INHIBITION OF RNA POLYMERASES FROM RAT LIVER BY THE SEMI-SYNTHETIC RIFAMPICIN DERIVATIVES

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1. Introduction

The antiviral drug rifampicin inhibits DNA-dependent RNA polymerase of *E. coli* (E.C. 2.7.7.6) in the preinitiation phase [1–4] without effecting the act of phosphodiester bond formation, responsible for the chain growth of the RNA molecules. This property of the drug enabled a detailed study of the mechanism of the polymerisation reaction in the bacterial system. While rifampicin is without influence on the RNA polymerase of eukaryotic origin, semi-synthetic derivatives of the drug, effecting these enzymes have been developed.

In order to determine the true chain growth rate of mammalian polymerases, the foremost prerequisite was the verification that the rifampicin derivatives act only on the preinitiation stages, without interfering with the elongation process. Data indicating that the latter condition may be operative were put forward by Butterworth et al. [5] and more recently by Meilhac et al. [6]. Published data [6] and comparative results presented in this report show that derivatives exemplify inhibitory potency related to their degree of apolarity.

The present report was adressed to the question dealing with the possible effect of semi-synthetic rifampicin derivatives AF/05, AF/013 and PR/19 on the chain growth rates catalysed by RNA polymerases from rat liver tissue.

2. Materials and methods

[γ-^{32}P]ATP and [γ-^{32}P]GTP, [^{3}H]UTP (1 Ci/mmole) were obtained from the Radiochemical Centre Amersham, nucleoside triphosphates, creatine phosphate and creatine phosphokinase from Boehringer (Mannheim). Rifampicin derivatives AF/05 (3-formyl-rifamycin SV:*o*-(diphenylmethyl) oxim), AF/013 (3-formyl-rifamycin SV:*o*-*n*-octyloxime) and PR/19 (3′-acetyl-1′-benzyl-2′-methylpyrrolo 3,2-*c*-4-deoxy-rifamycin SV) were generous gifts of Prof. L.G. Sylvestri (Gruppo Lepetit, Milano). All other chemicals used were reagent grade obtained from Merck (Darmstadt).

Nuclear polymerases A and B, and a RNA polymerase species recently isolated from the cytoplasma of the liver cell and designated as C, were isolated and purified as described by Seifart et al. [7]. RNA polymerase was measured in an *in vitro* system, incubated for 15 min at 37°, consisting of 0.2 μmoles each of ATP, GTP, CTP, 0.05 μmoles of UTP and 1 μCi [^{3}H]UTP. In cases where 5′-termini were labelled by [γ-^{32}P]ATP or -GTP, the concentration of the radioactive purine nucleotide was lowered to 0.05 μmole/assay thus adjusting the specific activity to approx. 1000–1500 dpm/nmole. In addition, the assay contained 0.5 μmoles $MnSO_4$ (in the case of enzyme A: 3.0 μmoles $MgSO_4$), 1 μmole β-mercaptoethanol and in the case of enzymes B and C, $(NH_4)_2SO_4$ at an ionic strength of 0.2. Each assay contained about 5 μg enzyme protein in a final volume of 150 μl. The enzyme, DNA and required ions were mixed and preincubated for 20 min at 0° to obtain rifampicin resistant preinitiation complexes, followed by the addition of a complete mixture of nucleoside triphosphates.

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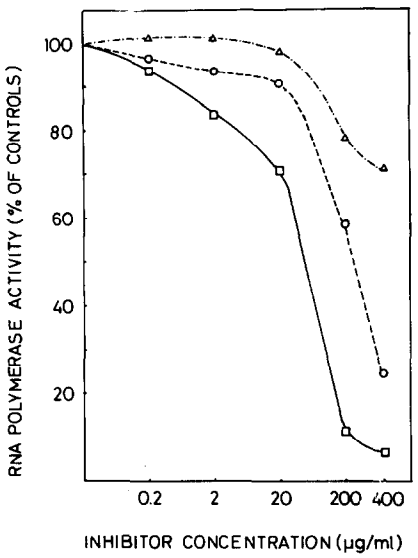

Fig. 1. Inhibitory effect of various concentrations of the rifampicin derivatives on the incorporation of [^{3}H]uridine triphosphate into acid insoluble polynucleotides catalysed by rat liver polymerase B. (△-·-·-△) AF/05; (□—□—□) AF/013; (○- - -○- - -○) PR/19. In this case the inhibitor was added to the enzyme and the reaction started by the addition of all other components.

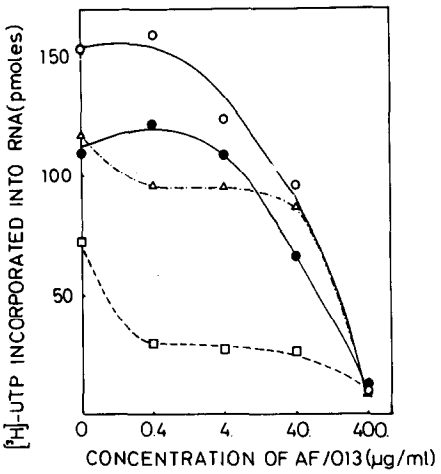

Fig. 2. Inhibition of different rat liver RNA polymerases by AF/013. Polymerase B was also tested in presence of a stimulatory protein factor [8] enhancing the chain elongation. (□- - -□- - -□) Polymerase A; (●—●—●) polymerase B without E- factor; (○—○—○) B with factor; (△-·-△-·-△) polymerase C.

At the end of the incubation, aliquots of 120 μl were pipetted onto filter paper discs (Schleicher u. Schuell, 2043b paper), fixed and decontaminated in 5% trichloroacetic acid as described [8]. To remove the remaining antibiotic giving rise to colour-mediated, optical quench of varying degree, the decontaminated paper discs were additionally washed in acetone–diethylether (3:1), followed by 3 rinses in diethylether. It is noteworthy that the paper-disc method employed yields more reproducible results when compared to nitrocellulose filtration in the presence of a carrier, possibly due to the lower ultraviolet absorption of the non-modified pure cellulose and a lower self-absorption of the tritium emissions.

For the calculation of the average chain length, it was assumed that ATP and GTP initiation is equally frequent, and that UTP is incorporated at a frequency of 25%. Under all conditions, controls from which DNA was omitted, were conducted for each experimental measuring point, in order to reduce error arising from DNA independent events. These controls were deducted from each value, ensuring that only DNA-dependent incorporation values were taken into account.

In those experiments only measuring [^{3}H]UTP incorporation, the concentration of both purine nucleoside triphosphates was 0.2 μmoles/assay and in cases assessing the time: temperature requirements for the formation of rifampicin resistant complexes, the exact experimental conditions are appropriately indicated.

3. Results

Fig. 1 represents the dose-response curves of the rifampicin derivatives AF/013, AF/05 and PR/19 on RNA synthesis catalysed by rat liver RNA polymerase B *in vitro*. If the drug is allowed to interact with the free enzyme, an almost complete degree inhibition is observed for AF/013 at 400 μg/ml, allowing only a residual synthesis of approx. 5%, whereas this figure is approx. 72 and 24% for AF/05 and PR/19, respectively. These data are somewhat different from those presented by Chambon and associates [6] for the calf thymus enzymes, who found complete inhibition for both derivatives AF/105 and AF/013. The concentration of AF/013 required to achieve complete inhibition

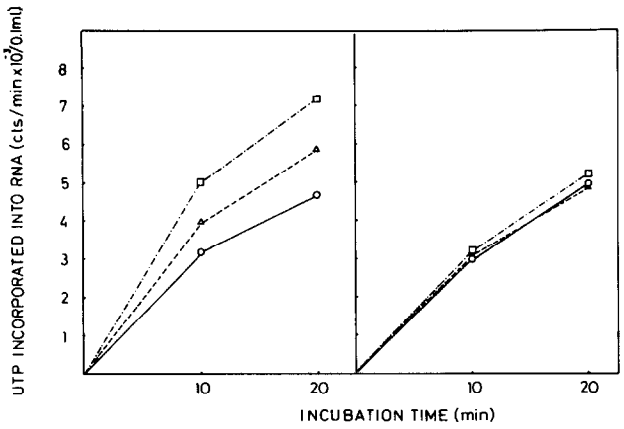

Fig. 3. Effect of preincubating polymerase B with DNA on the residual polymerase activity in presence of 400 μg/ml AF/013. Part A: Effect of preincubation temperature. The enzyme and native DNA were preincubated for 20 min at 37° (□-·-·-□), 18° (△- - - -△), or 0° (○——○). The reaction was then started by the addition of a complete mixture of nucleoside triphosphates and required ions. Part B: Effect of preincubation time at 0° for 5 (○——○), 10 (△—— △) or 20 (□-·-·-□) min on the residual polymerase activity.

is higher than communicated by Butterworth et al. [5] although these differences are possibly explained by differences in the amount of enzyme employed effecting the degree of inhibition for simple stoichiometric reasons. Subsequent studies were conducted with AF/013, being the most potent inhibitor of DNA-dependent RNA synthesis.

Fig. 2 demonstrates, as has very recently been shown for the enzymes from calf thymus [6], that both nuclear enzymes A and B as well as enzyme C are inhibited by AF/13. This inhibition is also observed if synthesis of enzyme B is conducted in the presence of a stimulatory factor from rat liver recently shown to act on the process of chain elongation [8].

The crucial question related to these experiments, centers on optimizing the conditions of preincubation, under which rifamycin resistant binary enzyme:DNA complexes are formed. In fig. 3 some of these conditions are outlined for enzyme B. It is evident that the temperature, at which enzyme and DNA are preincubated for 20 min plays an important role. The time of preincubation becomes of lesser importance, when

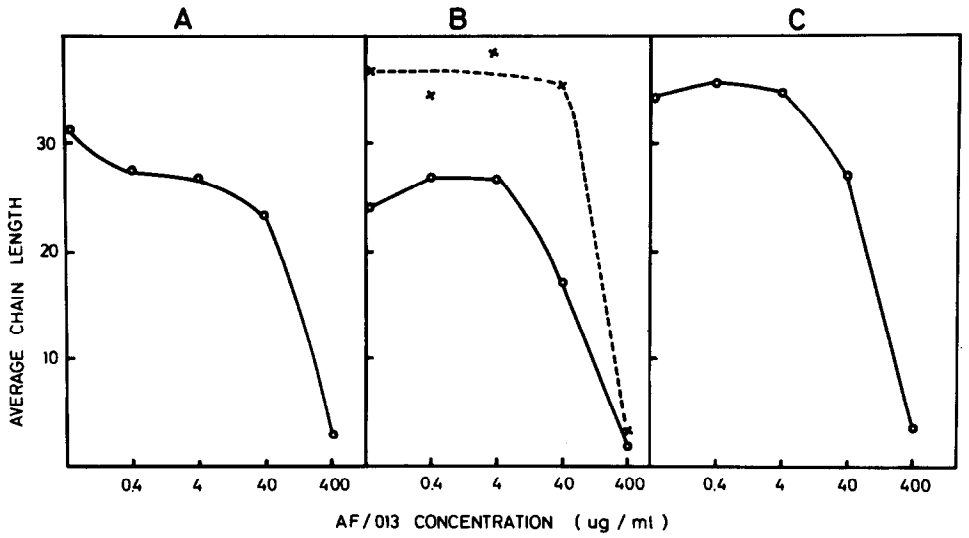

Fig. 4. Effect of different concentrations of AF/013 on the average chain length of RNA's synthesized *in vitro* by nuclear RNA polymerase A (part A); polymerase B (part B; (○—○—○) without elongation factor, (X- -X- -X) with elongation factor) and cytoplasmic polymerase C (part C).

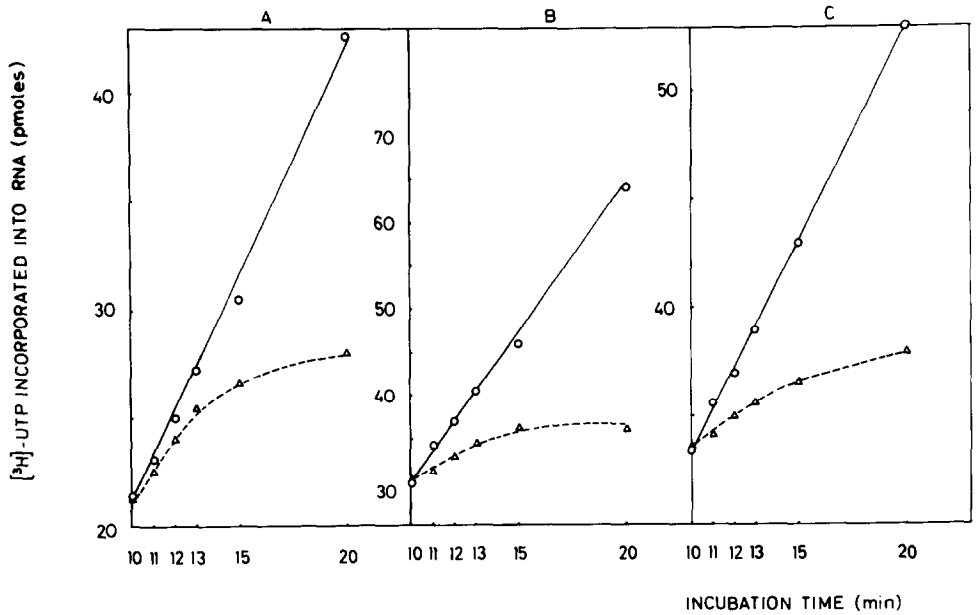

Fig. 5. Time kinetics of the inhibition of different RNA polymerases by 400 μg/ml AF/013, when the drug is added during enzymatic synthesis. Assays were started by adding a mixture of all components to the enzymes. After 10 min 5 μl dimethylformamide (○—○—○) or AF/013 in 5 ul DMF (△- - -△- - -△) was added. Part A: nuclear RNA polymerase A; Part B: nuclear RNA polymerase B; Part C: cytoplasmic RNA polymerase C [7].

conducted between 5 and 20 min (fig. 3 B).

To elucidate whether these initiation complexes, which are formed on DNA and demonstrate apparent rifamycin resistance at 0°, carry out RNA synthesis in a non-perturbed fashion even in the presence of the inhibitor, the average chain length of the remaining synthesis was calculated and compared to controls. The data of fig. 4 convey the conclusion that the average chain length of the RNA synthesized by all three enzymes after the formation of the binary DNA: enzyme complex, decreases as a function of inhibitor concentration. It should be pointed out that in the control experiments, containing only dimethylformamide, the average chain length is remarkably short, when compared to values estimated for the same enzymes under identical conditions, but in the absence of dimethylformamide [8]. It is conceivable, that the lipophilic solvent DMF required to apply the highly apolar antibiotics in sufficiently high concentrations, also has been an inhibitory action on the chain elongation process, since experiments have shown (data not presented here), that dimethylformamide clearly inhibits the overall rate of RNA synthesis.

When AF/013 is added to a system already actively engaged in transcription (fig. 5), a much higher proportion of residual synthesis is resistant to the inhibitor compared to its addition to the free enzyme. This is a confirmation of previously published data [6]. An exact analysis of the time sequence shows however, that the inhibition is manifested immediately after the addition of inhibitor.

4. Discussion

The data presented here demonstrate that rat liver RNA polymerases A, B and C can be inhibited by rifampicin derivatives PR/19, AF/05 and AF/013, the latter of which shows the highest degree of apolarity and highest inhibitory potency on RNA synthesis.

In their systematic study of the effect of rifampicin derivatives on the RNA polymerases from calf thymus, which appeared after the experimental part of this report had been completed, Meilhac et al. [6] have presented a detailed model of transcription analogous to that proposed for the bacterial enzyme [9]. Their

results demonstrate that the effective derivatives of rifampicin completely block the formation of the initiation complex, when the enzyme is added before the formation of the binary DNA:enzyme complex. If prior formation of the complex was permitted, addition of the apolar rifampicin derivatives caused only a partial inhibition of subsequent nucleotide polymerisation.

The data presented in this report in essence fully agree with the conclusions of Chambon and associates with respect to the effect of the synthetic rifamycins on the inhibition of rat liver RNA polymerases A, B and C at the preinitiation stage [6]. However, it has been found that these inhibitors, if used at the concentrations employed to achieve complete inhibition of free enzyme, may also effect the act of chain elongation. It should be pointed out, that if the inhibitor is added to a system actively engaged in transcription, residual synthesis does occur, reinforcing the previously drawn conclusion, that initiated enzyme displays a much higher degree of resistance than free enzyme. This is in accord with previously published reports. However, careful examination of the data shows an immediate inhibition of RNA synthesis after addition of inhibitor without a phase of complete resistance as in the case for *E. coli* RNA polymerase and rifampicin. In this context, the rate of reinitiation obviously becomes of importance since the immediate depression of synthesis could obviously reflect the amount of reinitiation. It is questionable, however, whether a considerable degree of reinitiation occurs with enzyme B after 10 min. Taken together with the calculations of the average chain length, these data collectively indicate, that additional processes, other than the mere act of initiation may be impeded by these inhibitors.

The question of inhibitor concentration is of supreme importance. Fig. 4 shows that concentrations up to 40 μg/ml hardly effect the process of chain elongation, as has also been reported by Meilhac [6]. However, this concentration also does not completely inhibit the free enzyme in our hands and considerably higher concentrations may then begin to act non-specifically. Therefore, the balance point, at which these compounds can be used as specific inhibitors of initiation appears to be extremely labile.

This is an important consideration for the estimation of parameters like absolute chain growth velocities and these findings may be of importance to other investigators dealing with related questions.

Acknowledgements

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