EFFECT OF RIFAMPICIN ON THE GROWTH OF RNA BACTERIOPHAGE M12

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1. Introduction

In bacteria, rifampicin impairs the initiation of DNA dependent RNA synthesis [1], probably by direct attack of the core enzyme of RNA polymerase [2] and therefore blocks transcriptional RNA synthesis as well as protein synthesis. The replication of RNA phages should not depend on the transcription machinery of the host and in that case rifampicin should be a good tool to study by exclusive labelling RNA phage dependent RNA and protein synthesis. In fact, it could be shown [3] that rifampicin, added 5 min after infection, did not inhibit the synthesis of RNA coliphage f2. It was thought to be superior to another inhibitor of transcription, i.e. actinomycin D which has been known to affect strongly the growth of such phages [4–7]. Several laboratories using rifampicin, however, failed to obtain a normal yield of phages R17, Qβ, MS2 respectively, and even f2 [8–11]. Does rifampicin permit the study of normal replicative processes or rather interfere with some step in RNA phage specific RNA replication and/or translation.

Here we report experiments which demonstrate that the replication of RNA phage M12, closely related to f2, proceeds in a normal way only some 25 min after addition of the drug. Later on, both phage specific RNA and protein synthesis simultaneously come to a halt, leading us to the assumption that rifampicin causes a depletion of one or more host controlled factors necessary for both phage-RNA and -protein synthesis. If RNA phage replication is studied too late after addition of the drug, rather pathological than physiological phenomena are observed.

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2. Methods

D-medium [12] without the addition of phosphates was used throughout except incorporation of ^{35}S sulfate which was performed in M9-medium [13] containing $MgCl_2$ in place of $MgSO_4$. *E. coli* AB 301 (λ), met⁻, RNAase I less (R. Gesteland), was grown at 37° (generation time was about 25 min in both media used) to a titer of 2×10^8/ml. The culture was divided into three equal parts I, II, and III. I and II were infected with M12 at a m.o.i. of 15. Five min later, to I and III rifampicin (Mann) in sterile water was added to a final conc. of 50 μg/ml. Cells were lysed with lysozyme/EDTA and chloroform [3]. Crude lysates were incubated with 50 μg of DNAase (RNAase free, Worthington) per ml for 15 min at 37°. After removal of debris by low speed centrifugation, phage titers were determined by the standard agar overlay method. Phages were concentrated by quantitative precipitation with polyethyleneglycol (Carbowax 6000, Roth) in 0.5 M NaCl at 4° [14] and purified to homogeneity by subsequent sucrose and CsCl gradient runs [15]. Nucleic acids were extracted with phenol in the presence of SDS and precipitated with ethanol [16]. The precipitate was dissolved in a small volume of 0.01 M Tris-HCl, pH 7.8, 0.001 M EDTA, and 1% SDS and kept frozen at −20°.

Extraction of cells with phenol in the presence of SDS and work-up for proteins was as previously published [13]. Electrophoresis on 10% polyacrylamide gels, prepared in the presence of 4 M urea [7], was performed as already described [13].

3. Results

More than 90% of the cells could be shown to be

infected 5 min after addition of M12. Rifampicin was given at this time in order to obtain results comparable with those mentioned above [3,8–11]. As reported recently [10], in our experiments also, little or no lysis was evident in the presence of the drug. Progeny could be demonstrated only by treatment with lysozyme/EDTA. Fig.1 shows a single step like growth of M12 in the presence or absence of rifampicin. With the drug, phage synthesis stopped already about 30 min after infection yielding a titer of only 15% of the control. This result was highly reproducible including experiments with two other male strains of *E. coli*, C 3000 (D. Nathans) and K 38 (N. Zinder).

In an attempt to clarify the mechanism by which rifampicin affects the growth of M12 we studied the effect on (a) RNA and (b) protein synthesis and on (c) maturation.

(a) It is clear from fig.2 that overall RNA synthesis as studied by pulse labelling with [^{3}H]uracil followed a different way in rifampicin treated cells from that

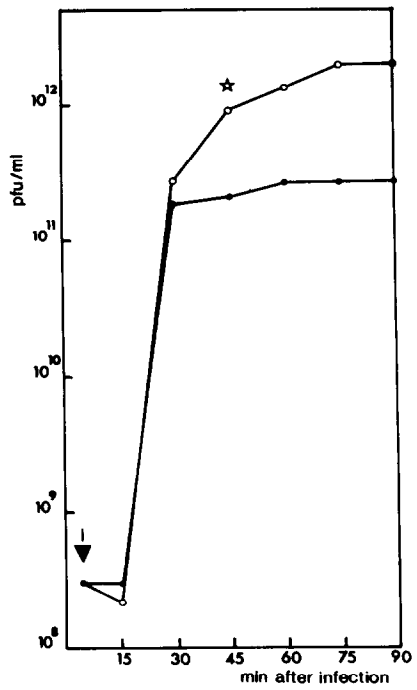

Fig. 1. Growth of M12 in the presence (●–●–●) or absence (○–○–○) of rifampicin. The drug was given 5 min after infection (arrow). Infectious titers were determined after completion of lysis (see Methods). *Marks the onset of spontaneous lysis in the control culture.

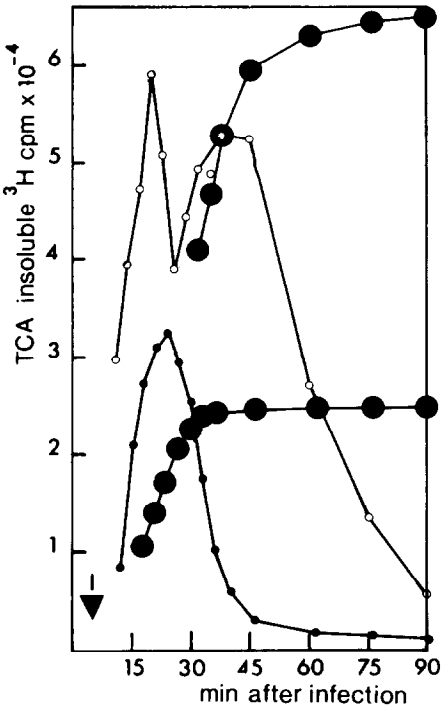

Fig. 2. Rate of incorporation of [^{3}H]uracil in the presence (●–●–●) or absence (○–○–○) of rifampicin. For each point, a sample of cultures I and II (see Methods) was incubated with 40 μCi of [^{3}H]uracil-(5) (specific activity 23.5 Ci/mM, Radiochemical Centre) per ml for 4 min at 37° under vigorous shaking. SDS was added to a final conc. of 2% (w/v) and the sample was rapidly chilled using an acetone/dry ice bath. After repeated freeze thawing, 50 μl were put onto a Whatman 3MM paper disk, washed with 10% (w/v), 5% cold TCA, ethanol/ether (1:1), and ether, and assayed for radioactivity. For comparison with fig.3, the pulse rate curves were integrated, part of which is given (large closed circles, lower curve corresponds to (●), upper curve to (○)).

in untreated cells. In inhibited cells, RNA synthesis was maximal at 25 min after infection and rapidly declined after 30 min after infection while still going on for about 15 min further in the uninhibited cells (the two peaks in fig.2 are due to the overlap with the rapidly declining host specific RNA synthesis). As more than two thirds of the RNA synthesized in the presence of the drug sedimented with 27 S in linear sucrose gradients (not shown), the overall synthesis curve reflects the production of M12 specific material quite well. In addition table 1 shows that both rifampicin treated and untreated cells made fairly

Table 1
Relative amounts of single stranded M12 RNA present at different times after infection.

Pulse (min after infect.)	I (with rifampicin)	II (control)
28–32	97	100
43–47	9	136

Pulse labelled nucleic acids from cultures I and II (see fig.2) were extracted, precipitated, and dissolved as given in Methods. 100 μl were mixed with 5 μl of a very dilute solution of ^{32}P-labelled homogeneous M12 RNA and layered onto 4.4 ml of a 5–20% (w/v) linear sucrose gradient in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.0, 0.001 M EDTA, and 0.1% SDS. Centrifugation was in the Spinco SW 56 Ti rotor for 2 hr at 47000 rpm and 11°. Tubes were punctured and fractions were assayed for radioactivity. Tracings of the 27 S (single stranded) M12 RNA were weighed and the value of the control at 30 min after infection was assumed to be 100%.

equal amounts of single stranded M12 RNA up to 30 min after infection; later on, however, rifampicin treated cells failed to synthesize any reasonable quantity of phage RNA, while control cells continued synthesis of M12 specific material.

(b) Protein synthesis in M12 infected cells was studied by means of incorporation of ^{35}S sulfate. Fig.3 shows that protein synthesis in rifampicin treated cells sharply decreased 30 min after infection, i.e. about the same time as RNA synthesis did so, while continuing in the uninhibited cells for at least a further 15 min. To determine phage specific protein synthesis we measured the production of M12 coat protein as corresponding to the large majority of phage specific proteins. 30 min after infection, the amount of coat protein was about the same in the presence or absence of the drug (fig. 4a). 90 min after infection, however, the amount of coat protein in the control was several times larger than that in the drug treated culture which showed no substantial increase in coat protein as compared with the value at 30 min (fig. 4b).

(c) Rifampicin inhibited M12 growth the less the later added. There was no longer inhibition to be observed when rifampicin was added 30 min after infection, i.e. when most of the phage coat protein and RNA was synthesized. The same percentage (30%) of all coat protein synthesized was incorporated into phage particles in the presence or absence of rifampicin (fig.

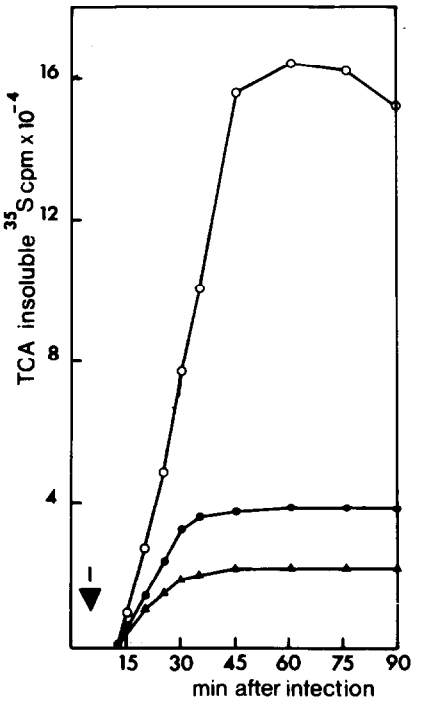

Fig. 3. Incorporation of ^{35}S sulfate. 13 min after infection, the cultures I–III (see Methods) were incubated with 20 μCi of carrier-free ^{35}S sulfate (Buchler) per ml. At times indicated 0.5 ml were removed and assayed for hot 5% TCA insoluble radioactivity in 100 μl as given in fig.2. (●–●–●), incorporation in the presence, (○–○–○), in the absence of rifampicin, and (▲–▲–▲), incorporation in an uninfected, but rifampicin treated culture.

4c). Purified phage preparations were identical with respect to the ratio viable/physical particles, RNAase resistance of infectivity, sedimentation pattern, and density distribution. All these results exclude a major effect of rifampicin on phage maturation. The only peculiarity found is the high sensitivity of intracellular "rifampicin" phages against lysis with 0.2% SDS, which is used as a convenient method to set free normal intracellular M12 phages [16]. It disappears as soon as the intracellular phages have become liberated by other methods (lysozyme/EDTA).

4. Discussion

The data presented above show that rifampicin,

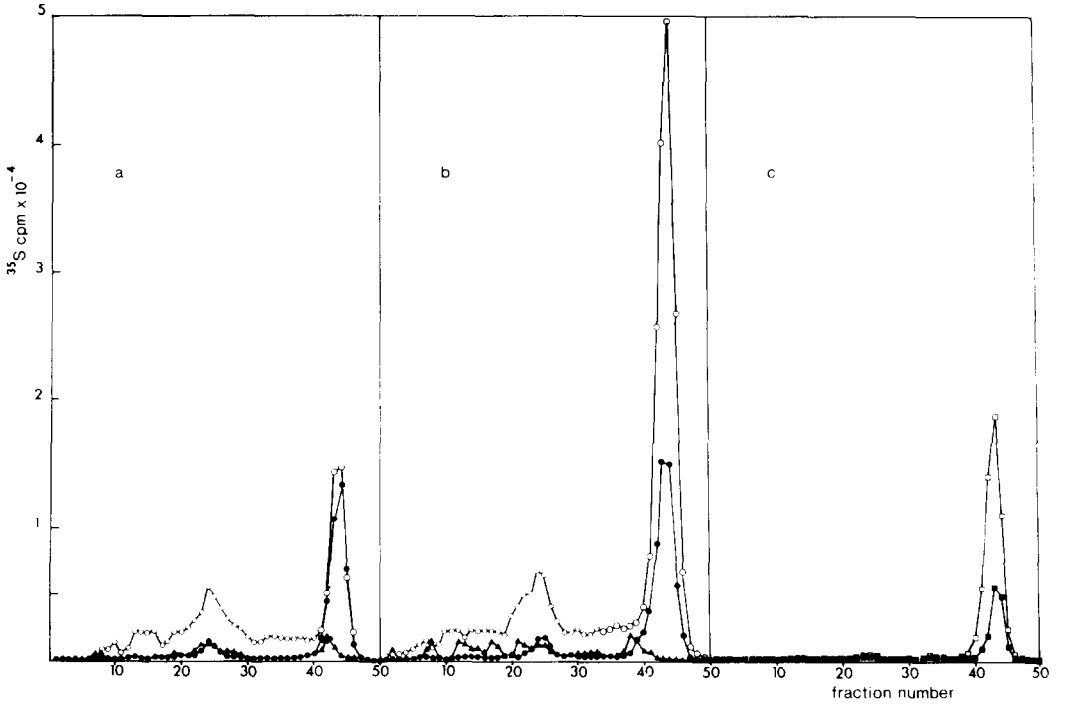

Fig. 4. Analysis of M12 coat protein. At 30 and 90 min, respectively, equal volumes were taken from the cultures I–III mentioned in fig.3, treated with SDS, and chilled. At 90 min also, an equal volume was taken from the cultures I and II. Cells were carefully lysed and phages were concentrated and purified as given in Methods. A volume equivalent to that taken from I–III at 30 and 90 min was equally treated and chilled. From all resulting 8 samples, proteins were extracted with phenol, worked up, and analyzed on 10% polyacrylamide gels in the presence of 4 M urea, 0.1% SDS, and 0.14 M 2-mercaptoethanol (see Methods). a) Proteins present at 30 min, b) at 90 min after infection, and c) in phage particles harvested 90 min after infection. Key symbols are as for fig.3.

added to a M12 infected culture of *E. coli* 5 min after infection, shuts off the synthesis of phage specific RNA and proteins quite simultaneously some 30 min after infection. As *in vitro* rifampicin does not affect the RNA dependent RNA synthesis by Qβ replicase [9,18,19] nor the M12 RNA dependent protein synthesis in a cell free extract from *E. coli* AB 301 [20], we favour the hypothesis that rifampicin must act in an indirect manner. This claim is supported by the finding that rifampicin at the same concentration had no effect at all on the growth of M12 in *E. coli* Hfr 2340/3 (G. Hartmann) bearing a rifampicin resistant DNA dependent RNA polymerase [17]. An easy explanation might be found in the activation of a nuclease. We found indeed some cleavage of M12 RNA into fragments of about 20 S and 15 S, respectively, but this activity appeared too late (50 min after infection) and was too little to account for the rather complete cessation of the synthetic processes at 30 min after infection. Therefore it is most likely that rifampicin inhibits the continued synthesis of one or more short-lived host controlled products necessary for phage RNA and protein synthesis. Clearly a candidate is one or more of the subunits of RNA replicase normally contributed by the host [18,19]. A most appealing explanation for the simultaneous breakdown of both RNA and protein synthesis might be found in the very recent observation [21] that two of the subunits of RNA phage Qβ replicase are identical with protein synthesis transfer factors T_u and T_s, the latter being very labile.

Our data do not agree with the recent proposal [22] that rifampicin might affect the phage yield via reduction of a host controlled phage maturation factor. The lability of rifampicin grown phages upon treatment with SDS cannot explain the great difference in macromolecular synthesis. Assembly inhibition should be only a minor effect of the drug.

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References

[1] G. Hartmann, K.C. Honikel, F. Knusl and J. Nusch, Biochim. Biophys. Acta 145 (1967) 843.
[2] D. Rabussay and W. Zillig, FEBS Letters 5 (1969) 104.
[3] H.P.M. Fromageot and N.D. Zinder, Proc. Natl. Acad. Sci. U.S. 61 (1968) 184.
[4] A.M. Haywood and J.M. Harris, J. Mol. Biol. 18 (1966) 448.
[5] M.R. Lunt and R.L. Sinsheimer, J. Mol. Biol. 18 (1966) 541.
[6] M.P. Oeschger and D. Nathans, J. Mol. Biol. 22 (1966) 235.
[7] E. Vinuela, I.D. Algranati and S. Ochoa, European J. Biochem. 1 (1967) 3.
[8] L.A. Phillips, J.A. Truden, W.J. Iglewski, B. Hotham-Iglewski and R.M. Franklin, Virology 39 (1969) 781.
[9] E. Bandle and Ch. Weissmann, Biochim. Biophys. Acta 199 (1970) 551.
[10] H. Engelberg and E. Soudry, J. Virol. 7 (1971) 847.
[11] J.D. Friesen, J. Mol. Biol. 46 (1969) 349.
[12] B. Francke and P.H. Hofschneider, J. Mol. Biol. 16 (1966) 544.
[13] R. Ward, R.N.H. Konings and P.H. Hofschneider, European J. Biochem. 17 (1970) 106.
[14] K.R. Yamamoto, B.M. Alberts, R. Benzinger, L. Lawhorne and G. Treiber, Virology 40 (1970) 734.
[15] R. Hehlmann and P.H. Hofschneider, J. Mol. Biol. 51 (1970) 225.
[16] S. Hattman and P.H. Hofschneider, J. Mol. Biol. 29 (1967) 173.
[17] D. Meier, unpublished results.
[18] M. Kondo, R. Gallerani and Ch. Weissmann, Nature 228 (1970) 525.
[19] R. Kamen, Nature 228 (1970) 527.
[20] R.N.H. Konings, personal communication.
[21] K. Weber, personal communication.
[22] J. Passent and P. Kaesberg, J. Virol. 8 (1971) 286.