the complex, enzyme · AcNHEtS-Rif, can be followed in the presence of antibody in accordance with the reaction scheme:

$$E \cdot \text{AcNHEtS-Rif} \rightleftharpoons E + \text{AcNHEtS-Rif} \quad (1)$$

$$\text{Antibody} + \text{AcNHEtS-Rif} \rightleftharpoons \text{antibody} \cdot \text{AcNHEtS-Rif} \quad (2)$$

The reformation of the complex, E · AcNHEtS-Rif, will be prevented, provided that the antibody concentration is sufficiently high. Thus, the dissociation of the enzyme · antibiotic complex should lead to the appearance of enzymatic activity. This we indeed observed, and, as depicted in Fig. 8, large concentrations of antibody suppressed completely the inhibition of RNA polymerase by AcNHEtS-Rif.

Table 3. *Binding of [^{14}C]biotin and biotinylNHEtS-Rif to avidin-Sepharose*
Aliquots of a solution containing either [^{14}C]biotin or [^{14}C]biotin and biotinylNHEtS-Rif in a ratio 1:1 were added to a suspension of avidin-Sepharose in buffer A at 4 °C. After each addition the suspension was gently shaken for 5 min. The avidin-Sepharose was then allowed to settle and an aliquot of the supernatant was measured for radioactivity

Ligand in solution	[^{14}C]Biotin bound
	(nmol/ml avidin-Sepharose)
[^{14}C]Biotin	8.5
[^{14}C]Biotin + biotinylNHEtS-Rif (1:1)	4.9

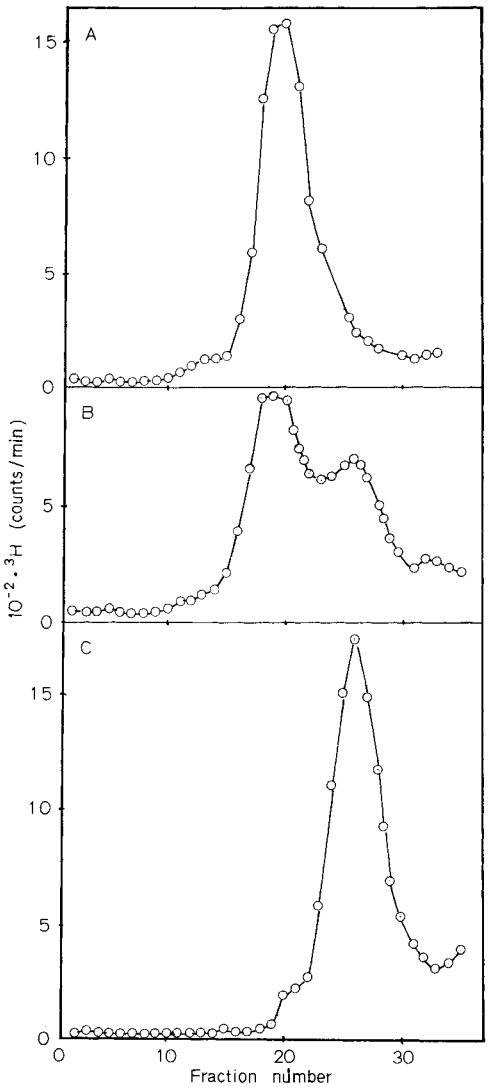

Fig. 6. *Interaction of RNA polymerase with AcNHEtS-Rif in presence of rifamycin-SV-specific immunoglobulins.* Experimental details of sucrose gradient ultracentrifugation as described in Methods. (A) RNA polymerase holo enzyme (2.2 μM) and [^{3}H]AcNHEtS-Rif (2 μM); (B) RNA polymerase holo enzyme (2.6 μM), [^{3}H]AcNHEtS-Rif (2 μM) and rifamycin-SV-specific immunoglobulins (6 mg/ml); (C) rifamycin-SV-specific immunoglobulins (6 mg/ml) and [^{3}H]-AcNHEtS-Rif (2 μM)

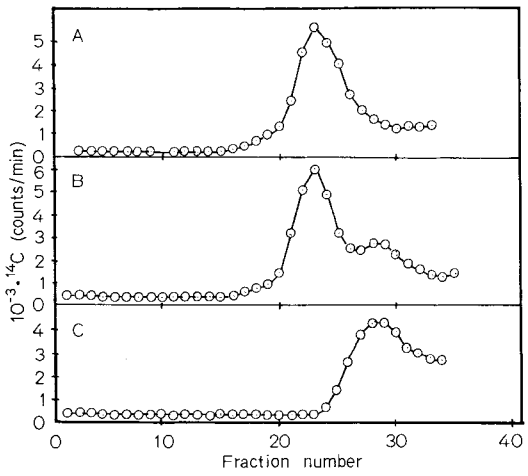

Fig. 7. *Interaction of RNA polymerase with N_2ph-Rif in presence of N_2ph-specific immunoglobulins.* Experimental details of sucrose ultracentrifugation as described in Methods. (A) RNA polymerase holo enzyme (2.2 μM) and [^{14}C]N_2ph-Rif (2 μM); (B) RNA polymerase holo enzyme (2.2 μM), [^{14}C]N_2ph-Rif (2 μM) and N_2ph-specific immunoglobulins (1.1 mg/ml); (C) N_2ph-specific immunoglobulins (1.1 mg/ml) and [^{14}C]N_2ph-Rif (2 μM)

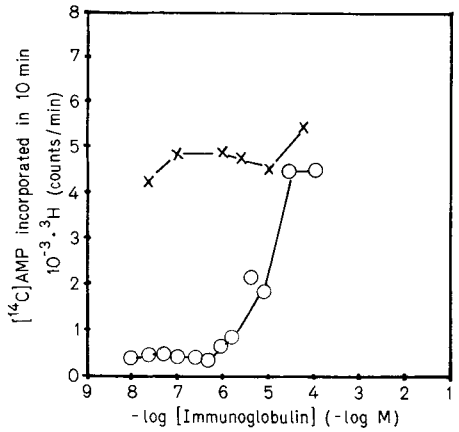

Fig. 8. *Suppression of inhibition by AcNHEtS-Rif of RNA polymerase employing rifamycin-SV-specific immunoglobulins.* The incubation mixtures contained RNA polymerase holo enzyme (27 nM), [^{3}H]-AcNHEtS-Rif (1.05 μM) and varying concentrations of immunoglobulins (○). For control experiments AcNHEtS-Rif was omitted from the incubation mixtures (×)

The data summarized in Table 4 show that all the synthesized N_2ph derivatives of rifamycin SV inhibited RNA polymerase to 50 %, at a concentration of approximately 0.15 μM. Thus, their affinities for RNA polymerase seem to be roughly comparable to the affinities of AcNHEtS-Rif or rifampicin for RNA polymerase.

Does this hold likewise for derivatives in which the N_2ph moieties are separated from the rifamycin SV part of the derivative by an aliphatic side chain of increasing length? We investigated this problem employing the following approach. Complexes between N_2ph derivatives of rifamycin SV and N_2ph-

Table 4. *Inhibition of RNA polymerase by derivatives of rifamycin SV*
Inhibition was measured in a standard assay with calf thymus DNA (100 μg/ml) and RNA polymerase holo enzyme (50 μg/ml). DNA, enzyme and rifamycin SV derivative were incubated for 5 min at 37 °C. RNA synthesis was started by addition of substrates and allowed to proceed for 10 min at 37 °C. Further experimental details as described in Methods. K_i denotes the concentration of rifamycin SV derivative necessary to inhibit the reaction to the extent of 50 %

Rifamycin SV derivative	$10^8 \times K_i$
	M
Rifampicin	5.5
AcNHEtS-Rif	17.8
Biotinyl · NHEtS-Rif	17
Rif-C_1-Rif	12.6
Rif-C_2-Rif	7.9
Rif-C_3-Rif	10.9
Rif-C_6-Rif	7.1
Rif-C_8-Rif	4.4
Rif-C_{11}-Rif	11.5
N_2ph-Rif	12
N_2ph-C_2-Rif	13.6
N_2ph-C_3-Rif	13
N_2ph-C_4-Rif	14.2
N_2ph-C_6-Rif	15.9
N_2ph-C_8-Rif	14
N_2ph-C_{11}-Rif	11.2

antibodies were performed at 0 °C, with an excess of antibodies. Then, RNA polymerase was added and the incubation was continued at 0 °C. The formation of a ternary complex was tested for indirectly by measuring the extent of inhibition of RNA polymerase in a standard assay. In agreement with the ultracentrifugation studies, the complex antibody · N_2ph-Rif does not inhibit RNS polymerase, indicating that no ternary complex is formed. However, complexes between antibodies and N_2ph-NH$(CH_2)_n$CONHEtS-Rif with $n \geq 3$ do inhibit RNA polymerase, and to approximately the same extent (Fig. 9).

The Interaction of Enzyme · BiotinylNHEtS-Rif Complexes with Avidin

Avidin is able to recognize the biotinyl residue in enzyme · biotinylNHEtS-Rif. This was confirmed by ultracentrifugation studies. The absorption profile of corresponding sucrose gradients, as well as analysis of the fractions by sodium dodecyl sulfate gel electrophoresis, reveal the formation of species containing enzyme and avidin (Fig. 10). The major part sediments with a velocity comparable to that of monomeric RNA polymerase core enzyme and is assumed to be enzyme · biotinylNHEtS-Rif · avidin. A smaller portion sediments with a velocity comparable to dimeric RNA polymerase core enzyme. We assume that this

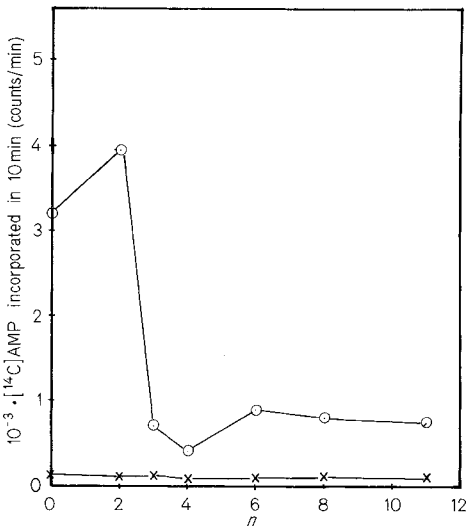

Fig. 9. *Inhibition of RNA polymerase by N_2ph-$(C)_n$-Rif · antibody complexes.* The incubation mixtures contained RNA polymerase holo enzyme (27 nM), rifamycin SV derivative (0.55 μM) and N_2ph-specific immunoglobulins (1.36 mg/ml). Complexes N_2ph-$(C)_n$-Rif · antibody were preformed by incubation for 15 min at 0 °C. Enzymic activity was measured in a standard assay employing poly[d(A-T)] as template. In control experiments, immunoglobulins were omitted from the incubation mixtures (×)

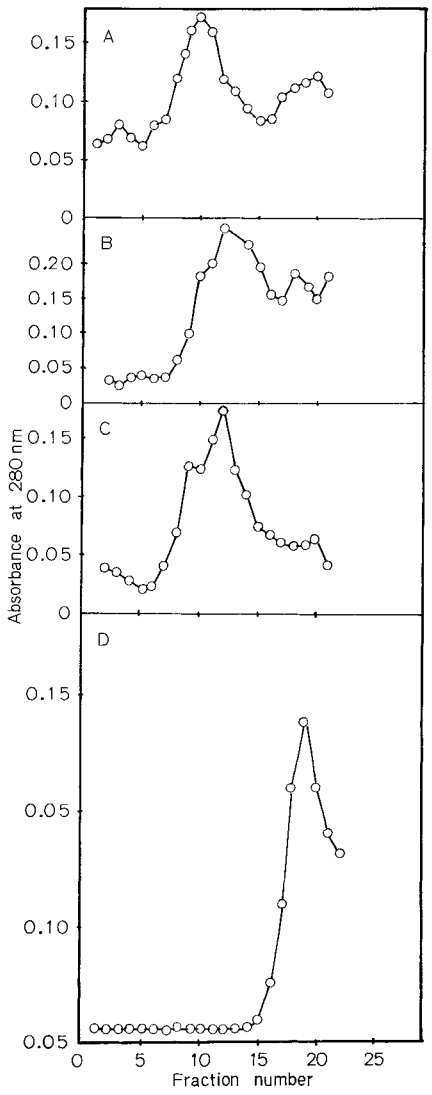

Fig. 10. *Interaction of RNA polymerase with biotinylNHEtS-Rif in presence of avidin.* Experimental details of sucrose gradient ultracentrifugation as described in Methods. (A) RNA polymerase core enzyme in buffer containing 0.05 M NaCl. (B) RNA polymerase core enzyme (5 μM) and avidin (2.5 μM) in buffer containing 0.4 M NaCl; (C) RNA polymerase core enzyme (5 μM), biotinylNHEtS-Rif (5 μM) and avidin (2.5 μM) in buffer containing 0.4 M NaCl; (D) avidin (5 μM)

species might be the complex (biotinylNHEtS-Rif · enzyme)$_2$ · avidin. The existence of such a complex seems likely in view of the observation by Green *et al.* [16, 17]. They established the formation of a similar type of complex: (antibody · 2,4-dinitrophenylbiotin)$_2$ · avidin. Consequently, the complex avidin · biotinyl-NHEtS-Rif was expected to inhibit RNA polymerase. Results shown in Fig. 11 demonstrate that biotinyl-NHEtS-Rif bound to avidin inhibits RNA polymerase to the same extent as does biotinylNHEtS-Rif. The affinity of RNA polymerase to the complex avidin · biotinylNHEtS-Rif is further established by an experiment described in Fig. 12. RNA polymerase core enzyme is absorbed out of solution onto avidin-Sepharose previously saturated with biotinylNHEtS-Rif.

The Interaction of RNA Polymerase with Dimeric Derivatives of Rifamycin SV

It has been shown by Silvestri *et al.* [33] that RNA polymerase is inhibited by a dimeric derivative of rifamycin SV. Employing ultracentrifugation they failed to demonstrate the existence of a ternary complex enzyme · Rif-C_n-Rif · enzyme. We suspected that a ternary complex would not form because the link between the two rifamycin SV molecules was too short. Therefore, we synthesized a series of dimeric derivatives of rifamycin SV linked by aliphatic chains ranging from 0.50 – 2.50 nm. The concentrations of the various dimeric rifamycin SV derivatives necessary to achieve 50% inhibition of RNA polymerase are listed in Table 4. These data show that the dimeric derivatives and AcNHEtS-Rif or rifampicin have similar affinities for RNA polymerase. The inhibition data do not indicate that both rifamycin SV residues are utilized in the dimeric derivatives for the inhibition of RNA polymerase. Sedimentation velocity analysis of complexes between RNA polymerase and Rif-C_{11}-Rif or Rif-C_{12} furnishes evidence for the exclusive formation of binary complexes. As shown by Wehrli *et al.* [10], the binding of radioactive rifamycin SV derivatives to RNA polymerase can be measured using charcoal to separate free antibiotic. Employing

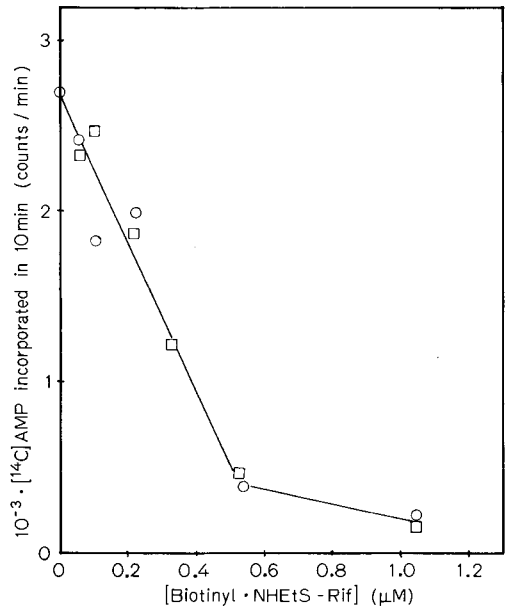

Fig. 11. *Inhibition of RNA polymerase by avidin · biotinylNHEtS-Rif.* The incubation mixtures (□) contained RNA polymerase core enzyme (30 nM), avidin (2 μM) and biotinylNHEtS-Rif in varying concentrations. Complexes avidin · biotinylNHEtS-Rif were performed by incubation for 15 min at 0 °C. Enzymatic activity was determined in a standard assay as described in Methods. Control experiments have been carried out in the absence of avidin (○)

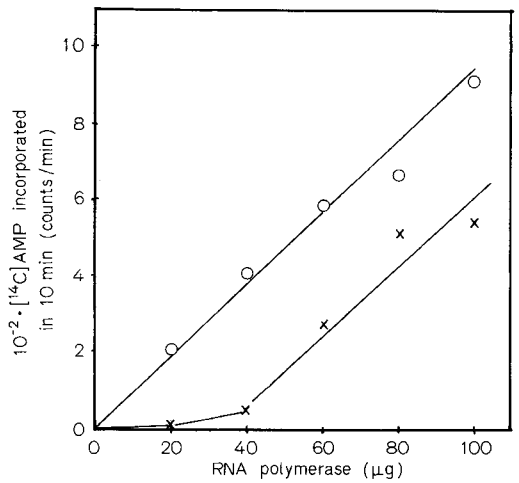

Fig. 12. *Binding of RNA polymerase core enzyme to avidin-Sepharose saturated with biotinylNHEtS-Rif.* (×) To 1 ml avidin-Sepharose saturated with biotinylNHEtS-Rif in 1 ml buffer A were added increasing amounts of RNA polymerase core enzyme. After gentle shaking of the suspension for 5 min at 37 °C, the suspension was centrifuged and the enzymatic activity of the supernatant measured by subjecting an aliquot to a standard assay. In a control experiment untreated avidin-Sepharose was employed (○)

this approach, we investigated the competition of AcNHEtS-Rif and Rif-C_{10}-Rif for the binding of [^{14}C]AcNHEtS-Rif to RNA polymerase. The results given in Fig. 13 demonstrate that AcNHEtS-Rif and Rif-C_{10}-Rif are similar with respect to their affinity for RNA polymerase.

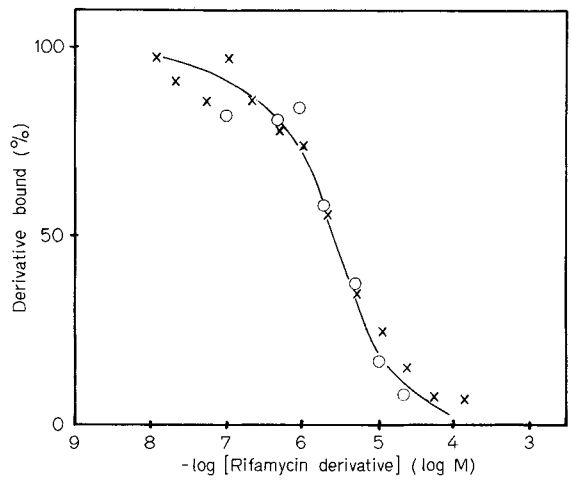

Fig. 13. *The binding of AcNHEtS-Rif and Rif-C_{12}-Rif to RNA polymerase core enzyme.* The experimental procedure of Wehrli *et al.* [10] was closely followed. The incubation mixtures contained RNA polymerase core enzyme (1.25 μM), [^{3}H]AcNHEtS-Rif (1.25 μM) and varying concentrations of either AcNHEtS-Rif (×) or Rif-C_{12}-Rif (○). The incubations have been carried out at 37 °C. Separation of unbound rifamycin SV derivatives by means of charcoal was performed at 0 °C

DISCUSSION

The dimensions of the N_2ph-binding site in N_2ph-antibodies has been measured by various means [15–20]. According to these results the binding site for the N_2ph residue seems to have a depth of 1.00 nm. The functional parts of N_2phNH$(CH_2)_n$CONHEtS-Rif (N_2ph and rifamycin SV) can only be recognized simultaneously by N_2ph-antibodies and RNA polymerase if $n \geq 3$. The extension of the N_2ph-NH-$(CH_2)_2$CONHEtS-Rif molecule ranges from 2.40–2.90 nm as measured on Corey/Pauling/Koltun model. Thus, we conclude that the binding site of RNA polymerase for rifamycin SV extends 1.40–1.90 nm deep into the interior of the enzyme structure. The results indicate furthermore, that the ansa chain of the antibiotic extends the furthest into the enzyme. The experiments with biotinylNHEtS-Rif and avidin support this interpretation. According to investigations by Green *et al.* [16], the biotin binding site on avidin seems to be 1.00 nm deep and the bound biotin molecule is oriented such that the carboxyl group is accessible to the outside. The inhibition of RNA polymerase by avidin · biotinylNHEtS-Rif complexes clearly indicates that the interaction between avidin and biotin does not involve the regions of the ligand where the carboxylic group is located. Since the distances of the N_2ph and biotinyl groups from the rifamycin SV part of the respective derivatives are nearly identical,

the results obtained with the complex avidin · biotinylNHEtS-Rif further support the above dimensions of the rifamycin SV binding site on RNA polymerase.

We observe that dimeric derivatives of rifamycin SV, even those with a spacer of approximately 3.00 nm, did not form a ternary complex with RNA polymerase. A possible explanation could be that RNA polymerase has two binding sites for a dimeric rifamycin SV derivative: the genuine site of the enzyme for the antibiotic and a topographically closely related unspecific second site for hydrophobic ligands. The association constant for the dimeric rifamycin SV molecule would then be the product K_a (specific) · [1 + K_a (unspecific)]. If K_a (unspecific) is > 10, the affinity of RNA polymerase for the dimeric rifamycin SV should be significantly higher than for AcNHEtS-Rif. This, however, was not observed. Therefore, the hypothesis of a second binding site for a dimeric rifamycin SV molecule cannot be valid. From the interactions of enzyme · N_2ph-C_3Rif with N_2ph-antibodies, and of enzyme · biotinylNHEtS-Rif with avidin we could expect that the second rifamycin SV moiety of the complex enzyme · Rif-C_{11}-Rif would be accessible for another RNA polymerase molecule from a stereochemical point of view. A ternary complex, however, might not be formed, because complex formation would involve close contacts between the two enzyme molecules possibly at a highly charged site of the protein structure.

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W. Stender and K. H. Scheit*, Abteilung Molekulare Biologie,
Karl-Friedrich-Bonhoeffer-Institut, Max-Planck-Institut für Biophysikalische Chemie,
Am Faßberg, D-3400 Göttingen-Nikolausberg, Federal Republic of Germany

* To whom reprint requests should be addressed.