The Effect of Rifampicin on the Stability of the Messenger Ribonucleic Acid of *Bacillus amyloliquefaciens* as determined by DNA:RNA Hybridization

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SUMMARY

Rifampicin at a concentration of 10 µg/ml completely inhibited protein synthesis in exponential-phase cultures of *Bacillus amyloliquefaciens*. At this same concentration the drug was shown to have no effect on the stability of mRNA, determined as the functional and hybridizable material, when compared with hybridizable mRNA in an uninhibited system. In each case, the half-life of the mRNA had a value in the range 5 ± 1 min, at 30 °C.

INTRODUCTION

The antibiotic compound rifampicin has been known for some time as a specific inhibitor of the initiation of transcription (Sippel & Hartmann, 1968). In this role it has been used in studies on the synthesis and stability of bacterial macromolecules (Gray & Midgley, 1971; Leighton & Doi, 1971).

Leighton & Doi (1971) showed that the inhibitory effect of rifampicin on *Bacillus subtilis* closely resembled the inhibition observed by raising the temperature of a rifampicin-resistant temperature-sensitive RNA polymerase mutant of the same organism. Whilst attempting to repeat the experiments of Leighton & Doi (1971), Coote, Wood & Mandelstam (1973) observed that rifampicin had a general cytotoxic effect causing fall in oxygen consumption, gross physical damage and loss of viability in *B. subtilis*. These findings led Coote *et al.* (1973) to infer, since no direct measurements were made, that no reliable conclusions about the lifetime of mRNA can be drawn from experiments with so toxic a drug.

In view of the possible seriousness of the repercussions of such a conclusion, it would seem necessary to carry out certain control experiments, omitted by Coote *et al.* (1973), as a preliminary to studies on macromolecule synthesis and stability using rifampicin. The results of such experiments with *Bacillus amyloliquefaciens* are reported below.

METHODS

Organism. *Bacillus amyloliquefaciens* strain T (Welker & Campbell, 1967) was used.

Growth conditions. The composition of the medium was: (NH₄)₂HPO₄, 34 mM; KCl, 5 mM; MgSO₄, 1 mM; FeCl₃, 0.5 mM; CaCl₂, 0.125 mM; ZnSO₄, 0.0125 mM; sodium citrate,

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2.125 mM; trace metal ion solution (Coleman & Elliott, 1965), 0.25 ml/l; casein hydrolysate (BDH) 0.5%; yeast extract (Difco), 0.05%; maltose, 1% (w/v). The medium was adjusted to pH 7.2 with 10% (v/v) phosphoric acid. Batches (50 ml), contained in 250 ml conical flasks, were loop-inoculated from an aqueous spore suspension. The cultures were incubated at 30 °C in a gyrotory incubator-shaker (model G25, New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.).

Measurement of [¹⁴C]uracil incorporation. The effect of rifampicin (Lepetit Pharmaceuticals Ltd, Maidenhead, Berkshire) on ¹⁴C-labelled uracil incorporation into RNA was determined by adding 10 µCi [2-¹⁴C]uracil (62 mCi/mmol) to a 10 ml sample of exponential-phase (18 h) bacterial culture contained in a 100 ml conical flask, followed 90 s later by the drug, to a final concentration of 10 µg/ml. Samples (0.1 ml) of the culture were taken at short intervals after the addition of radioactive uracil, during incubation with shaking at 30 °C, and pipetted into ice-cold 5% (w/v) trichloroacetic acid (TCA) (3 ml) containing unlabelled uracil (0.5 mg/ml). The precipitates formed were collected and counted as described by Coleman & Elliott (1965).

Measurement of L-[¹⁴C]leucine incorporation. The effect of rifampicin on protein synthesis was determined by incubating a 10 ml sample of exponential-phase culture (18 h) in a 100 ml conical flask, at 30 °C, with 4 µCi L-[U-¹⁴C]leucine (25 mCi/mmol) followed 5 min later by the drug, to a final concentration of 10 µg/ml. Samples of the culture (0.1 ml) were taken at intervals throughout the experiment and pipetted into 3 ml of 5% (w/v) TCA containing 0.1% casein hydrolysate. The resulting preparations were heated in a water bath at 90 °C for 30 min, cooled and prepared for counting as described by Coleman & Elliott (1965).

Rapid-labelling experiments. Exponential-phase cultures (18 h) were incubated for 90 s with [5-³H]uracil (21 Ci/mmol) or [2-¹⁴C]uracil (62 mCi/mmol) at 1 µCi/ml, after which the cultures were immediately centrifuged for 90 s at 6500 g. The bacterial pellets sedimented were then frozen in liquid nitrogen. Cell-free extracts were subsequently prepared from the frozen pellets and the labelled RNA was isolated for hybridization studies.

Similar results were obtained irrespective of whether ¹⁴C-labelled or ³H-labelled uracil was used. It was found possible, however, to achieve higher specific radioactivities using the tritiated precursor.

'Pulse-chase' experiments. These were carried out in the same manner as the rapid-labelling experiments with the exception that a 'chase', consisting of a large excess of unlabelled uracil (50 µg/ml) with or without rifampicin (10 µg/ml), was added immediately after the 90 s 'pulse' of radioactive uracil incorporation.

Formation of bacterial cell extracts. Bacterial cell extracts, required as the first stage in the preparations for both rapidly-labelled RNA and unlabelled rRNA, were prepared as described by Coleman (1967).

Preparation of rapidly-labelled RNA. ¹⁴C- and ³H-labelled RNA were prepared from samples of *B. amyloliquefaciens* extracts using the procedure described by Pigott & Midgley (1968).

Preparation of unlabelled rRNA. rRNA was prepared from extracts of exponential-phase *B. amyloliquefaciens* as described by Pigott & Midgley (1968).

Preparation of denatured bacterial DNA. DNA was isolated and denatured as described by Pigott & Midgley (1968).

Preparation of DNA-bearing filters. Nitrocellulose filters (Sartorius Membranfilter MF50, 2.5 cm diameter; V. A. Howe & Co. Ltd, London) were presoaked for at least 24 h in ice-cold 0.01 × SSC buffer (SSC buffer is 0.15 M-sodium chloride–0.015 M-sodium citrate,

pH 7.0). They were then transferred to a Millipore sampling manifold and each washed with 50 ml of $6\times$ SSC buffer at 4 °C. Samples of denatured DNA, diluted with 2 vol. of cold $10\times$ SSC buffer, were allowed to percolate through the filters, without suction, again at 4 °C to maximize the DNA binding. The loaded filters were washed with 50 ml $6\times$ SSC buffer on each side and allowed to dry at room temperature overnight. They were then dried for a further 4 h at 80 °C in a vacuum oven over P_2O_5 .

It was found that amounts of DNA up to 150 μ g would bind with 100% efficiency to the filters. With larger amounts there was a reduction in efficiency, so that to prepare a filter containing 300 μ g of immobilized DNA it was found necessary to load on to the filter a sample containing 350 μ g DNA.

DNA-RNA hybridization. The hybridization of RNA to denatured DNA, immobilized on nitrocellulose membrane filters, was carried out as described by Pigott & Midgley (1968). Thus, unless otherwise stated, a DNA-bearing filter containing either 100 or 300 μ g denatured DNA was incubated with 10 or 1 μ g RNA, respectively, in 1 ml $6\times$ SSC buffer contained in a stoppered scintillation vial. The vials were then immersed in a water bath at 66 °C for 20 h to allow the interaction between DNA and RNA to reach equilibrium. After incubation the vials were cooled in ice and the filters removed and washed on each side with 50 ml $6\times$ SSC buffer. The filters were then incubated for 1 h in $2\times$ SSC buffer containing 20 μ g pancreatic ribonuclease (Sigma) and 10 units T_1 ribonuclease (Sigma)/ml, both of which had been heated at 80 °C to destroy deoxyribonuclease activity. The filters were then rewashed as described above and dried in an oven at 60 °C. The dry filters were placed in vials with scintillation fluid and counted.

Each variable in this assay was subjected to an independent examination, particular attention being paid to the suggestions of Kennell & Kotoulas (1968). The conditions used, therefore, were those which resulted in the maximum efficiency of hybridization in the *B. amyloliquefaciens* system.

In all the hybridization experiments, an efficiency of hybridization correction factor was applied to all results, and each result represents the mean value from duplicate assays.

Estimation of DNA and RNA. The concentration of nucleic acid in the various preparations was calculated from measurements of E_{260} in 1 cm light-path silica cuvettes in a Unicam SP500 spectrophotometer. The extinction coefficient was always taken as $E_{1cm}^{1\%} = 200$.

Radioactivity measurements. Radioactivity measurements were made using a Packard Tri-Carb liquid scintillation spectrometer (model 3375). The scintillation fluid used consisted of 0.4% PPO and 0.01% POPOP in xylene (BDH, sulphur-free). Efficiency of counting was calculated by the channel ratios method, a standard curve being prepared for each isotope using known amounts of isotope and different quantities of quenching agent.

RESULTS

Kinetics of [^{14}C]uracil incorporation in the presence of rifampicin

The characteristics of labelled uracil incorporation into total RNA, in the presence of rifampicin, were first determined, as a necessary preliminary to an examination of the effect of the drug on the stability of mRNA under the same conditions.

An exponential-phase bacterial culture was incubated with ^{14}C -labelled uracil for 90 s, after which rifampicin was added to a concentration of 10 μ g/ml. After the addition of rifampicin, incorporation of labelled uracil continued at a declining rate until it reached a maximum value 90 s later (Fig. 1). This was consistent with the known role of rifampicin as

Fig. 1

Fig. 2

Fig. 1. Time course of ^{14}C -uracil incorporation by exponential-phase bacteria in the presence of rifampicin. Rifampicin ($10 \mu\text{g/ml}$) was added at 90 s (arrow).

Fig. 2. The progress of L- ^{14}C -leucine incorporation into the proteins of exponential-phase bacteria in the presence of rifampicin. Rifampicin ($10 \mu\text{g/ml}$) was added at 5 min (arrow).

an inhibitor of the initiation of transcription. Further, the time scale is that which would be expected if the drug exerted its inhibitory effect without a lag and allowed incorporation to continue until all the partially-formed RNA chains present at the time of addition of inhibitor were completed. Subsequently, there was a loss of radioactivity, a plateau level being reached at 61% of the maximum value. This distribution of radioactivity incorporated during the first 3 min between unstable and stable RNA species in the ratio of approximately 1:2 was similar to the pattern of labelling over short time periods described by Midgley (1969).

Kinetics of L- ^{14}C -leucine incorporation in the presence of rifampicin

Evidence that the unstable RNA which decayed in the presence of rifampicin corresponded to functional mRNA was obtained from a study of the effect of the drug on the capacity of the bacteria to incorporate ^{14}C -labelled leucine into protein. Exponential-phase bacteria incorporated ^{14}C -labelled leucine in a linear manner in the absence of inhibition (Fig. 2). The addition of rifampicin at a concentration of $10 \mu\text{g/ml}$ was sufficient completely to inhibit protein synthesis, since there was a reduction in the rate of incorporation in its presence which reached a plateau level within a 30 min incubation period. At this stage, provided that it was the limiting component of the protein synthesizing system, no functional mRNA remained. The decay of unstable RNA and the loss of capacity to incorporate leucine into protein, both in the presence of rifampicin, have somewhat similar characteristics (compare Figs. 1 and 2).

*DNA:RNA hybridization as a direct means of measuring *B. amyloliquefaciens* mRNA*

Since the ability to make meaningful direct measurements of mRNA was a key requirement in this investigation, it was of importance to demonstrate that the technique of DNA:RNA hybridization represented a specific and selective method of distinguishing mRNA from other nucleic acid components and of measuring relative changes in the mRNA content of rapidly-labelled RNA in *B. amyloliquefaciens*.

Table 1. *Effect of concentration on the hybridization of rapidly-labelled RNA at low DNA:RNA ratios*

Rapidly-labelled RNA was isolated from an exponentially-growing culture of *B. amyloliquefaciens*. The RNA was labelled with [14 C]uracil for 90 s and hybridized with denatured DNA at DNA:RNA ratios of 5:1 and 10:1 in 1 ml incubation mixtures.

Concentration of rapidly-labelled RNA (μ g/ml)	Percent of rapidly-labelled RNA hybridized at DNA:RNA ratios of:	
	5:1	10:1
1	15	22
5	24	31
10	32	37
20	36	36
30	35	38

Fig. 3

Fig. 4

Fig. 3. Time course of hybridization of rapidly-labelled RNA to denatured DNA immobilized on nitrocellulose membrane filters. The RNA was rapidly labelled with [14 C]uracil for 90 s and hybridization was carried out at DNA:RNA ratios of 300:1 (○) and 10:1 (●).

Fig. 4. Hybridization of rapidly-labelled RNA from exponential-phase bacteria with increasing amounts of immobilized, denatured DNA. The RNA was rapidly labelled for 90 s with [14 C]uracil.

The basic method and conditions adopted were those of Pigott & Midgley (1968), whilst the precautions suggested by Kennel & Kotoulas (1968) were applied, particularly in relation to the nucleic acid concentration effect. The percentage of the radioactivity of rapidly-labelled RNA hybridized at a DNA:RNA ratio of 10:1 reached a maximum value in the presence of a minimum RNA concentration of 10 μ g/ml (Table 1). The details given in Methods represent the optimal conditions for hybridization in the *B. amyloliquefaciens* system as defined by Brown (1974). Under these conditions hybridization at both high and low DNA:RNA ratios was complete after 16 h at 66 °C (Fig. 3).

The amount of the rapidly-labelled RNA which hybridized increased with increase in DNA:RNA ratio from 39% at 5:1 to 100% at 200:1 and above (Fig. 4). This is in agreement with the findings of Midgley (1969) that at a low DNA:RNA ratio only mRNA, accounting for one-third of the rapidly-labelled material, is hybridized, all the rapidly-labelled RNA being hybridized at high DNA:RNA ratios. It can be seen in Fig. 5 that by

Fig. 5

Fig. 6

Fig. 5. Effect of competition for hybridization sites on denatured DNA between rapidly-labelled total RNA and unlabelled rRNA. The RNA was rapidly labelled with [^{14}C]uracil for 90 s and hybridization was carried out at DNA:RNA ratios of 300:1 (○) and 10:1 (●).

Fig. 6. Time course of loss of ^3H -labelled hybridizable mRNA from exponential-phase bacteria in the absence (○) and presence (●) of rifampicin (10 $\mu\text{g/ml}$). Hybridization was carried out at a DNA:RNA ratio of 10:1.

taking the same preparation and adding increasing amounts of unlabelled ribosomal RNA there was a reduction in the hybridizable RNA detected, a plateau being reached in the presence of a 15-fold excess of unlabelled rRNA when 39% of the input RNA was hybridized; this corresponds to the mRNA component of the rapidly-labelled material. The hybridization at a low DNA:RNA ratio was unaffected by the presence of exogenous ribosomal RNA, remaining constant at the plateau level of 39% reached by competing out all the radioactive rRNA in the high DNA:RNA system.

Effect of rifampicin on the stability of mRNA as determined by DNA:RNA hybridization

The results already presented show that the mRNA component of rapidly-labelled RNA can be selectively bound during hybridization at a DNA:RNA ratio of 10:1. The means is therefore provided for assessing, by direct measurement, the effect of protein synthesis-inhibiting concentrations of rifampicin on mRNA stability.

Thus, a culture of exponential-phase bacteria was divided into two identical halves each of which was given a 90 s 'pulse' of ^3H -labelled uracil. This was followed by a 'chase' consisting of a large excess of unlabelled uracil, with or without the concomitant addition of rifampicin (10 $\mu\text{g/ml}$). At the time of addition of the 'chase', as expected, 39% of the 'pulse-labelled' material consisted of mRNA, as determined by DNA:RNA hybridization (Fig. 6). The progress of decay of this mRNA fraction was followed, in each case, over a 60 min period; rifampicin had no detectable effect on the characteristic exponential decay pattern of the hybridizable mRNA (Fig. 6).

DISCUSSION

Over the time period of these studies (60 min), rifampicin at a concentration of 10 $\mu\text{g/ml}$ had no detectable effect on the lifetime of functional and hybridizable mRNA at 30 $^{\circ}\text{C}$,

when compared with hybridizable mRNA in an uninhibited system. Thus, the mRNA half-life in each case was in the range 5 ± 1 min. Since the half-lives were the same with and without rifampicin, the decline in ability to synthesize protein shown in Fig. 2 was probably due to loss of mRNA rather than energy supply being the limiting component of the system.

In each of the present studies, carried out at a temperature of 30 °C, all the useful kinetic data was obtained well within a 1 h period. In contrast, Coote *et al.* (1973), working at 37 °C, found that at rifampicin concentrations of up to 30 µg/ml there was little detectable effect of the drug on the cellular morphology or oxygen uptake by the bacteria during the first hour after treatment; at this higher temperature the only dramatic short-term effect was a 95% loss of cellular viability which occurred during the first 2 min after addition of inhibitor. Thus, the principal claims of Coote *et al.* (1973) apply only when subjecting the bacteria to extremely high concentrations of rifampicin (> 30 µg/ml) and after prolonged incubation periods (greater than 1 h and as much as 4 h).

In the absence of further experiments this provides little support for the far-reaching claim that 'no reliable conclusions about the lifetime of mRNA can be drawn from experiments with so toxic a drug'. Perhaps, a more sensible interpretation of the data would be that great care must be taken when using toxic drugs in studies involving the stability of mRNA, particularly when high concentrations are employed over prolonged incubation periods. Nevertheless, with the exercise of caution, drugs such as rifampicin can be useful tools in the study of bacterial nucleic acid metabolism when used under conditions which have been defined on the basis of the results obtained from suitable control experiments.

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