THE EFFECT OF RIFAMPICIN ON MITOCHONDRIAL DNA SYNTHESIS

Cecilia SACCONE and Maria N. GADALETA
Institute of Biological Chemistry, University of Bari, 70126 Bari, Italy

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1. Introduction

In 1971 Stavrianopoulos et al. [1] demonstrated that DNA polymerase from chicken embryo acting on a denatured calf-thymus DNA could be stimulated by the concomitant transcription of the template with *E. coli* RNA polymerase. The synergism between DNA and RNA polymerase was subsequently confirmed by reports from other laboratories with studies *in vitro* and *in vivo* [2—4]. From these studies many authors have suggested that probably the transcription process is coupled to duplication either in bacteria as well as in higher organisms, although the biological significance of this mechanism is still under investigation.

Dependence of DNA synthesis on transcription in bacteria has often been demonstrated using the inhibitor rifampicin which specifically acts on the DNA-dependent RNA polymerase of prokaryotic organisms [2, 3]. Since the mitochondrial RNA polymerase of rat liver is sensitive to this antibiotic [5, 6] an eventual synergism between the two mitochondrial polymerase enzymes could be demonstrated by studying the action of rifampicin on mitochondrial DNA synthesis.

This paper reports an inhibitory effect of rifampicin on DNA synthesis in isolated rat liver organelles. This fact suggests that probably RNA and DNA synthesis are jointly occurring events also in mitochondria.

2. Materials and methods

Labeled and unlabeled deoxyribonucleoside triphosphates were purchased from Schwarz Bio-Research, pancreatic ribonuclease from C.F. Boehringer, Mannheim, W. Germany; all other reagents were of analytical grade.

Male albino rats weighing about 200 g and fed ad libitum were used in all experiments. Mitochondria were isolated from rat liver under sterile conditions as already reported [7]. The homogenization and the suspension medium was 0.25 M sucrose plus 2 mM EDTA (pH 7). Isolated mitochondria were kept at 37°C for 10 min in order to alter their permeability. The reaction mixture for DNA polymerase activity contained: 50 mM Tris–HCl buffer; 20 mM sodium phosphate; 20 mM sodium succinate; 4 mM KCl; 0.5 mM ATP; 7 mM $MgCl_2$; 60 mM sucrose; 4 μM ^{3}H-labeled deoxyribonucleotides and 15 μM of the other deoxyribonucleoside triphosphates; final pH, 7.4. Incubation was carried out at 37°C. Reactions were started by adding the mitochondrial protein and stopped by 4 ml of ice-cold 10% trichloroacetic acid. The precipitate was collected on membrane filters, washed four times with 5 ml ice-cold 5% trichloroacetic acid and, after drying, counted in a PPO–POPOP scintillation mixture, in a Packard scintillation counter; ^{3}H efficiences were about 30%. The acid-insoluble radioactivity was solubilized by DNAase but not by RNAase treatment.

Protein was determined by the biuret method [8], with serum albumin as standard.

3. Results

Rat liver mitochondria isolated as described in Materials and methods were kept at 37°C for 10 min in order to make the organelle's membrane permeable to some inhibitors like rifampicin or actinomycin D which are known to be unable to pass through the in-

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Table 1
[^{3}H]dTTP or [^{3}H]dATP incorporation into acid insoluble material by rat liver mitochondria.

System	Activity (pmoles/mg protein/30 min)	
	[^{3}H]dTTP	[^{3}H]dATP
Complete	0.19 ± 0.03	0.20 ± 0.03
+ calf thymus DNA (100 μg/ml)	0.20 ± 0.03	0.21 ± 0.03
− dGTP, dCTP, dATP	0.08 ± 0.02	–
+ Actinomycin D (200 μg/ml)	0.06 ± 0.02	–

All incubations were performed under standard conditions (see Materials and methods). 2.5 mg of mitochondrial protein were incubated in 0.5 ml of the reported reaction mixture. Zero time controls were already subtracted.

Table 2
Effect of some inhibitors of RNA and protein synthesis on [^{3}H]dTTP incorporation.

Additions	Incorporation (% of control)
Rifampicin (25 μg/ml)	65
Rifampicin (50 μg/ml)	50
Pancreatic RNAase (50 μg/ml)	30
Chloroamphenicol (90 μg/ml)	90
Cycloheximide (270 μg/ml)	82

Inhibitors were added to the incubation mixture to reach the final concentrations reported in the table. 100% control activity was equivalent to 0.2 ± 0.03 pmoles of [^{3}H]dTMP incorporated/mg protein/30 min. The same results were obtained when [^{3}H]dATP was the labeled precursor.

tact inner membrane [5, 7]. DNA synthesis was studied by measuring the incorporation of labeled deoxyribonucleoside triphosphates into acid-insoluble material as described by others [9, 10]. Some of the characteristics of this reaction are presented in table 1. The incorporation of either dATP or dTTP is mostly dependent on the presence of the other three dNTP's, the remaining incorporation being presumably supported by the endogenous pool of dNTP's. The reaction is not dependent on the addition of exogenous DNA since the polymerase uses the endogenous DNA since the polymerase uses the endogenous DNA as template but is sensitive to actinomycin D. The incorporation of the two labeled nucleotides is the same, as reported by others [9, 10].

Table 2 shows the effect of rifampicin and some other inhibitors of mitochondrial DNA synthesis. After 30 min incubation the reaction is inhibited by about 35% using 25 μg/ml rifampicin (5 μg/mg protein) and by 50% using 50 μg/ml rifampicin (10 μg/mg protein). The reaction is inhibited also by RNAase clearly showing that synthesis or the presence of RNA molecules are necessary for DNA synthesis. Since rifampicin inhibits mitochondrial protein synthesis by acting on the synthesis of the messenger RNA [11, 12], we have studied whether another specific inhibitor of mitochondrial protein synthesis like chloramphenicol [13] could affect the reaction. As is shown in table 2, up to 90 μg/ml of chloramphenicol does not inhibit the incorporation of labeled precursor into mitochondrial DNA. Cycloheximide which is an inhibitor of cytoplasmic protein synthesis [13] also does not affect the reaction. These results indicate that the effect of rifampicin on DNA synthesis is specific and not secondary to its inhibition of mitochondrial protein synthesis.

In order to investigate the effect of rifampicin on mitochondrial DNA polymerase better, the time course of DNA synthesis in the presence or absence of rifampicin was studied. Fig. 1 shows that the percentage of inhibition by rifampicin increases with time either when the drug is present from the beginning or when added after 15 min incubation. This probably means that DNA polymerase is able to elongate the already initiated chains and therefore synthesis of new RNA chains is required in order to obtain inhibition by rifampicin.

4. Discussion

The results reported in this paper indicate that rifampicin inhibits mitochondrial DNA synthesis in rat liver. Since it has been demonstrated that this drug affects mitochondrial RNA synthesis [5, 6] and a direct effect of rifampicin on DNA polymerase itself has never been reported, it is conceivable to suppose that the inhibition of DNA synthesis is dependent on the inhibition of RNA synthesis in the organelles. According to several authors, in bacteria as well as in higher organisms, DNA synthesis requires the collaboration of a system synthesizing RNA or the presence of a preformed oligonucleotide primer hydrogen-bonded to the template. According to Karkas [3] DNA polymer-

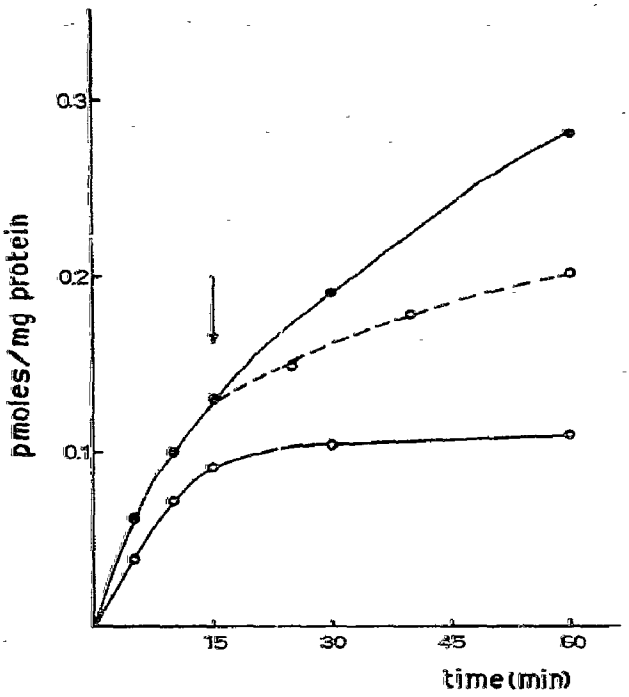

Fig. 1. Time course of [^{3}H]dTTP incorporation in the presence or in the absence of rifampicin. A 10-fold standard assay mixture was prepared (final volume 5 ml) in an Erlenmeyer flask (25 ml) and incubation was started by the addition of 25 mg of mitochondrial protein; 0.5 ml samples were removed into tubes containing 4 ml of 10% Cl_3CCOOH at 0, 5, 10 and 15 min. At 15 min (arrow), 2 ml of the remaining reaction mixture was transferred into a flask containing 100 µl of rifampicin solution (1 mg/ml). At times indicated, 0.5 ml samples were removed from each flask into 4 ml of 10% Cl_3CCOOH. (•——•) Control; (o— — —o) with rifampicin added at 15 min. The curve (o——o) was obtained in an experiment similar to the control one except that in the reaction mixture rifampicin at the final concentration of 50 µg/ml was present.

ase requires primer sites with free 3′-OH and therefore only those RNA molecules that have been released by the RNA polymerase can serve as primer for DNA synthesis. The experiments on the time course of the reaction in the presence of the drug reported in fig. 1 suggest that rifampicin inhibits DNA polymerase also in our system by the mechanism postulated above. Probably mitochondrial DNA polymerase, as in other systems, is able to elongate DNA chains already initiated and therefore the inhibitory effect of the drug, presumably due to lack of primer sites for the DNA polymerase enzyme, becomes more significant as the reaction proceeds. Bosmann [14] has reported the presence in rat liver mitochondria of an enzyme with properties of an RNA dependent DNA polymerase. Even if the presence of this enzyme in our preparation cannot be excluded, the effect of rifampicin seems to indicate that in our system continuous synthesis of primer RNA is more necessary for DNA polymerase than the presence of the template RNA. In conclusion our results demonstrate that in rat liver mitochondria rifampicin inhibits DNA synthesis probably interfering at the level of the transcription of the mitochondrial genome. Such results, besides, indicate that the synergism between DNA and RNA polymerase is probably a generally occurring phenomenon.

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