EFFECT OF TUNICAMYCIN ON MICROORGANISMS: MORPHOLOGICAL CHANGES AND DEGRADATION OF RNA AND DNA INDUCED BY TUNICAMYCIN

AKIRA TAKATSUKI, KEN-ICHI SHIMIZU and GAKUZO TAMURA

Laboratory of Microbiology, Department of Agricultural Chemistry, the University of Tokyo, Bunkyo-ku, Tokyo, Japan

(Received for publication April 28, 1971)

The treatment of gram-positive bacteria and yeasts with tunicamycin induces morphological changes such as elongation, conversion from rod-form to cocci-form and enlargement of microbial cells. Even when bacteria grew normally or to a somewhat lesser extent than controls as determined by measurement of optical density, the increase in viable cells was suppressed to a great extent. Such effects of tunicamycin are observed without inhibition of protein, DNA and RNA synthesis in *Bacillus subtilis* W 23 cells. RNA and DNA degradation occurred subsequent to the morphological changes of *B. subtilis* cells, and the former degraded to a larger extent than the latter. No difference was detected between control and tunicamycin-treated cells in acid-solubilization of the cellular macromolecules labeled with radioactive amino acids or glucosamine.

Tunicamycin is a glucosamine-containing antibiotic active against animal and plant viruses, gram-positive bacteria, yeasts and fungi[1]. The antibiotic was found to induce various morphological changes in microorganisms. Studies on the action mechanism of tunicamycin employing Newcastle disease virus and cell cultures of chick embryo fibroblasts suggest that the antibiotic affects membrane synthesis and function (manuscript in preparation). Morphological changes observed with some microorganisms have suggested that tunicamycin influences cell surface synthesis in microbial cells as in animal cells. Studies on cell surfaces have attracted interest lately and conditional morphological mutants have been mainly used in some of the studies[2]. Tunicamycin induced morphological changes in many bacteria and yeasts. These results indicate that tunicamycin might become a useful tool in studies of cell surface synthesis and cell division. This paper deals with induction of morphological changes in microorganisms by tunicamycin and its effect on synthesis and degradation of cellular macromolecules.

Materials and Methods

Culture media. Bouillon containing 7.0 g meat extract, 10 g Polypeptone and 3.0 g NaCl per 1,000 ml (Kyokuto Pharmaceutical Industries, Tokyo) was used for the cultivation of bacteria except mycobacteria which were grown in DUBOS medium (Nippon Eiyo Kagaku Kaisha, Tokyo). Yeasts were grown in bouillon supplemented with 2 % glucose.

Effect of tunicamycin on microbial growth. Cultures incubated overnight were diluted 10-fold with prewarmed fresh medium in L-tubes and grown on a shaker at 30°C. During the middle log phase of growth, 1 ml of the cultures was mixed with 9 ml fresh medium containing specified concentrations of tunicamycin. Growth was followed by measuring the optical density at 550 mμ ($OD_{550\,m\mu}$) of the cultures. Viable counts were determined in some cases. Photographs of microorganisms were taken without fixation.

Synthesis of macromolecules. Synthesis of the cellular macromolecules in *Bacillus subtilis* W 23 was assessed by the incorporation of appropriately labeled precursors for the macromolecular fractions. Specifically, 0.5 μCi of ^{14}C-amino acid mixture was added per 10 ml of culture medium to follow protein synthesis, 10 μCi of ^{3}H-uridine per 10 ml of broth was added to follow ribonucleic acid (RNA) synthesis, 10 μCi of ^{3}H-thymidine per 10 ml of broth was added to follow deoxyribonucleic acid (DNA) synthesis, and 0.5 μCi of ^{14}C-glucosamine per 10 ml of broth was added to follow synthesis of glucosamine-containing macromolecules. In these labeling experiments, cultures of *B. subtilis* W 23 at the middle log phase of growth were diluted with medium containing one-tenth of the normal concentration of the constituents, supplemented with phosphate-buffered (0.05 M, pH 7.3) physiological saline. Precursors and tunicamycin were added during the early log phase of the growth cycle. Sample portions (0.5 ml) were withdrawn from the cultures at appropriate time intervals. The samples were made 5 % with cold trichloroacetic acid (TCA), the acid-insoluble fractions collected on membrane filters (pore size 0.45 mμ; Millipore Corp.), dried, and counted in 10 ml scintillation liquid containing 6.0 g PPO and 0.4 g dimethyl-POPOP per 1,000 ml toluene.

Stability of macromolecular cellular fractions in the presence of tunicamycin. To assess stability of the cellular protein, DNA, RNA and glucosamine-containing macromolecular fractions in *B. subtilis* W 23 cells in the presence of tunicamycin, cells were prelabeled for two to three generations at the early log phase by the addition of 0.1 μCi of ^{14}C-amino acid mixture, 1.0 μCi of ^{3}H-thymidine, 1.0 μCi of ^{3}H-uridine or 0.1 μCi of ^{14}C-glucosamine per 10 ml of culture medium. After the labeling period, cells from 10 ml of culture medium were recovered by centrifugation, washed and resuspended in 40 ml of fresh culture medium. This suspension was divided into four 10-ml portions, and tunicamycin (0.5, 1.0 or 10 μg/ml) was added to three. The other served as control. The cultures were reincubated, and sample portions (0.5 ml) were withdrawn into 1 ml cold 10 % TCA at regular time intervals. Acid-insoluble fractions were collected on Millipore filters, washed, dried, and counted in 10 ml scintillation liquid.

Extraction and sucrose density-gradient centrifugation analysis of RNA. The results presented in this paper show that tunicamycin induces extensive degradation of RNA in *B. subtilis* W 23 cells. The nature of the degradation was analyzed by sucrose density-gradient centrifugation. *B. subtilis* W 23 was prelabeled with ^{3}H-uridine (1.0 μCi/ml), harvested and washed as described above. Cells from 30 ml of culture medium were resuspended in 30 ml of fresh medium and divided into three 10-ml portions. Tunicamycin (0.05 or 0.1 μg/ml) was added to two and the other served as control. At designated time intervals, 3 ml of the cultures were sampled and made 10^{-2} M in respect to ethylenediaminetetraacetic acid (EDTA) and 100 μg/ml in respect to lysozyme. RNA was extracted by the SDS-phenol method, precipitated with ethanol, recovered by centrifugation, washed, and dissolved in 1.0 ml of phosphate buffer containing 0.05 M K-PO_4, pH 6.7; NaCl 0.1 M; and EDTA 0.01 M. A portion (0.5 ml) of the solution was loaded on sucrose gradients containing the same buffer. Sucrose gradients (5~15 w/v %, 13 ml per tube) were formed on a 1.0 ml cushion of 40 w/v % sucrose. The gradients were run at 25,000 rpm (77,000×*g*) for 19 hours in a RPS 25-3 A rotor and a Model 65 P preparative ultracentrifuge (Hitachi, Tokyo). Gradients were fractionated dropwise by puncturing the bottom of the tubes, bovine serum albumin (200 μg) was added to each fraction (*ca.* 0.5 ml), and 10 % TCA-insoluble radioactivity was counted.

Chemicals. Tunicamycin was prepared according to the method reported previously[1] and the same lot of the antibiotic was used throughout these experiments. Uridine-5-^{3}H (specific activity, 5.0 Ci/mM), thymidine-6-^{3}H (specific activity, 25.3 Ci/mM) and D-glucosamine-1-^{14}C (specific activity, 55.6 mCi/mM) were purchased from the Radiochemical Centre, Amersham, England. ^{14}C-Amino acid mixture was obtained from Dai-Ichi Chemicals, Tokyo.

Results

I. Effect of Tunicamycin on Growth and Morphology of Microorganisms

The effect of tunicamycin on the growth and morphology of microorganisms was examined with 138 strains of bacteria and 8 strains of yeasts. Microorganisms grown in the presence of tunicamycin showed various responses to the antibiotic.

Extensive inhibition of cell division, *i.e.*, elongation, was observed with *B. subtilis* IAM 1069 (Plate 1) and IAM 1214, and cell length was more than 30-fold that of the controls. Spherical conversion was detected with some rod-type bacteria, and *B. cereus* IAM 1110 was a typical example (Plate 2). The most frequent change observed with *B. subtilis* cultures were chain-formation and tadpole-like appearance, *i.e.*, cells grew in chains and some of the rod-shaped forms would then converted to cocci, giving the cultures a tadpole-like appearance when observed under the microscope.

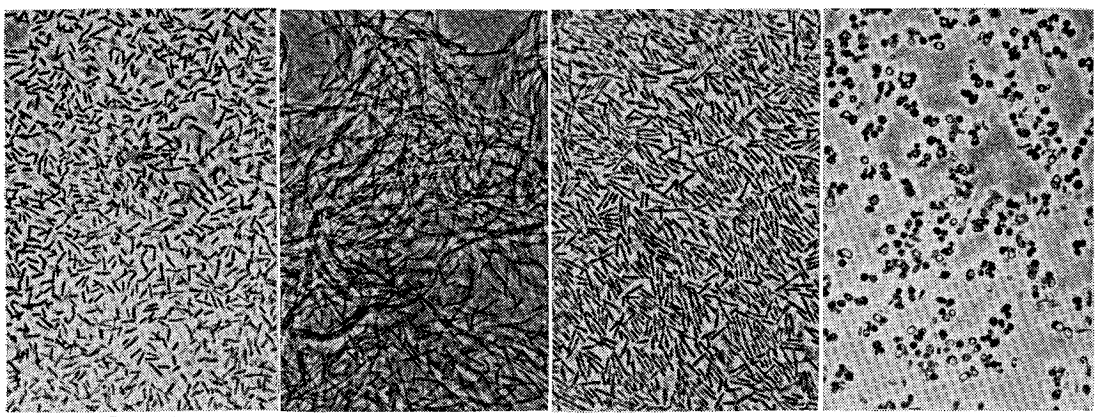
Some morphologically altered bacteria grew to a similar extent as controls as followed by measurement of $OD_{550\,m\mu}$ (*e.g.*, *B. megaterium*, Fig. 1), while others showed decreased growth rates (*e.g.*, *B. subtilis* and *B. sphaericus*, Fig. 1). But increase in $OD_{550\,m\mu}$ did not reflect increase in viable cells. In the case of *B. subtilis* W 23, the $OD_{550\,m\mu}$ increased for the first 3~4 hours after the addition of tunicamycin (0.5~10 μg/ml), but viable cell counts either did not increase or actually decreased (Fig. 1). The decrease in $OD_{550\,m\mu}$ after 5~6 hours of treatment may be a reflection of cell lysis. In the presence of 15 w/v % of sucrose, lysis was prevented. Microscopic observations showed that tunicamycin-treated cells appeared more rigid in comparison with typical protoplasts of *B. subtilis* W 23 prepared by treatment with lysozyme.

Plate 1. Elongation of *B. subtilis* cells by tunicamycin.

B. subtilis IAM 1069 was treated with tunicamycin (0.5 μg/ml) at 30°C, and the photographs were taken after a 10-hour period of treatment.

Plate 2. Spherical conversion of *B. cereus* cells by tunicamycin.

B. cereus IAM 1110 was grown at 30°C in the presence of tunicamycin (2.0 μg/ml), and the photographs were taken after a 3-hour period of treatment.



Control | Treated with tunicamycin | Control | Treated with tunicamycin

Fig. 1. Effect of tunicamycin on bacterial growth.

Cultures of *B. subtilis* W 23, *B. megaterium* IFO 3003 and *B. sphaericus* IFO 3525 incubated overnight were diluted 10-fold with prewarmed fresh medium and reincubated in the presence of tunicamycin (0.5, 1.0 or 10 μg/ml). Effect of tunicamycin on bacterial growth was determined by measuring $OD_{550\,m\mu}$ and viable cell counts.

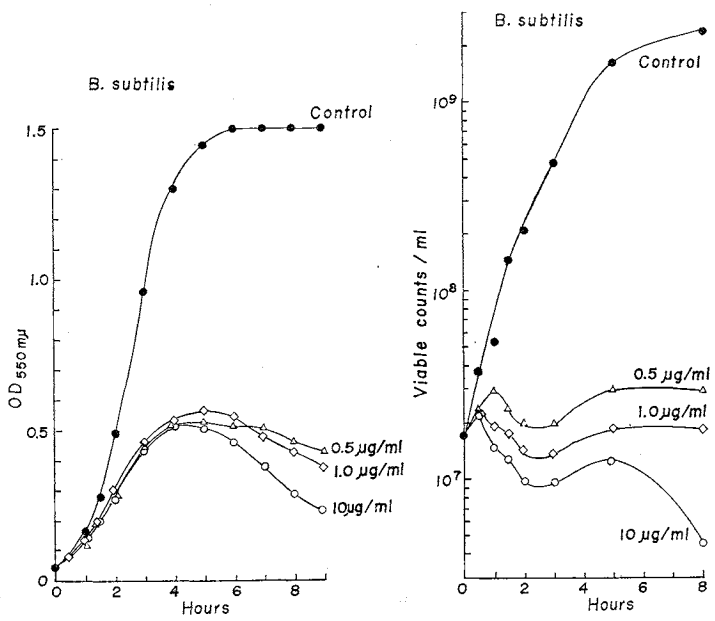
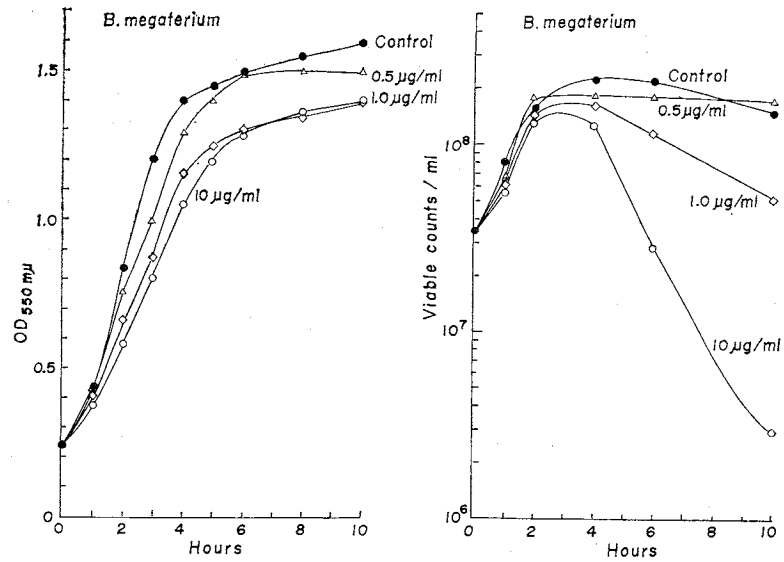


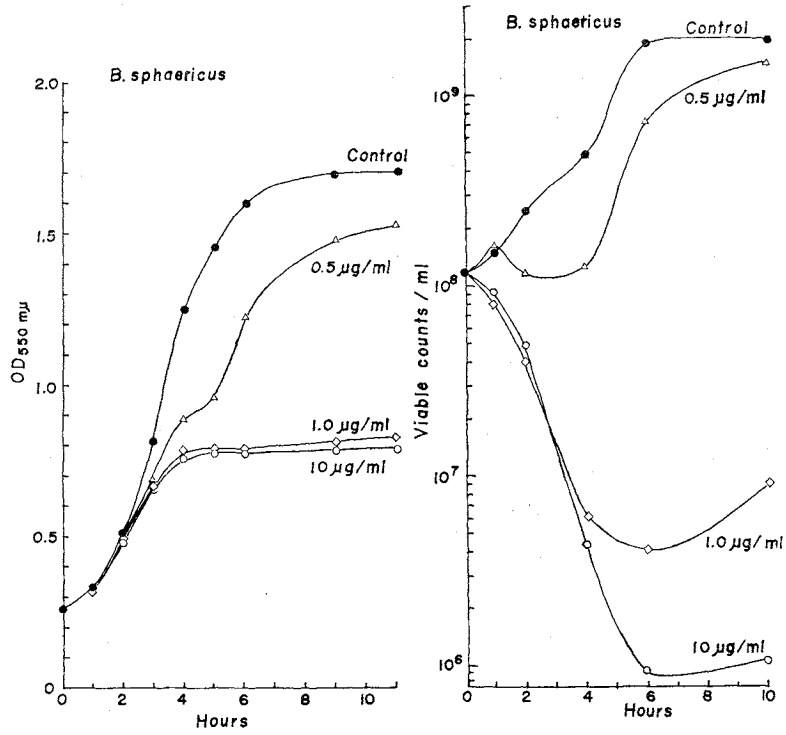
Fig. 1-B



The most significant discrepancy between $OD_{550\,m\mu}$ and viable cell counts was observed with *B. sphaericus* (Fig. 1).

All the yeasts tested including *Saccharomyces*, *Candida*, *Hansenula* and *Torula* were sensitive to tunicamycin. The most frequent morphological change observed

Fig. 1-C



was enlargement of cell volume. In this case yeasts lost their rigidity and appeared opaque, but cell lysis was not detected during the 10-hour period of observation.

II. Effect of Tunicamycin on Cellular Macromolecular Biosynthesis in *B. subtilis* W 23

A change to spherical forms was observed after 3-hour treatment of *B. subtilis* W 23 with tunicamycin (1.0 μg/ml). The effects of tunicamycin on synthesis and stability of the cellular macromolecular fractions were investigated with this strain of *B. subtilis*.

The effects of tunicamycin on bacterial growth and cellular macromolecular synthesis are shown in Figs. 2 and 3. Incorporation of ^{3}H-uridine, ^{3}H-thymidine and ^{14}C-amino acids, reflecting RNA, DNA and protein biosyntheses, respectively, was partially inhibited by the antibiotic. The extent of incorporation nearly paralleled or somewhat exceeded the increase of $OD_{550\,m\mu}$ (Table 1). Inhibition of incorporation of ^{14}C-glucosamine was most extensive among all examined. Glucosamine is a main constituent of the cell wall. Microscopic observations and labeling experiments suggest altered synthesis of cell surfaces in the presence of tunicamycin. Studies on the effect of tunicamycin on the synthesis of cell surfaces are now in progress employing bacterial and animal cells.

III. Stability of Cellular Macromolecular Fractions in *B.subtilis* W 23

B. subtilis W 23 changed its morphology after 3 hours of incubation and began to lyse 5 hours after the addition of tunicamycin (1.0 and 10 μg/ml) as shown in

Fig. 2. Effect of tunicamycin on the growth of *B. subtilis* and synthesis of RNA and protein.

A culture of *B. subtilis* W 23 in the log phase of the growth cycle was mixed with fresh medium containing tunicamycin (0.5, 1.0 or 10 μg/ml). Ten microcuries of ^{3}H-uridine and 0.5 μCi of ^{14}C-amino acids were added per 10 ml of broth to follow the process of RNA and protein synthesis, respectively. Precursors and tunicamycin were added at the same time. Sample portions (0.5 ml) were withdrawn from the cultures at appropriate time intervals. The samples were made 5% with cold TCA; the acid-insoluble fractions collected on membrane filters and counted in 10 ml of scintillation liquid. Bacterial growth was followed by measuring $OD_{550\,m\mu}$ of the cultures.

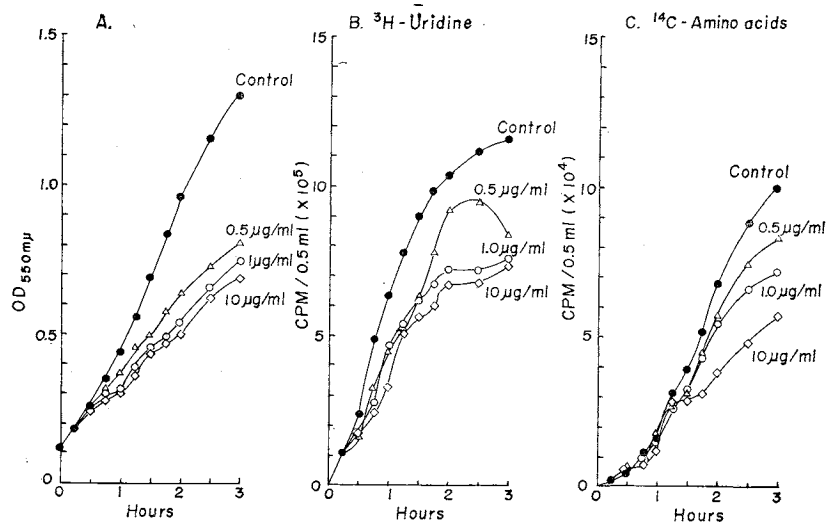


Fig. 3. Effect of tunicamycin on the growth of *B. subtilis* and synthesis of DNA and glucosamine-containing cellular macromolecules.

B. subtilis W 23 was treated with tunicamycin (0.5, 1.0 or 10 μg/ml), and synthesis of DNA and glucosamine-containing cellular macromolecules was assessed by the incorporation of ^{3}H-thymidine (1.0 μCi/ml) and ^{14}C-glucosamine (0.05 μCi/ml), respectively, into the acid-insoluble fractions. The procedure was the same as described in the legend to Fig. 2.

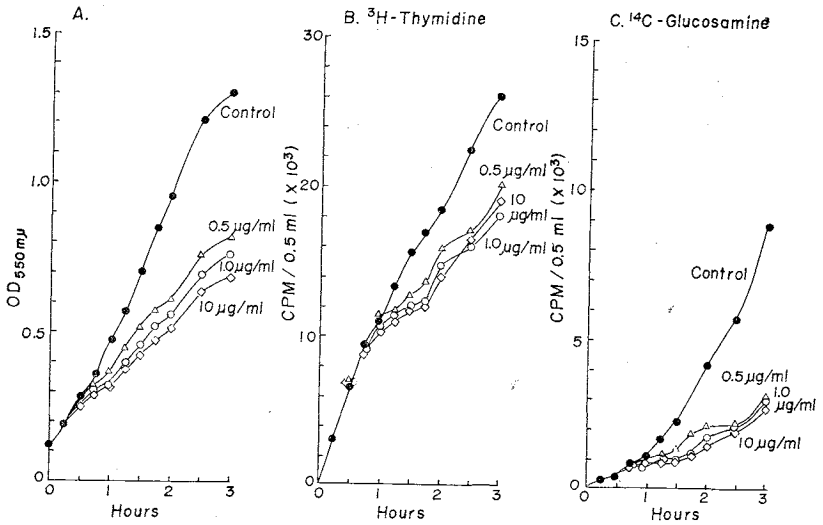


Table 1. Degree of growth and incorporation of uridine, thymidine, amino acids and glucosamine in the presence of tunicamycin

		% Growth or incorporation			
		Control	Tunicamycin-treatment		
			0.5 μg/ml	1.0 μg/ml	10 μg/ml
Exp. 1	$OD_{550\,m\mu}$	100	61	58	52
	^{3}H-Uridine	100	72	65	63
	^{14}C-Amino acids	100	82	72	51
Exp. 2	$OD_{550\,m\mu}$	100	62	58	52
	^{3}H-Thymidine	100	77	73	69
	^{14}C-Glucosamine	100	33	31	30

S. subtilis W 23 was grown in the presence of tunicamycin(0.5, 1.0 or 10 μg/ml). $OD_{550\,m\mu}$ and the incorporation of ^{3}H-uridine, ^{3}H-thymidine, ^{14}C-amino acids or ^{14}C-glucosamine into the acid-insoluble macromolecular froctions were compared after 3-hour period of treatment. The figures in the Table were calculated from Fig. 2 (Exp. 1) and Fig. 3 (Exp. 2).

Figs. 4 A and 5 A. The stability of the cellular macromolecular fractions during this period was examined. Cellular macromolecular fractions were prelabeled with ^{3}H-uridine, ^{3}H-thymidine, ^{14}C-amino acids or ^{14}C-glucosamine as described in Materials and Methods. Loss of acid-insoluble radioactivity was then used as a parameter to measure the stability of the cellular macromolecular fractions.

Relatively little loss of acid-insoluble radioactivity was observed in the control cultures during an incubation of 5~6 hours (Figs. 4 and 5). In the presence of tunicamycin, extensive degradation of RNA and DNA fractions was observed. RNA and DNA began to degrade at nearly the same time. Extensive RNA degradation took effect before cell lysis became evident in the presence of 10, 1 or 0.5 μg/ml of tunicamycin (Fig. 4 A, B). Significant DNA degradation was initiated at the onset of cell lysis in the presence of 10 or 1 μg/ml of tunicamycin. At 0.5 μg/ml of tunicamycin, DNA degradation was insignificant. Only trace amounts

Fig. 4. Effect of tunicamycin on stability of prelabeled RNA or protein fractions in *B. subtilis*.

B. subtilis W 23 cells were prelabeled for two to three generations in the early log phase of the growth cycle with 1.0 μCi of ^{3}H-uridine and 0.1 μCi of ^{14}C-amino acids per 10 ml of culture medium. After the labeling period, cells were recovered by centrifugation, washed, and resuspended in 40 ml fresh culture medium. The suspension was divided into four equal portions, and tunicamycin (0.5, 1.0 or 10 μg/ml) was added to three. The cultures were reincubated, and sample portions (0.5 ml) were withdrawn at regular time intervals. Cold TCA-insoluble fractions were collected on filters and counted in 10 ml scintillation liquid.

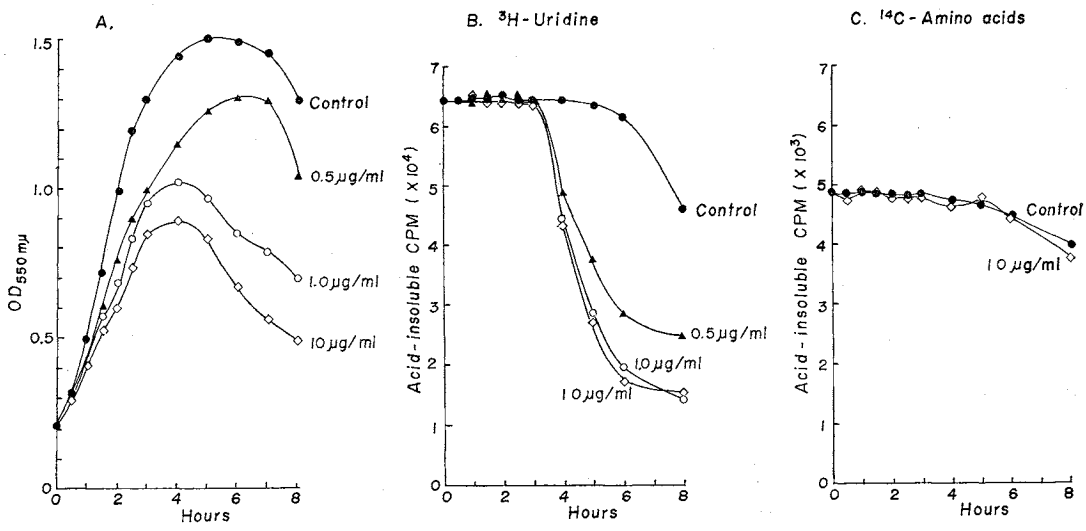


Fig. 5. Effect of tunicamycin on stability of prelabeled DNA or glucosamine-containing cellular macromolecular fractions in *B. subtilis*.

B. subtilis W 23 cells were prelabeled for two to three generations in the early log phase of the growth cycle with 1.0 μCi of ^{3}H-thymidine and 0.1 μCi of ^{14}C-glucosamine per 10 ml of culture medium. Stability of DNA and glucosamine-containing cellular macromolecular fractions was assessed by measuring radioactivity in the acid-insoluble fractions as described in the legend to Fig. 4.

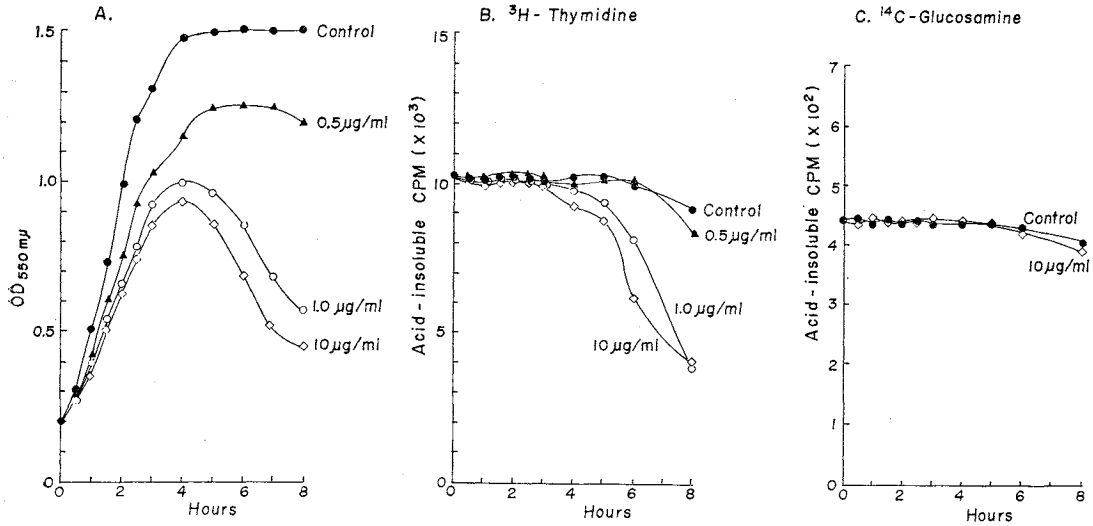
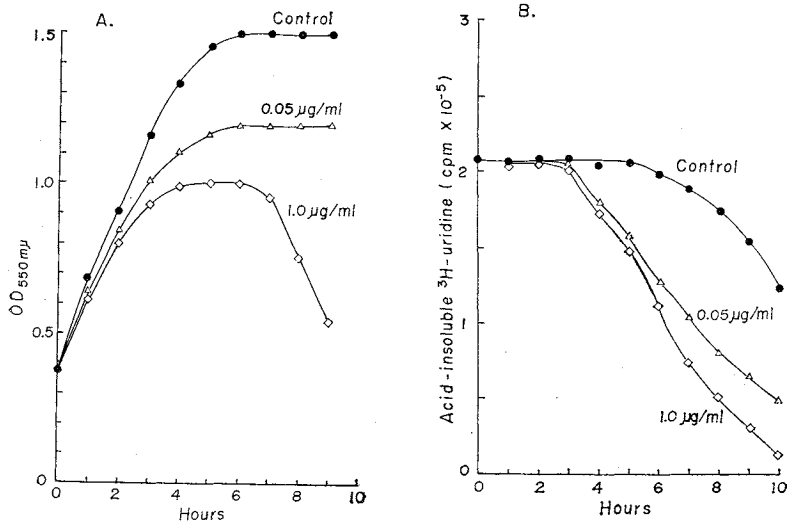


Fig. 6. Nature of the degradation of *B. subtilis* W 23 RNA by tunicamycin as revealed by sucrose density-gradient centrifugation.

B. subtilis W 23 cells were prelabeled with ^{3}H-uridine for two to three generations. Tunicamycin was then added (0.05 or 1.0 μg/ml) and the cultures were reincubated. The time course of degradation of RNA was measured by the same method as described in the legend to Fig. 4. RNA was extracted by the SDS-phenol method and 0.5 ml portions of RNA solutions were applied to 13 ml of 5% to 15% sucrose gradients. The gradients were centrifuged at 25,000 rpm for 19 hours at 4℃. Acid-precipitable fractions were collected and assayed for radioactivity.



of the cellular macromolecules labeled with ^{14}C-amino acids or ^{14}C-glucosamine degraded regardless of the presence of tunicamycin (Figs. 4 and 5).

IV. Nature of the Degradation of *B. subtilis* W 23 RNA by Tunicamycin

The nature of the degradation of *B. subtilis* W 23 RNA caused by treatment with tunicamycin was analyzed by sucrose density gradient centrifugation. *B. subtilis* W 23 prelabeled with ^{3}H-uridine was grown in the presence of tunicamycin (0.05 or 1.0 μg/ml), portions of the cultures were sampled at 3, 4, 6.5 and 9 hours of treatment, and RNA was extracted. These times correspond to detection of morphological changes and RNA degradation (3 hours), lack of increase of $OD_{550\,m\mu}$ (4 hours), detection of decrease of $OD_{550\,m\mu}$ (6.5 hours) and profound cell lysis (9 hours) in the presence of 1.0 μg/ml of tunicamycin (Figs. 6-1, 6-2).

Even when bacterial growth began to slow down as revealed by $OD_{550\,m\mu}$ (Fig. 6-A) and degradation of RNA was dectected in the presence of tunicamycin (Fig. 6-B), no significant difference was observed with the sucrose density-gradient profiles of ^{3}H-uridine labeled *B. subtilis* W 23 RNA (Fig. 6-C, 6-D). When the degradation of RNA proceeded rapidly, the profiles of ^{3}H-RNA prepared from tunicamycin-treated cells showed abnormality (Fig. 6-E, 6-F). Radioactivity of degraded ^{3}H-RNA was detected with tunicamycin-treated cells between the 16S and 4S RNA regions. At these times decrease of radioactivity at the 4S transfer RNA region was less than that at the 23S and 16S ribosomal RNA regions.

Fig. 6-B.

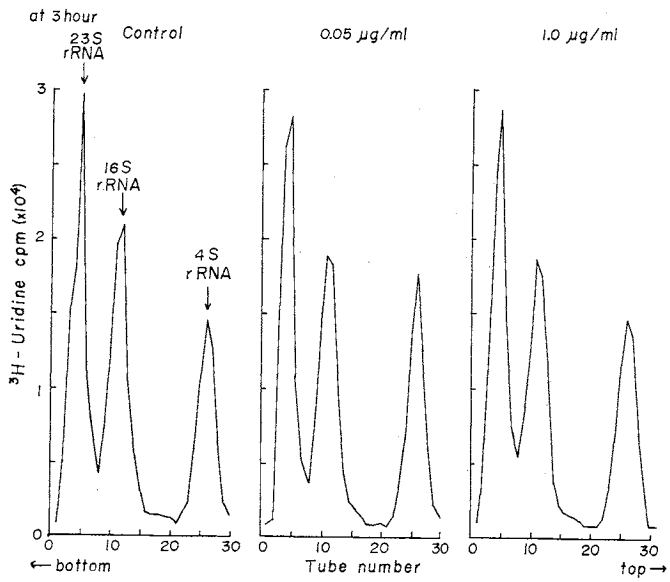
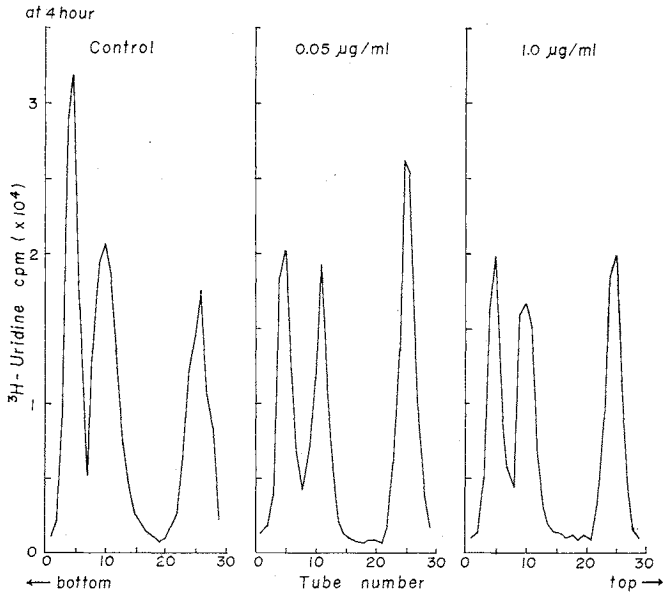


Fig. 6-C.



When the degradation of 23S and 16S RNA was compared, the former was more significant than the latter and radioactivity at the 16S RNA region was greater than that at the 23S RNA region. A similar instability of 23S ribosomal RNA was observed with the control cells after 6.5- and 9-hour period of incubation.

Fig. 6-D.

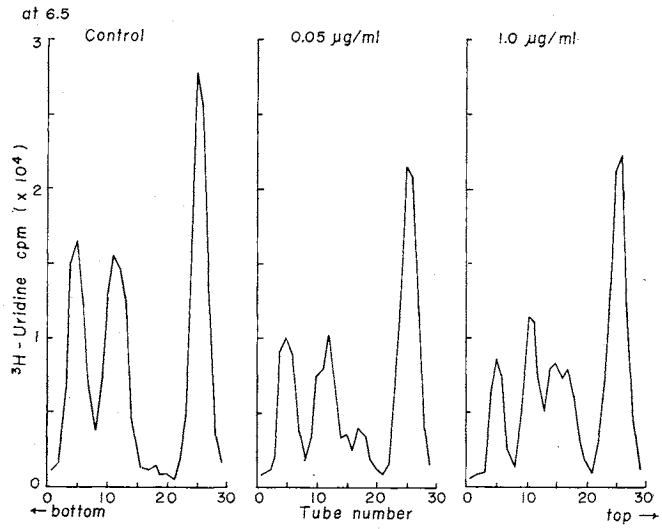
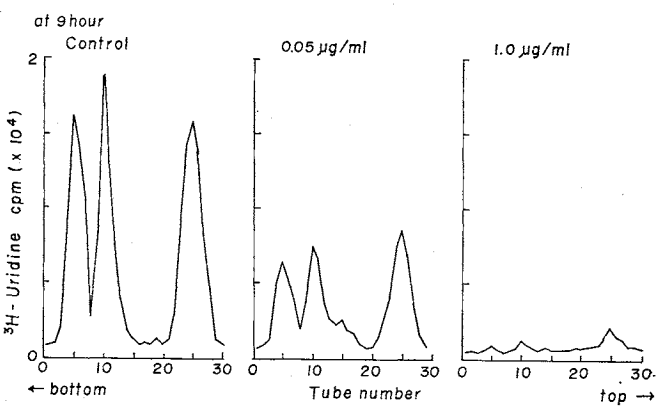


Fig. 6-E.



Discussion

Various temperature-sensitive mutants of *B. subtilis* in morphology have been prepared[2]. A mutant of *B. subtilis* whose cellular division is sensitive to high temperature is known to recover its ability to divide at high temperature after addition of extract of the wild strain[3]. This indicates that some product present in the wild strain is absent in the mutant. Tunicamycin inhibited cell division of a few strains of *B. subtilis*. Thus tunicamycin may offer another tool, in addition to conditional mutants, for elucidation of the mechanism of cell division in *B. subtilis*.

Tunicamycin affected the syntheses of protein, RNA and DNA in *B. subtilis* W 23 slightly, but incorporation of glucosamine into the acid-insoluble macromolecular fractions was strongly inhibited. A similar effect of the antibiotic was observed with animal cells, and it was found that tunicamycin interferes with membrane synthesis, especially the plasma membrane rather than the endoplasmic reticulum (ref. 4 and manuscript in preparation). Morphological changes of *B. subtilis* cells by tunicamycin with little effect on the biosyntheses of protein, RNA and DNA suggest that a similar inhibition of cell envelope synthesis takes place in microorganisms as in animal cells. Glucosamine is one of the main constituents of the cell wall but not of the cell membranes of bacteria. Appropriate precursors of the cell membranes of bacteria are not known at present, and the separation of the cell membranes is required for the analysis of the effect of tunicamycin on membrane synthesis. Such studies are now in progress.

Significant degradation of RNA was observed in morphologically changed *B. subtilis* W 23 cells in the presence of tunicamycin regardless of the lysis of cells. Degradation of DNA was detected at nearly the same time as that of RNA, but it was significant only when cells lysed. Colicins are known to induce degradation of DNA and RNA[5]. Colicins

exert their effect by adsorbing onto sensitive bacterial cells, and the membrane plays an important role in this case. The nature of the degradation of RNA induced by colicin E_2[6] differs from that induced by tunicamycin. On challenge with colicin E_2 both 23S and 16S ribosomal RNA were degraded promptly. No normal-sized RNA peaks were detected and a very heterogenous radioactivity region was observed between the 23S and 4S RNA regions.

Acknowledgement

This investigation was financially supported by grant-in-aid from the Waksman Foundation of Japan.

References

1) TAKATSUKI, A.; K. ARIMA & G. TAMURA: Tunicamycin, a new antibiotic. I. Isolation and characterization. J. Antibiotics 24: 215~223, 1971
2) ROGERS, H. J.: Bacterial growth and the cell envelope. Bacteriol. Rev. 34: 194~214, 1970
3) MACH, F.: Thermosensitive filamentous mutant of *Bacillus subtilis* non-affected in processes of DNA and protein synthesis. in Abstract of Xth International Congress for Microbiology, Mexico, 1970, p. 12
4) TAKATSUKI, A. & G. TAMURA: Effect of tunicamycin on the synthesis of macromolecules in cultures of chick embryo fibroblasts infected with Newcastle disease virus. J. Antibiotics 24: 780~789, 1971
5) NOMURA, M.: Colicins, in "Antibiotics, vol. 1, Mechanism of Action" p. 696 (D. GOTTLIEB & P. D. SHAW ed.) Springer-Verlag, 1967
6) YANAI, A.; T. BEPPU & K. ARIMA: Nature of DNA and RNA degradation in *Escherichia coli* induced by colicin E_2. Agr. Biol. Chem. 34: 149~151, 1970