BIKEN JOURNAL Vol. 9, 125-130 1966

PREFERENTIAL INHIBITION OF RNA SYNTHESIS IN *BACILLUS SUBTILIS* BY ACTINOMYCIN S[1]

TAKESHI YAMADA[2] and JUNICHI KAWAMATA

Department of Experimental Chemotherapy, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
(Received March 23, 1966)

SUMMARY In Bacillus subtilis SB 15, the incorporation of ^{14}C-uracil into messenger RNA with a large sedimentation coefficient was inhibited preferentially by a low concentration of actinomycin S.

The incorporation of ^{14}C-uracil into 23 S ribosomal RNA was much more sensitive to a growth-inhibitory concentration of actinomycin than incorporation into 16 S ribosomal RNA.

The net synthesis of 23 S ribosomal RNA was considerably inhibited by a concentration of actinomycin less than that required for complete suppression of growth.

INTRODUCTION

It has been reported that actinomycin inhibits DNA dependent RNA synthesis (REICH *et al.*, 1962; HURWITZ *et al.*, 1962; LEVINTHAL *et al.*, 1962), presumably by interacting with DNA molecules (KWAMATA *et al.*, 1960; RAUEN *et al.*, 1960; KIRK, 1960). Reports have also indicated that a low concentration of actinomycin inhibits messenger RNA with a large sedimentation coefficient in mammalian cells (PERRY, 1962; GEORGIEV, 1963) and bacterial cells (YAMADA, *et al.*, 1964). Furthermore it has also been reported that in mammalian cells a low concentration of actinomycin inhibits 28 S and 18 S ribosomal RNA completely but not 4 S RNA (PERRY, 1962; GEORGIEV, 1963; FRANKLIN, 1963). However, in bacteria, we observed that a low concentration of actinomycin inhibits 23 S ribosomal RNA preferentially and more than 16 S ribosomal RNA and 4 S RNA (YAMADA *et al.*, 1964). In this paper detailed evidence is presented for the preferential inhibition of RNA with a larger sedimentation coefficient in *B. subtilis* SB 15.

MATERIALS AND METHODS

1. *Actinomycin*

The actinomycin S used in this study was purchased from Daiichiseiyaku Pharmaceutical Co., Ltd.

2. *DNase*

Crystalline pancreatic DNase was obtained from Worthington Biochemical Co., Ltd.

1 This work was supported in part by Scientific Research Grants from the Ministry of Education, Japan and from the National Cancer Institute, U.S.A., CA-05075-CY.

2 Present address: Department of Tuberculosis II

3. *Organism*

The organism was grown at 37°C with shaking in nutrient broth (meat extract 10 g, polypeptone 10 g, NaCl 2.0 g per liter, pH 7.0). The organism used in this study was *B. subtilis* strain SB 15.

4. *Labeling of RNA*

The labeling of RNA with ^{14}C-uracil (4.8 mc/m. mol, purchased from Daiichi Pure Chemical Co., Ltd.) was carried out by quickly adding ^{14}C-uracil (0.02 μc/OD), when the optical density of the exponentially growing culture reached $OD_{660}=1.0$.

5. *Preparation of RNA*

For preparation of RNA, the cells were collected by centrifugation for 15 minutes at 8,000 rpm and washed once with 10^{-2} M tris-HCl buffer containing 10^{-3} M Mg-acetate at pH 7.3. These cells were suspended in 3 vol. of the above buffer containing lysozyme (500 μg per ml.) and DNase (30 μg per ml.). After incubation for 20 minutes at room temperature, sodium dodecylsulphate was added at a final concentration of 2.5 per cnet and incubation was continued for a further 20 minutes. Then the extract was deproteinized by shaking vigorously with an equal volume of 90 per cent phenol for 20 minutes at room temperature and partially purified RNA was recovered by precipitating the aqueous phase with 3 vol. of cold ethanol at −20°C. The precipitate collected by centrifugation for 20 minutes at 10,000 rpm was redissolved in 10^{-2} M tris-HCl buffer containing 10^{-3} M Mg-acetate at pH 7.3. The treatment with DNase, deproteinization and cold alcohlic precipitation were repeated. The precipitate with ethanol was finally dissolved in 10^{-2} M tris-acetate buffer, pH 7.4, containing 10^{-4} M Mg-acetate at a final OD_{260} of 50–100 per ml. The OD_{260}/OD_{230} ratio was 2.1. The OD_{280}/OD_{260} ratio was 0.5 and the ε(P) was 10,000.

6. *Sucrose gradient analysis*

For analysis, 0.2–0.3 ml of the sample was subjected to linear sucrose density gradient analysis. A linear density gradient of sucrose (5–20 per cent) in 0.01 M tris-acetate buffer (pH 5.0) containing 0.1 M NaCl was prepared according to the procedure of MONIER *et al.*, 1962). Measurements were made of radioactivity and absorbancy at 260 mμ of the drop fractions collected after centrifugation of the samples in a RPS 40–130 rotor (Hitachi 40 P ultracentrifuge) at 39,000 rpm for 270 minutes. The radioactivity was assayed in a Nuclear Chicago gas flow counter.

RESULTS

1. *Effect of actinomycin on the incorporation of ^{14}C-uracil into messenger RNA*

When the OD_{660} of the exponentially growing *B. subtilis* SB 15 culture reached 1.0, the whole culture was divided into five parts. One part was pulse labeled with ^{14}C-uracil for 3 minutes. The second part was pulse labeled with ^{14}C-uracil for 3 minutes and then the organism was collected, washed once at 4°C and resuspended in fresh medium without ^{14}C-uracil and the incubation was continued for a further 30 minutes. To the remaining three parts, ^{14}C-uracil and various final concentrations of actinomycin S (0.01, 1.0 and 10 μg per ml) were added and the incubation

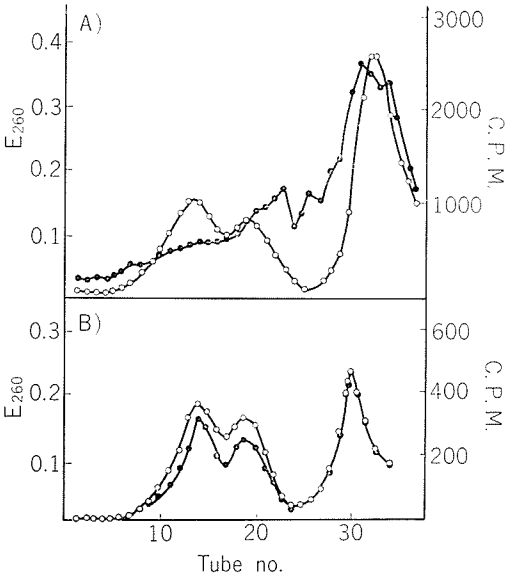

FIGURE 1 A) Linear sucrose gradient analysis of rapidly labeled RNA from *B. subtilis* SB 15 cultured in the absence of actinomycin.
B) The pattern of RNA after further incubation for 30 minutes in the absence of ^{14}C-uracil after labeling with ^{14}C-uracil for 3 minutes.
○——○ E_{260}
●——● C.P.M. per tube

was continued for 3 minutes.

After incubation, these culture were quickly chilled in ice. RNA was extracted from the cells and analysed in a sucrose gradient after lysis with lysozyme.

As shown in Fig. 1A), rapidly labeled radioactive peaks, which did not coincide with the UV absorbing peaks, were observed between 16 S ribosomal and 4 S soluble RNA. From Fig. 1B), it is seen that these peaks moved to the positions of 23 S, 16 S and 4 S RNA after rapidly labeled organisms had been incubated for a further 30 minutes in the absence of ^{14}C-uracil. These results indicate that the radioactive peaks seen in this experiment seem to be those of messenger RNA. In the presence of a low concentration of actinomycin, the incorporation of ^{14}C-uracil into messenger RNA with a large sedimentation coefficient was inhibited preferentially, although the synthesis of messenger RNA's were inhibited equally regardless of their sedimentation coefficients in the presence of 10 μg per ml of actinomycin (Figs. 2, 3 and 4).

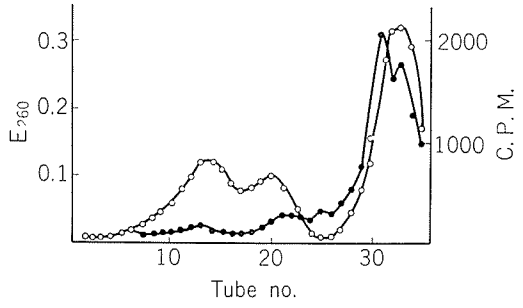

FIGURE 2 Linear sucrose gradient analysis of rapidly labeled RNA from *B. subtilis* SB 15 cultured in the presence of 0.01 μg per ml of actinomycin.
○———○ E_{260}
●———● C.P.M.

2. *The effect of a growth-inhibitory concentration of actinomycin on the incorporation of ^{14}C-uracil into 23 S and 16 S ribosomal RNA's*

When the OD_{660} of a culture reached 1.0, it was incubated with ^{14}C-uracil in the presence of 0.1 μg per ml of actinomycin for 30 minutes. The control culture was incubated with ^{14}C-uracil in the absence of actinomycin for 30 minutes when the OD_{660} reached 1.0.

After incubation, the organism was collected, washed and resuspended in fresh medium without either ^{14}C-uracil or actinomycin. Then the incubation was continued for a further 30 minutes. The RNA was extracted from each culture after lysis of the cells with lysozyme and subjected to sucrose gradient analysis.

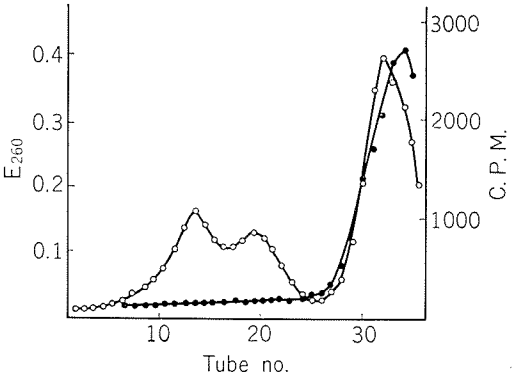

FIGURE 3 Linear sucrose gradient analysis of rapidly labeled RNA from *B. subtilis* SB 15 cultured in the presence of 1 μg per ml of actinomycin.
○———○ E_{260}
●———● C.P.M. per tube

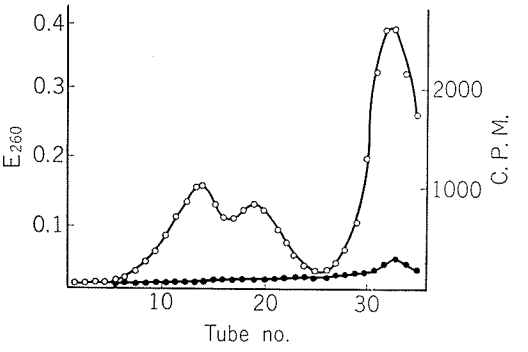

FIGURE 4 Linear sucrose gradient analysis of rapidly labeled RNA from *B. subtilis*, SB 15 cultured in the presence of 10 μg per ml. of actinomycin.
○———○ E_{260}
●———● C.P.M. per tube

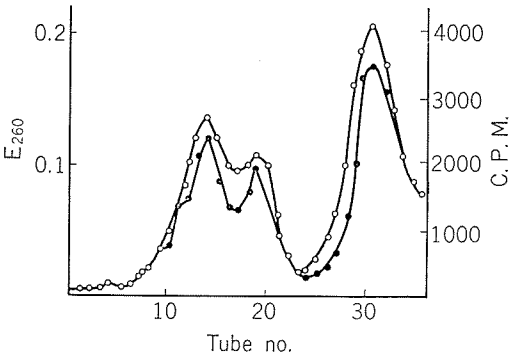

FIGURE 5 Linear sucrose gradient analysis of RNA from *B. subtilis*, SB 15 which was labeled with ^{14}C-uracil for 30 minutes in the absence of actinomycin, when the OD of the culture at 660 mμ reached 1.0, and then incubated for 30 minutes more in fresh medium without ^{14}C-uracil.
○———○ E_{260}
●———● C.P.M.

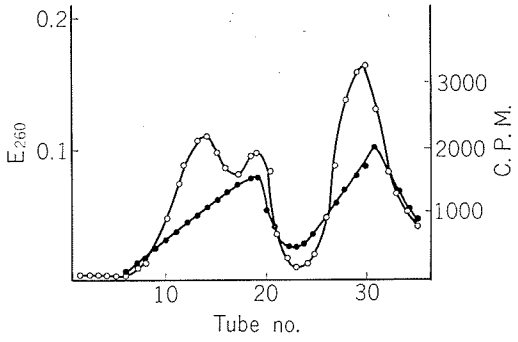

FIGURE 6 Linear sucrose gradient analysis of RNA from *B. subtilis*, SB 15 which was labeled with ^{14}C-uracil for 30 minutes in the presence of 0.1 μg per ml of actinomycin, when the OD of the culture at 660 mμ reached 1.0 and then incubated for a further 30 mintues in the absence of ^{14}C-uracil and ACT.
○———○ E_{260}
●———● C.P.M. per tube

As shown in Figs. 5 and 6, the incorporation of ^{14}C-uracil into 23 S ribosomal RNA was inhibited much more than that into 16 S ribosomal RNA. On the other hand, considerable inhibition of soluble RNA was also observed in this case.

The effect of a subinhibitory concentration

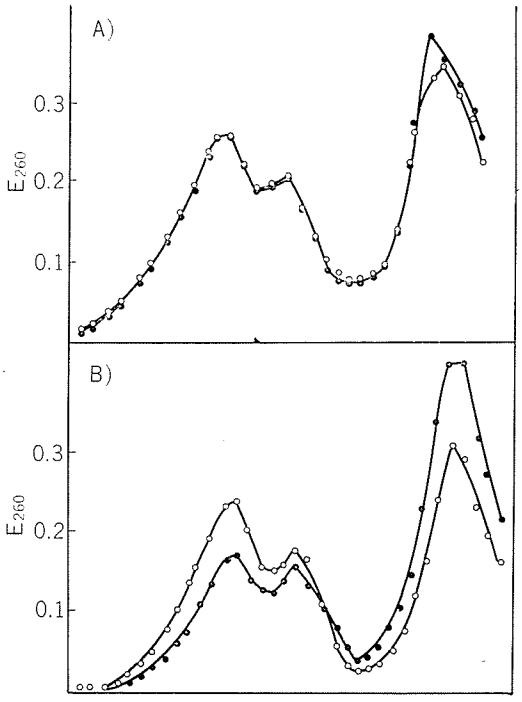

FIGURE 7 Sucrose gradient analysis of RNA from *B. subtilis*, SB 15 A) in the presence of 0(○———○) and 0.01 μg (●———●) per ml of actinomycin, B) in the presence of 0(○———○) and 0.025 μg (●———●) per ml of actinomycin.

of actinomycin on the net synthesis of 23 S ribosomal RNA was further investigated, since it seemed interesting to see whether the bacteria could grow when synthesis of their 23 S ribosomal RNA was inhibited by actinomycin.

3. *Effect of a concentration of actinomycin insufficient to inhibit growth on the net synthesis of 23 S ribosomal RNA in B. subtilis*

The organism was cultured in the presence of various final concentrations of actinomycin (0, 0.01 and 0.025 μg per ml respectively). When the OD_{660} of the exponentially growing organism reached 1.0, RNA was extracted from each culture and analyzed on a sucrose density gradient.

The results presented in Figs. 7 and 8 show

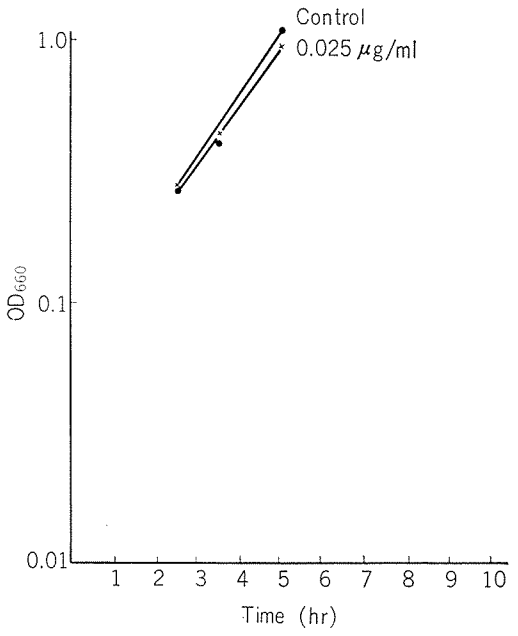

FIGURE 8 Growth curve of *B. subtilis*, SB 15 with or without actinomycin (0.025 μg per ml.). Growth was measured on the turbidity at 660 mμ.

that, although the growth is not suppressed at the concentration of actinomycin employed, considerable inhibition of net synthesis of 23 S ribosomal RNA appears to take place at a concentration of 0.025 μg per ml of actinomycin.

DISCUSSION

Differential inhibition of various classes of RNA by actinomycin D has been reported in mammalian cells (PERRY, 1962; GEORGIEV, 1963; FRANKLIN, 1963).

Perry reported that rapidly labeled RNA from L strain fibroblasts treated with a low level of actinomycin D was found to be reduced in the heavier RNA components but unaffected in the intermediate and 4 S fractions.

In bacteria, it has been reported in a preliminary report that in *Corynebacterium diphtheriae* the incorporation of radioactive precursors into RNA around the 23 S region (including messenger RNA and 23 S ribosomal RNA) was inhibited at a low concentration of actinomycin S (YAMADA *et al.*, 1964). In this work, at a low concentration of actinomycin it was shown that messenger RNA with a larger sedimentation coefficient was preferentially inhibited in *B. subtilis*.

Consequently, it may well be that preferential inhibition of messenger RNA with a larger sedimentation coefficient by a low concentration of actinomycin may be a general phenomenon occurring not only in mammalian cells but also in bacteria which are sensitive to actinomycin.

POLLOCK (1963) and COLLMAN *et al.* (1964) reported that the formation of enzymes in *B. subtilis* and *B. cereus* is selectively inhibited by actinomycin D. KADOWAKI *et al.* (1965) reported similar results. We Observed, in *C. diphtheriae*, that formation of toxic protein was partially inhibited while growth was not inhibited by actinomycin (YAMADA unpublished data). The intrinsic mechanisms of these phenomena are not yet clear. However, as previously considered (YAMADA *et al.*, 1964), it may be that the frequency of binding of the antibiotic molecules to the guanine moiety of DNA increases in parallel with increase in the size of the cistron, particularly at a low concentration of the antibiotic and this may result in preferential inhibition of DNA dependent synthesis of RNA with a larger molecular size. If this is so, one possible explanation of the phenomena might be that the differential inhibition of messenger RNA with a larger sedimentation coefficient by a low concentration of actinomycin may result in differential inhibition of synthesis of various proteins.

With respect to ribosomal RNA, PERRY (1962) reported that in mammalian cells (L fibroblasts) treated with actinomycin D, no labeling of 28 S or 18 S ribosomal RNA occurred although 4 S RNA continued to increase. In L cells, FRANKLIN (1963) reported that after exposure to low doses of actinomycin D, the synthesis of ribosomal RNA (28 S and

18 S compoments) was completely inhibited and synthesis of soluble RNA (4 S) was partially inhibited. The residual incorporation of radioactive precursors into 4 S RNA that was resistant to an increased concentration of actinomycin appeared to be caused by terminal group turnover of the CCA sequence in soluble RNA.

In this paper, however, it was shown that the incorporation of radioactive precursors into 23 S ribosomal RNA was much more sensitive to a growth inhibitory concentration of actinomycin than incorporation into 16 S ribosomal RNA and soluble RNA. It is of particular interest that the net synthesis of 23 S ribosomal RNA was considerably inhibited by actinomycin even when growth was not inhibited completely.

This observation suggests that the bacteria may be able to grow under conditions when 23 S ribosomal RNA is remarkably inhibited. Indeed, preliminary experiments recently showed that an actinomycin resistant strain was able to grow when 23 S ribosomal RNA was strongly inhibited by actinomycin (YAMADA, 1965). However, it is still possible that there was a low yield of 23 S RNA from the resistant strain for some unknown reason.

ACKNOWLEDGEMENTS

We are much indebted to Profs. M. YONEDA and M. HORI for their advice and encouragement throughout this work.

REFERENCES

COLEMAN, D. and ELLIOTT, W. H. (1964). Stimulation of extracellular ribonuclease formation in B. subtilis by actinomycin D. *Nature* **202**, 1083–1085.

FRANKLIN, R. M. (1963). The inhibition of ribonucleic acid synthesis in mammalian cells by actinomycin D. *Biochim. Biophys. Acta* **72**, 555–565.

GEORGIEV, G. P., SAMARINA, O.P., LERMAN, M. I., SMIRNOV, M. N. and SERVERTZOV, A. N. (1963). Biosynthesis of messenger and ribosomal ribonucleic acids in the nucleo-chromosomal apparatus of animal cells. *Nature* **200**, 1291–1294.

HURWITZ, J., FURTH, J. J., MALAMY, M. and ALEXANDER, M. (1962). The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin D and proflavin. *Proc. Natl. Acad. Sci. U.S.* **48**, 1112–1113.

KADOWAKI, K., HOSODA, J. and MARUO, B. (1965). Effects of actinomycin D and 5-fluorouracil on the formation of enzymes in *Bacillus subtilis*. *Biochim. Biophys. Acta* **103**, 311–318.

KAWAMATA, J. and Imanishi, M. (1960). Interaction of actinomycin with deoxyribonucleic acid. *Nature* **187**, 1112–1113.

KIRK, J. M. (1960). The mode of action of actinomicin D. *Biochim. Biophys. Acta* **42**, 167–169.

LEVINTHAL, C., KEYNAN, A. and HIGA, A. (1962). Messenger RNA turnover and protein synthesis in B. subtilis inhibited by actinomycin D. *Proc. Natl. Acad. Sci. U.S.*, **48**, 1631–1638.

MONIER, R., NAONO, S., HAYES, D., HAYES, F. and GROS, F. (1962). Studies on the heterogeneity of messenger RNA from *E. coli*. *J. Mol. Biol.* **5**, 311–324.

PERRY, R. P. (1962). The cellular sites of synthesis of ribosomal and 4 S RNA. *Proc. Natl. Acad. Sci. U.S.* **48**, 2179–2186.

POLLOCK, M. R. (1963). The differential effect of actinomycin D on the biosynthesis of enzymes in Bacillus subtilis and cereus. *Biochim. Biophys. Acta* **76**, 80–93.

RAUEN, H. M., KERSTEN, H. and KERSTEN, W. (1960). *Zur Wirkungsweise von Actinomycin Hoppe-Seyler's Z. Physiol. Chem.* **321**, 139–147.

REICH, E., FRANKLIN, R. M., SHATKIN, A. J. and TATUM, E. L. (1962). Action of actinomycin D on animal cells and viruses. *Proc. Natl. Acad. Sci. U.S.* **48**, 1238–1245.

YAMADA, T. (1965). A demonstration of preferential inhibition of net synthesis of 23 S RNA in growing bacteria by actinomycin S. *Biochim. Biophys. Acta* **108**, 158–159.

YAMADA, T., YONEDA, M. and KAWAMATA, J. (1964). A profile of ribonucleic acid synthesis in *Corynebacterium diphtheriae* treated with actinomycin S. *Biken J.* **7**, 131–136.