THE ACTION OF RIBONUCLEASE ON SENSITIVE AND NON-INDUCED LYSOGENIC CELLS OF *BACILLUS MEGATERIUM*

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SUMMARY

The rate of protein synthesis in *Bacillus megaterium* is decreased by ribonuclease under conditions where no cellular lysis occurs although the ratios existing between RNA, DNA and protein content of the cells are unaffected. Moreover, the same relation exists in control and in RNase-treated cells between uptake of [^{14}C]adenine in RNA and net RNA synthesis.

Nevertheless, the decrease of protein synthesis is accompanied by a modification in the base ratios of the RNA, an increase in cell volume and an irreversible and extensive decrease of the capacity of the cells to undergo several successive multiplications.

A hypothesis is put forward concerning the action of RNase which takes these facts into account.

INTRODUCTION

The inhibitory action of ribonuclease (RNase) on growth and division in many types of plant and animal cells has been the subject of several studies in recent years, more especially by BRACHET and his collaborators[1,2].

The action of RNase on bacterial cells is less well known[3–5]. In the course of a study on the influence of RNase on phage multiplication observations on the action of the enzyme on bacterial cells have been collected; they form the subject of the present paper.

The material chosen for study was *Bacillus megaterium* whose sensitivity to RNase was shown by GROTH[4] in BRACHET's laboratory.

MATERIAL AND METHODS

The lysogenic (899) and the non-lysogenic phage-sensitive strains (mutilat sensible) of *Bacillus megaterium* were supplied by Dr. A. LWOFF. From these, a certain number of strains were isolated, possessing different RNase sensitivities.

Cultures were grown on a yeast extract casein hydrolysate medium (LWOFF[6]). Agitation of the culture (maximum 200 ml in 1500-ml Erlenmeyer) provided the necessary aeration. A calibrated glass tube was welded onto the side of the culture flask. This device allowed readings of optical density without removing samples from the culture.

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Exponentially growing cultures were maintained in a continuous culture apparatus operating at constant optical density by means of a photoelectric control.

Viable counts were made by plating on Difco nutrient agar under strictly standardized conditions.

The crystalline ribonuclease utilized was a commercial preparation (Armour).

Inhibition of the enzyme by H_2O_2 and assay of the enzymic activity were performed according to LEDOUX[7].

Nitrogen insoluble in cold 5 % trichloracetic acid, alcohol and ether was determined after mineralisation in the apparatus described by MARKHAM[8]. RNA was assayed according to OGUR[9] and DNA according to CERIOTTI[10].

The specific activity of adenine recovered from the RNA of bacteria, having incorporated ^{14}C-labelled adenine, was determined by the following technique: the cold 5 % TCA-, alcohol- and ether-washed bacteria were extracted with cold 10 % perchloric acid. The extract was heated at 100° and the purines were precipitated as silver salts. Adenine was separated by paper chromatography[11] and recovered on a small glass plate for radioactivity determination. The amount of adenine present was estimated by ultraviolet spectrophotometry.

The ratio of the RNA bases was determined by the method described by DAVIDSON AND SMELLIE[12]. Extraction of RNA was performed with 10 % NaCl at 100°.

RESULTS

Cytolytic action of RNase

The lytic action of RNase on the *B. megaterium* cells introduces a serious source of errors in the analysis of the influence of this enzyme on protein synthesis and especially on phage synthesis. The conditions under which this lysis occurs, therefore, deserve particular notice.

It is possible to lyse growing cultures of lysogenic *B. megaterium*, and of sensitive *B. megaterium*, by the addition of 100 to 300 μg RNase/ml culture (Figs. 1 and 2). In this case, lysis begins immediately and is accompanied by a rapid decrease in the optical density.

If lesser amounts of RNase are added, growth is at first simply slowed down, whether the strain is lysogenic or not. Later, however, the behaviour of the two strains becomes entirely different (Figs. 1 and 2): the sensitive strain continues to grow, but the lysogenic strain lyses on a large scale 30 min to 120 min after addition of RNase. The time between addition of RNase and that of lysis depends on the strain utilized; but it is, for the same strain, independent of RNase concentration.

The immediate lysis initiated by a sufficient concentration of RNase in both types of bacteria and the delayed lysis in the lysogenic form might be attributable to different mechanisms.

Nevertheless, the two types of lysis have a certain number of features in common.

In both cases, lysis seems to be an all-or-none type of effect that overtakes at random one or the other cell in any of the long chains of cells. The neighbouring cells remain normal in appearance; they are basiphilic, capable of division and they continue to grow without lysis. If the surviving cells are numerous, the chances of selection of mutants remain small and, in this case, the surviving cells have the same RNase sensitivity as the cells before treatment. The shock effect of RNase is thus

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apparently dependent upon physiological differences in cells within a genetically homogeneous population.

That the RNase sensitivity of the cells depends on slight modifications of their physiological state is brought to light by the fact that delayed lysis of lysogenic *B. megaterium* does not occur when they are cultured at 30° instead of 37°. This particular feature has been of great use to us.

The sensitivity of *B. megaterium* to the lytic action of RNase is dependent also on the genetic constitution of the strain studied. Threshold concentrations of RNase, which

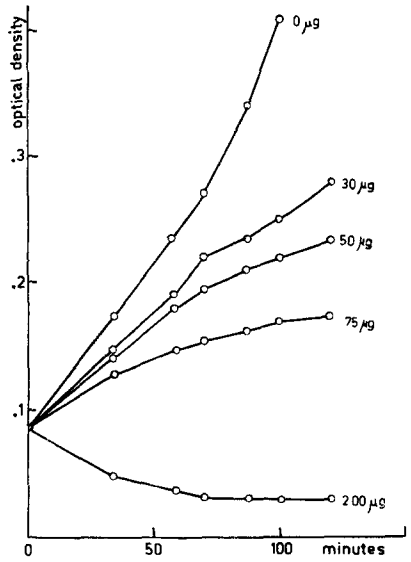

Fig. 1.

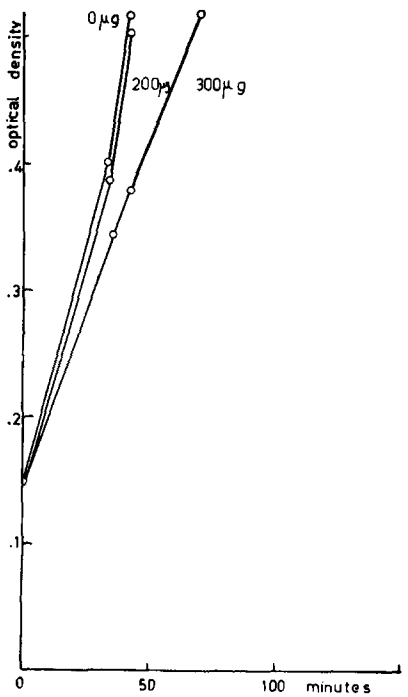

Fig. 3.

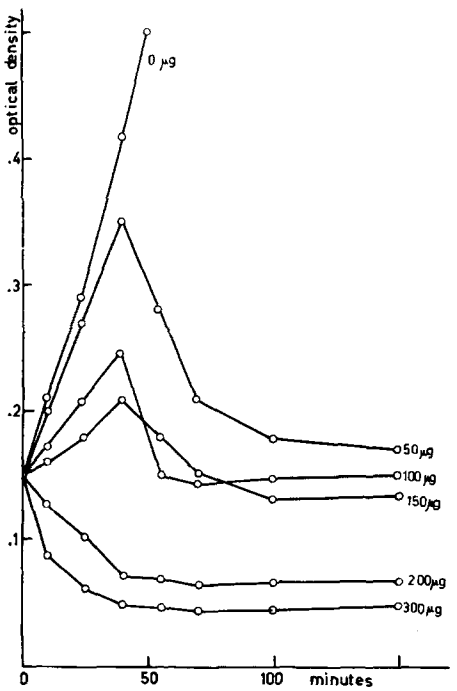

Fig. 2.

Fig. 1. Action of RNase at various concentrations (given as μg/ml) on sensitive *B. megaterium*. RNase added at time 0.

Fig. 2. Action of RNase at various concentrations (given as μg/ml) on lysogenic *B. megaterium*. RNase added at time 0.

Fig. 3. Action of RNase at various concentrations (given as μg/ml) on a strain of surviving cells obtained during the experiment reported in Fig. 2 after addition of 300 μg/ml RNase.

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bring about delayed lysis of lysogenic bacteria, vary between 5 and 150 μg/mlfor the strains isolated in this laboratory. The concentration of enzyme required for immediate lysis does not show such a wide variation (100 to 300 μg/ml).

On the other hand, we often have isolated colonies derived from cells which have grown overnight in the presence of a high concentration of RNase (300 μg/ml), after apparently total lysis of the parent culture. These cells were always more resistant to the lytic action of RNase than the parent strain (Fig. 3). This resistance to RNase was stable and remained unaltered after 35 generations in a chemostat or after 2 months on solid medium, the culture being transferred every second day. It seems thus likely that we have been able to select mutants resistant to the lytic action of RNase.

This great variability of resistance to RNase lysis has permited us to study the action of this enzyme on cells whose structure remains intact. A choice of strain and experimental conditions is necessary.

In the following part of this paper, it is to be understood that cellular lysis due to RNase does not occur. The absence of lysis was confirmed by microscopic observation and establishing the absence of free protein, resulting from lysis, in the culture medium.

Finally, we may add that the cytolytic action of RNase is apparently bound to its enzymic activity. A decrease of the enzymic activity by 90 %, after moderate hydrogen peroxide oxidation, results in a suppression of the cytolytic action for concentrations which were lytic before treatment.

Inhibition of cellular growth

The results described in this part of the paper were obtained with sensitive *B. megaterium*. RNase was added when cultures reached an optical density of 0.200. At the time of addition of RNase, the culture was diluted twofold and the change in optical density was followed for several hours.

After addition of the RNase at a sufficient concentration, the growth rate, measured by optical density, decreased immediately and the growth curve became linear and remained so for several hours. Higher concentrations of RNase, permitting growth without partial lysis provoke a progressive inflection of the growth curve, which finally becomes horizontal. The RNA and DNA content of the cells increases in the same manner as the optical density, although it is possible that the DNA/protein N ratio slightly increases (max. 10 %).

The same ratio exists, whether RNase is present or not, between the uptake of ^{14}C-labelled adenine in RNA and the increase in optical density. Consequently, RNase does not modify the turnover of RNA which is probably very low in *B. megaterium* as well as in other bacteria[13].

Since the RNA content of the cells remains constant and since its renewal rate is unaffected by the presence of RNase, we must conclude that no large-scale breakdown of RNA occurs in the presence of the enzyme inside the inhibited cells.

Nevertheless, the following experiment shows that RNase does have an appreciable action on cellular RNA. The enzyme is added to a final concentration of 100 μg/ml to a growing culture. After an increase of 50 % of the RNA content, both in control and in inhibited cells, the bacteria are harvested and their RNA is submitted to a base analysis. The RNA of the treated cells showed an increase of 10 % in uridylic

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acid in two experiments performed. Eight paper ionophoresis analyses, were performed for each sample of RNA studied. In all cases, the observed differences were statistically significant (Table I).

These differences are quite small, but one must bear in mind that only one third of the RNA analysed was synthesised after addition of RNase; the increase of uridylic acid might very well be threefold greater in the newly synthesised RNA. If this RNA constitutes the template for protein synthesis, a modification of its structure might result in the synthesis of abnormal unactive enzymes.

TABLE I

EFFECT OF RNASE (100 μg/ml) ON THE MOLAR RATIO OF URIDYLIC ACID TO THE FOUR NUCLEOTIDES OF RNA

The optical density of the culture increased from 0.33 to 0.50 during the action of RNase. Eight electrophoreses were performed on each sample.

Control	*Culture grown in presence of RNase*
0.253	0.284
0.235	0.255
0.250	0.261
0.258	0.251
0.251	0.276
0.239	0.273
0.243	0.282
0.213	0.263
Mean = 0.243	Mean = 0.268
S.d. = 0.004	S.d. = 0.005

The two means are significantly different at the 5 % significance level.

In order to test this hypothesis, we have measured oxygen consumption in the presence of different concentrations of RNase. This experiment gave negative results: the oxygen consumption per mg protein N showed no decrease for concentrations of RNase lowering the growth rate by 80 %.

Aside from the slight already mentioned alteration of the RNA composition, the observations reported above do not reveal any serious lesions in the inhibited cells, although their growth rate is reduced at least by 50 to 75 %.

However, if the observations are extended for a longer period, the cells appear to be in a less satisfactory condition than seems to be at first apparent. Indeed, their "viability" is severely reduced if they are plated on the same medium containing 2 % agar and without RNase (Fig. 4). The proportion of cells capable of giving rise to a colony is even smaller than is apparent from Fig. 4. Indeed, the "viable count" determines the number of chains of bacteria present in the culture. In *B. megaterium*, the chains are composed of a dozen cells. If only one of these is capable of successive divisions, the chain will give rise to a colony.

Microscopic observations on cells taken a short time after addition of RNase (for instance 1 hour) indicate that the suppression of cellular division is the result of early damage to an essential mechanism. These cells are very basiphilic and have a mean diameter 60 % greater than normal cells cultured under similar conditions. Giemsa's staining, after elimination of RNA by hydrochloric acid, shows that the

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nuclei, larger and better stained than usual, are spaced further apart within the chain of cells. In one instance, 191 nuclei were counted in the treated cells and 350 in the control for an equal chain length. The cytoplasmic volume per nucleus is thereby several fold greater than in the untreated cells. It looks as if the cells merely increased in volume, with the relative proportions of RNA, DNA and protein remaining constant. This result is to be expected if division rate were more affected by RNase than the synthesis of cellular constituents.

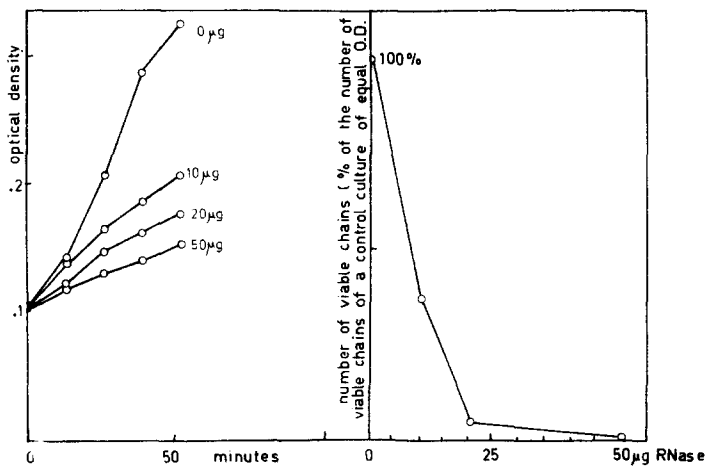

Fig. 4. Action of RNase on viability. Left: growth of the cultures; RNase added at time o; concentrations given as μg/ml. Right: number of viable chains of cells one hour after addition of RNase.

The action of RNase on different types of living cells is accompanied by an important decrease in their basophily (after fixation) although their RNA content barely changes[1,2]. This observation has been made also on *B. megaterium*. Unfortunately, the proportion of cells that do not stain with toluidine blue varies too much from one experiment to another to permit to establish a relation between the degree of growth inhibition and the proportion of cells which do not take the stain. We have frequently obtained inhibitions of growth of 70 % without observing an increase in non-basophilic cells in the treated cultures.

DISCUSSION

The action of RNase on different cells having recently been the subject of a review by BRACHET[1], we shall limit the discussion of this paper to a comparison of our results with other known observations on bacteria.

The action of RNase has been described by GROTH[4], in the case of *B. megaterium* and by JERNE AND MAALØE[5], in the case of *E. coli*: it is accompanied by an important reduction of the RNA content of the cells.

We have obtained the same effect in *B. megaterium* only under conditions where RNase provoked cellular lysis and liberation of large amounts of protein into the culture medium.

We therefore share the opinion of JERNE AND MAALØE[5] that RNase may have a

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destructive effect on cellular organisation, thereby hindering biochemical activity in a manner wich is not easily analysable. However, the results described in this paper were obtained with cells which were normal with respect to respiration and which continued to grow. No protein was released into the culture medium and the ratios between RNA, DNA and protein N remained normal. Consequently breakdown of cellular structure does not seem to have occurred in our experiments.

The action exerted by RNase on the growth of either uninfected phage-sensitive *B. megaterium* or uninduced lysogenic *B. megaterium* is most readily accounted for if one supposes that the enzyme acts as such on the RNA and consequently affects the RNA-dependent protein synthesis.

Two hypotheses concerning its mode of action are conceivable.*

The first postulates a preferential breakdown of the RNA molecules assembled in the presence of the enzyme, which presumably are more liable to breakdown than the pre-existing RNA molecules bound to protective protein structures. This might explain why the rate of accumulation of RNA is slowed down in the presence of RNase, although the ratio existing between uptake of ^{14}C-labelled adenine and net RNA synthesis is not modified by the presence of the enzyme.

The second hypothesis implies that the RNase alters the structure of the RNA molecules possibly by provoking a rearrangement of ribonucleotides by interconversion of polynucleotide chains (HEPPEL, WHITEFELD AND MARKHAM[14]). These altered RNA molecules would be unable to fulfil their role of template in protein synthesis; growth would thus be slowed down while no breakdown of RNA is observed.

It is possible that either of these two effects, or both concurrently, are differently exerted on various RNA fractions. The above hypothesis would thus explain why the composition of the entire RNA is modified in the presence of RNase.

It seems unlikely, at least in our case, that RNase exerts its inhibitory influence by acting as a basic protein which combines with nucleic acids: in fact, the action of RNase on the growth of *B. megaterium* decreases simultaneously with its enzymic activity, when the enzyme is treated with hydrogen peroxide. On the other hand, cells of *B. megaterium* whose growth has been greatly inhibited by RNase, might still present a normal basophily.

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* The hypothesis according to which the RNase would be active solely on those RNA molecules that are involved in the transfer of activated amino acids onto the genetic determinants (Hoagland) will be examined in the discussion of a forthcoming paper concerning the inhibition of phage-protein synthesis by RNase.