MODE OF ACTION OF TOMAYMYCIN

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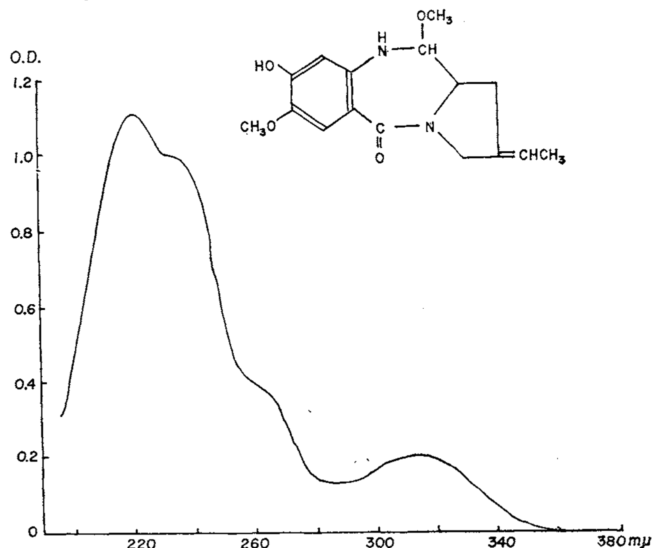
(Received for publication July 17, 1972)

The mode of action of tomaymycin (TM) which is a new antibiotic with antitumor activity was studied. Inhibition of the incorporation of labeled precursors into the acid-insoluble fraction of *Bacillus subtilis* cells indicated that TM inhibits nucleic acids biosynthesis, while having relatively little effect on protein synthesis. TM displaced methyl green (MG) from a MG-DNA complex and a TM-DNA complex was isolated by gel filtration chromatography. The formation of the TM-DNA complex resulted in (1) shifts of UV absorption profiles of TM and the corresponding difference spectra, (2) increase in the melting temperature of DNA, (3) protection of native DNA from degradation by nuclease O. These findings are compatible with the proposition that TM exerts its action as a growth inhibitor by forming a complex with DNA which in turn prevents the DNA from participating as a template in the biosynthesis of nucleic acids.

A new antitumor antibiotic tomaymycin (TM) was isolated from the culture filtrate of *Streptomyces achromogenes* var. *tomaymyceticus* by K. ARIMA *et al*[1]. and its structure was determined[2] (Fig. 1). In addition to antiphage and antibacterial activity, TM has a strong inhibitory effect on Leukemia L 1210 cells. We report in this paper that TM forms a complex with DNA. This conclusion was obtained by studying the physicochemical effects of TM on DNA in various *in vitro* systems.

Fig. 1. Structure and UV spectrum of tomaymycin.



Materials and Methods

Materials

TM was donated from Dr. H. SAKAI, Fujisawa Pharmaceutical Co., Ltd., Osaka. ^{3}H-TM was obtained from Shin-Roihi Co., Ltd., Kamakura. Uracil-2-^{14}C, thymine-2-^{14}C and L-methionine-$^{14}CH_3$S were purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo.

DNA was extracted from *Escherichia coli* B by the

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method of MARMUR[7]. The purity of DNA was confirmed by the absorption ratios at 260 mμ, 230 mμ and 280 mμ which were found to be 1 : 0.468 : 0.530.

Bacillus subtilis Marburg strains 168 (thy⁻, met⁻) and W 23 (ade⁻, met⁻) were provided by Dr. H. SAITO., Institute of Applied Microbiology, Tokyo.

Methods

Effect of TM on the biosynthesis of DNA, RNA and protein

Cells of *B. subtilis* 168 grown in a synthetic medium (10 % glucose, 0.3 % K_2HPO_4, 0.1 % KH_2PO_4, 0.01 % $MgSO_4$, 0.05 % sodium citrate, 1.5 % $(NH_4)_2SO_4$, 1 mcg/ml biotin and vitamin B_1, 50 mcg/ml of thymine and methionine) with shaking at 37°C and collected in early log phase, washed and starved of methionine for 30 minutes in the same medium and then suspended in medium to 1×10^9 cells per ml. This cell suspension was used for the incorporation of labeled precursors.

Displacement of methyl green from the methyl green-DNA complex

At pH 7.5, the methyl green (MG)-DNA complex is intensively green, whereas unbound MG is colorless.[4] Therefore, a compound which displaces MG from its complex with DNA decreases the intensity of the green-colored solution. The effect of TM on the MG-DNA complex was studied according to the method of BATES *et al*[5].

Change in melting temperature of DNA

The melting temperature of DNA was determined in Gilford 240 Spectrophotometer by the method of MANDEL and MARMUR[6] with the modification that 0.01×SSC (0.0015 M NaCl +0.00015 M sodium citrate) was used instead of 1.0×SSC. The temperature in the cuvette chamber was increased at a rate of 5°C per hour.

Effect of TM on nuclease O

Nuclease O, which is an endonuclease produced by *Aspergillus oryzae*, was presented by Dr. T. UOZUMI of this department and nuclease O-catalyzed DNA degradation was performed as described by T. UOZUMI[8].

DNA degradation induced by TM

Cells of *B. subtilis* 168, prelabeled with ^{14}C-thymine were washed twice and starved for 30 minutes. The cells were suspended in the medium described above to make 1×10^9 cells per ml and 50 mcg/ml of TM was added to the suspension. The radioactivity appearing in trichloroacetic acid-soluble fraction was measured in a liquid scintillation counter.

Results

1. Effect of TM on the Growth of Bacteria

When TM was added to a growing culture of *B. subtilis* W 23, optical density continued to increase for 2 or 3 hours and then a rapid cell lysis was observed (Figs. 2 and 3 a), lysis was not induced in *E. coli* B (Fig. 3 b). Fig. 3 shows that TM has a strong bactericidal effect on both *B. subtilis* W 23 and *E. coli* B and 99.9 % of cells were killed within 30 minutes.

2. Effect of TM on the Biosynthesis of DNA, RNA and Protein

As shown in Fig. 4, incorporation of ^{14}C-thymine and ^{14}C-uracil into the acid-insoluble

Fig. 2. Influence of TM on the growth of *B. subtilis* W 23.

Cells were incubated in a nutrient broth (1 % peptone, 1 % meat extract, pH 7.2) with shaking at 37°C. The absorbancy at 550 mμ was measured.

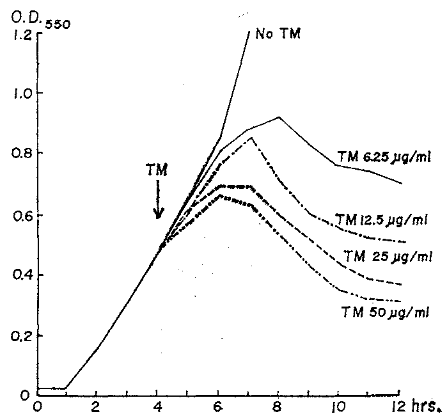


Fig. 3. Influence of TM on the growth of (a) *B. subtilis* W 23 and (b) *E.coli* B.
Cells were incubated in a nutrient broth (1% peptone, 1% meat extract, pH 7.2) at 37°C. The number of living cells was counted by the dilute plating method.

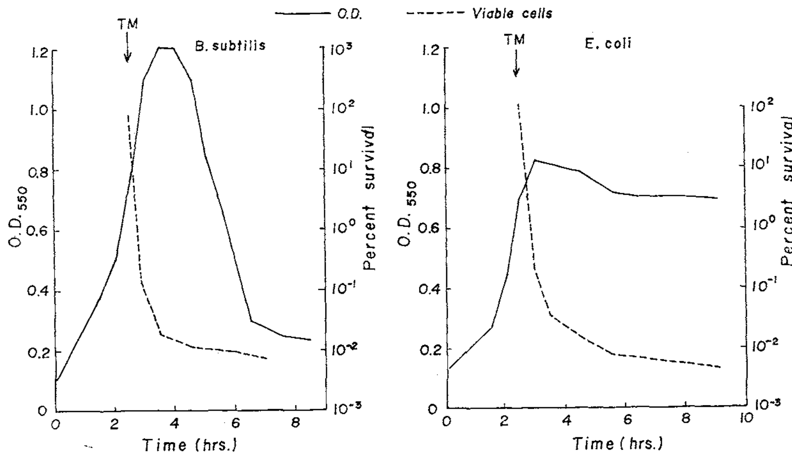
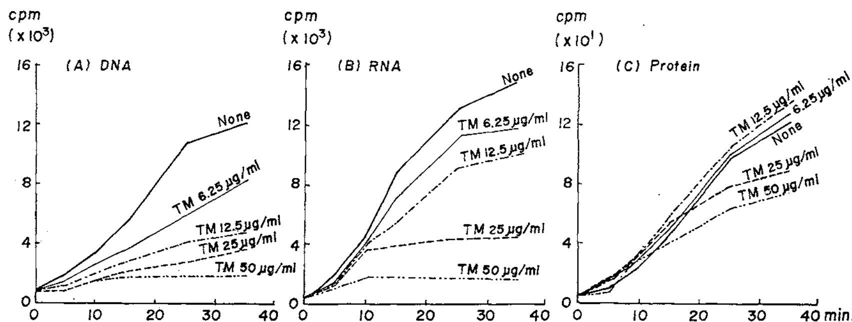


Fig. 4. Effect of TM on the synthesis of DNA, RNA and protein.
After the cell suspension described in Methods was preincubated for 20 minutes at 37°C, TM and (A) ^{14}C-thymine (0.1 μCi/ml), (B) ^{14}C-uracil (0.1 μCi/ml) or (C) ^{14}C-methionine (0.2 μCi/ml) were added. The incorporation was terminated by adding 1 ml of 10% TCA to 1 ml of the reaction mixture. The precipitate was collected on Millipore filter and washed three times with 5 ml of 5% TCA and the radioactivities were counted by a gas flow counter.

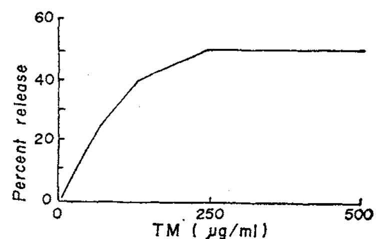


fraction of *B. subtilis* 168 was strongly inhibited by TM at a concentration of 25 mcg/ml. Incorporation of ^{14}C-methionine was reduced only slightly at the same concentration. As this inhibitory pattern is common to antibiotics which bind to DNA[9], these results suggested, as one of several possibilities, that TM binds to DNA and prevents the DNA from functioning as a template in the biosynthesis of nucleic acids.

3. Displacement of Methyl Green from the MG-DNA Complex

The specific inhibition of DNA and RNA synthesis in *B. subtilis* 168 implied that TM might interfere with DNA primer function. With this

Fig. 5. Release of MG from an MG-DNA complex.
A solution containing the MG-DNA complex was prepared by placing 15 mg of the MG-DNA compound into 100 ml of 0.05 M Tris-HCl buffer (pH 7.5). The assay was carried out by incubating 4 ml of the MG-DNA solution and 1 ml of the drug solution for 24 hours at 20°C in the dark. The absorbancies of the solution at 640 mμ were measured spectrophotometrically.



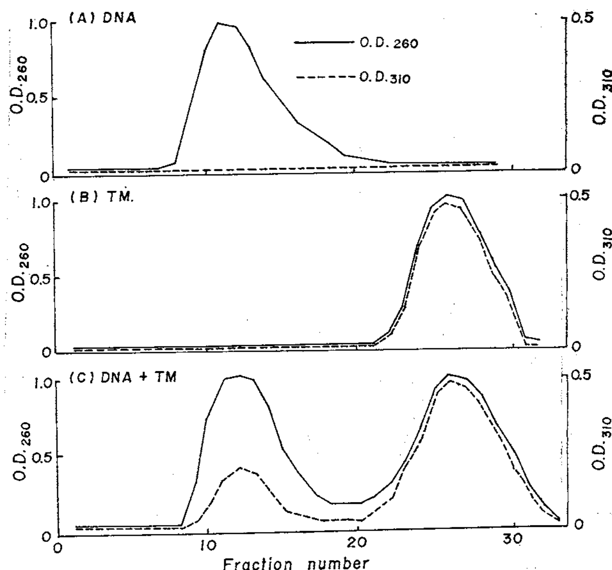
in mind, we looked for evidence of a direct interaction between TM and DNA. MG forms a complex with DNA and compounds, which bind to DNA, *e.g.* quinacrine[10], have been reported to displace MG from its complex with DNA. Fig. 5 shows that TM was effective in displacing about 50 % of the MG from the MG-DNA complex. This result strongly suggests that TM bind to DNA.

4. Isolation of the TM-DNA Complex by Gel Filtration Chromatography

In order to isolate the TM-DNA complex, gel filtration chromatography on Sephadex G 50 was performed. The elution profiles are shown in Fig. 6. The binding ratio is one molecule of TM per 13 nucleotide pairs. The elution profile with ^{3}H-TM is shown in Fig. 7 and the binding ratio is one molecule of TM per 16 nucleotide pairs.

Fig. 6. Isolation of the TM-DNA complex by gel filtration chromatography on Sephadex G-50.

A, B and C are the elution profiles at pH 7.2 on Sephadex G-50 columns (30 mm × 350 mm) for DNA (2 mg), for TM (1 mg) and for a mixture containing the same quantities of DNA and TM that had been incubated for one hour at 26°C, respectively. The materials were dissolved in 2.0 ml of 0.1 × SSC, placed on the columns and eluted with the same solution. Fractions of 2.0 ml were collected (flow rate = 5 ml/hour) and the elution was followed by measuring the absorbancies at 260 mμ for DNA and at 310 mμ for TM.



5. Optical Studies of the TM-DNA Complex

The interaction of TM with DNA was accompanied by a change in a TM absorption peak which shifted from 310 mμ to 328 mμ (Fig. 8). Corresponding

Fig. 7. Elution profiles of a ^{3}H-TM-DNA complex on Sephadex G-50.

The procedure was the same as (C) in Fig. 6. except that ^{3}H-TM was used instead of TM. Radioactivity was determined in a liquid scintillation counter with PPO-dioxane system.

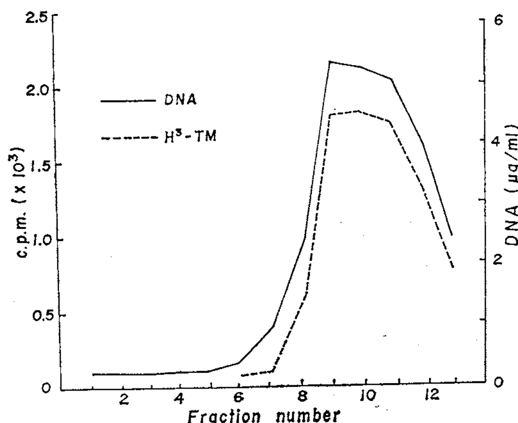


Fig. 8. Spectra of free and DNA bound TM.

A : 10 μg/ml of TM in 0.1 SSC, pH 7.2

B : TM-DNA complex isolated by gel filtration on Sephadex G-50.

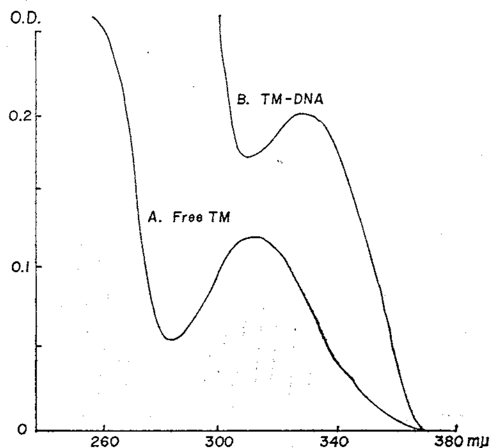


Fig. 9. Difference spectra

80 μg/ml of TM, 0.2 mg/ml of DNA, 0.1×SSC, pH 7.0.

(A) The difference spectra were obtained by placing, in one light-path, a cuvette containing a mixture of TM and DNA, and a cuvette containing 0.1×SSC; and in the other light-path, a cuvette containing TM and a cuvette containing DNA.

(B) The difference spectra at pH 3.5 and pH 10.0.

(C) The difference spectra in the presence of 10^{-3} M $CuSO_4$, $AgNO_3$ or $HgCl_2$.

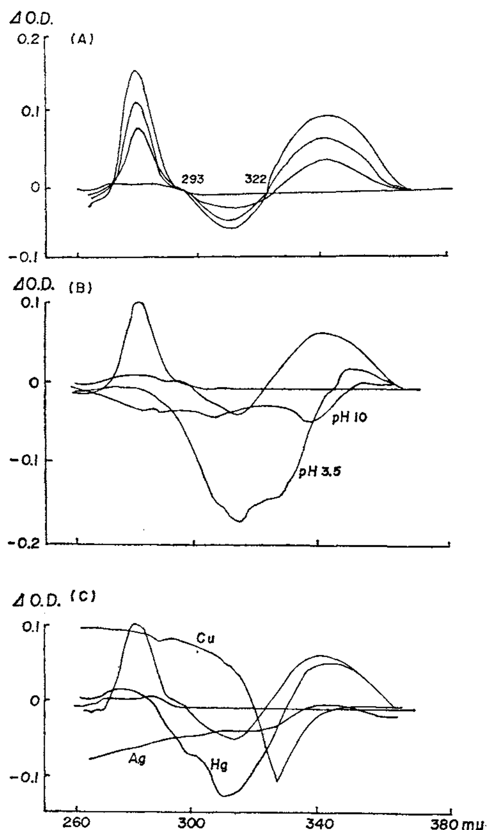
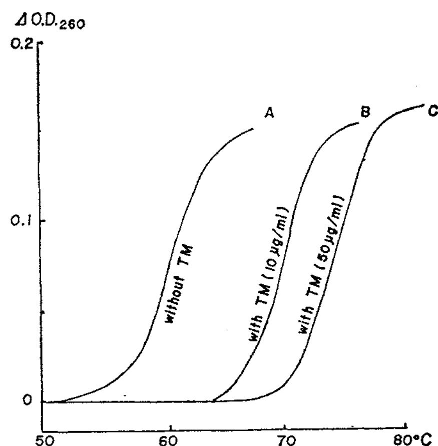


Fig. 10. Effect of TM on thermal denaturation of DNA.

In a glass-capped cuvette of 1 cm light-path, 1 ml of a test solution containing 0.01×SSC (0.0015 M NaCl, 0.00015 M Na-citrate), DNA (24 μg/ml) and TM was placed. Optical density at 260 mμ was read automatically in a Gilford 240 spectrophotometer against the above solution containing no antibiotic. The temperature in the cuvette chamber was increased at a rate of 5°C per hour.



difference spectra were observed which had isosbestic points at 293 mμ and 328 mμ (Fig. 9a). The difference spectrum altered in profile by a change of pH to 3.5 and 10.0 (Fig. 9b) or by an addition of metal ions such as Ag^{+}, Hg^{++} and Cu^{++} which have been reported to bind to DNA (Fig. 9c).

6. Increase of the Melting Temperature of DNA

Various antibiotics which bind to DNA, *e.g.* actinomycin D[11,12] and anthramycin[16], have been reported to increase the melting temperature of DNA, therefore it appeared desirable to determine whether TM might increase the resistance of DNA to the action of heat. As shown in Fig. 10, the melting temperature of *E. coli* DNA in 0.01×SSC was 61°C and it was raised to 74°C by the addition of TM. This result indicates that TM binds to DNA and makes the DNA double helical structure tighter and more resistant to heat denaturation.

7. Effect of TM on Nuclease O

BATES *et al.*[5] reported that anthramycin inhibits nuclease action *in vitro*, therefore the effect of TM on the enzymatic hydrolysis of DNA by nuclease O was studied. As shown in Fig. 11, nuclease O-catalyzed degradation of native *E. coli* DNA was strongly inhibited (up to 90 %) by TM, but nuclease O was not inhibited by TM when heat denatured DNA was used as a substrate. These results suggest that TM makes DNA double strands somewhat stable and insensitive to nuclease O.

Fig. 11. Inhibition of nuclease O by TM.

A mixture containing 1 mg/ml of *E. coli* DNA, 0.5 mM $MgCl_2$, 0.01 % gelatin, 0.15 M Tris-HCl (pH 7.7) and nuclease O (300 m units/ml) was incubated at 30°C. To follow the degree of DNA degradation, 0.5 ml of the mixture was sampled at appropriate time intervals and DNA was precipitated by addition of 0.1 ml of uranyl reagent (0.75 % uranyl acetate in 25 % perchloric acid) and centrifuged. Degradation products in the supernatant were measured by a spectrophotomer at 260 mμ. Degree of hydrolysis was expressed by percent solubility, taking O.D. value after comptete hydrolysis in 0.5 N perchloric acid at 90°C for 15 minutes as 100 %.

(A) Native DNA was used as a substrate.

(B) Heat denatured DNA was used as a substrate.

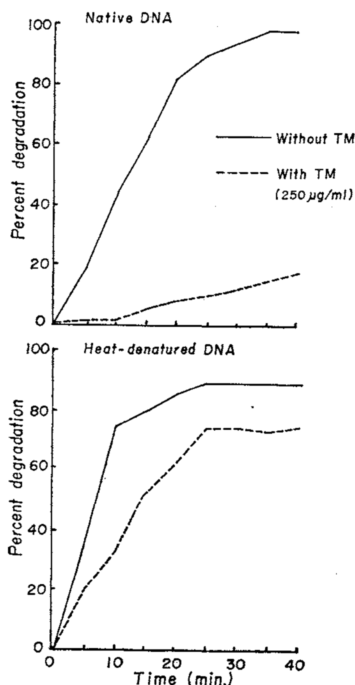


Fig. 12. DNA degradation by TM.

A) Cells of *B. subtilis* 168, prelabeled by C-thymine were washed twice and starved for 30 minutes. Then cells were suspended in the medium described in Methods to 1×10^9 cells per ml and 50 mcg/ml of TM (tomaymycin), MC (mitomycin C) or SM (streptomycin) was added to the suspension. The radioactivity in the acid-soluble fraction was determined in a liquid scintillation counter using a PPO-dioxane system.

B) Influence of temperature on DNA degradation by TM was studied by determining the radioactivity in acid-soluble fraction at one hour after TM was added.

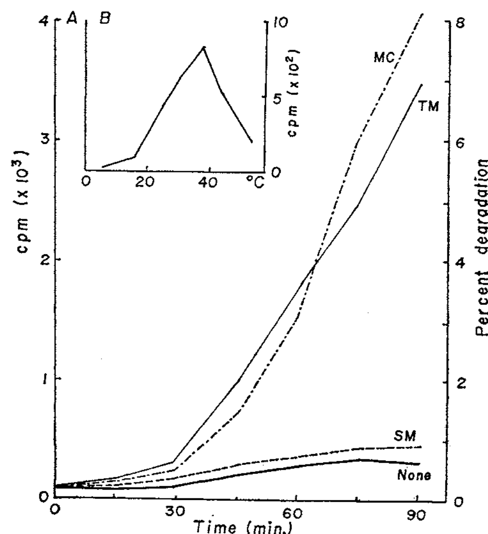
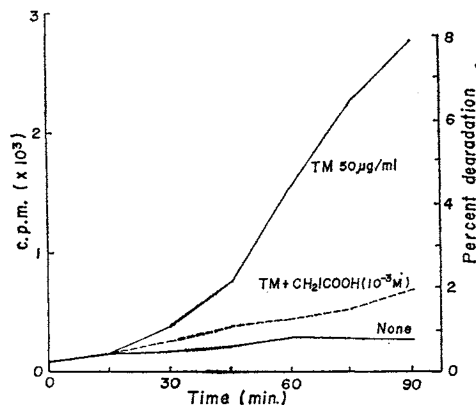


Fig. 13. Effect of iodoacetic acid on degradation of DNA by TM.

The procedure was the same as previously described.



8. DNA Degradation Induced by TM

Mitomycin C has been reported to degrade DNA.[13,14] We observed that the addition of TM to *B. subtilis* 168 whose DNA was previously labeled with ^{14}C-thymine also brought about DNA degradation and the radioactivity appeared in the acid-soluble fraction (Fig. 12). DNA degradation induced by TM was a slow reaction which could be detected 30 minutes after the addition of TM and only 7.5 % of the total DNA degraded at 90 minutes. This reaction was maximum at about 40°C and was strongly inhibited by 10^{-3} M of iodoacetic acid. These findings led us to think that TM first binds to DNA and then activates specific nucleases which excise those parts of DNA where TM attaches.

Discussion

KOHSAKA *et al*[3]. showed that T_3 phage treated with TM absorbed to *E. coli* B cells and injected their DNA into bacterial cells but the injected DNA did not serve as a template for RNA polymerase. Moreover, λ-DNA treated with TM was inactivated and consequently lost its transforming activity in KAISER's system[15]. They suggested that phage inactivation by TM resulted from the formation of biologically inactive complex between phage DNA and TM.

In this paper, evidence is presented which supports the assumption that TM forms a complex with DNA which prevents the DNA from participating as a template in the biosynthesis of nucleic acids.

TM has the same chromophore as anthramycin[2], an antitumor antibiotic, which was reported to form a complex with DNA[16,17]. Anthracylines, such as nogalamycin and daunomycin, are known to displace MG from the MG-DNA complex and increase the melting temperature of DNA[9]. As an intercalation model for daunomycin-DNA complex has been suggested[9], it is possible that TM also intercalates into DNA double strands.

Mitomycin C (MC) was reported to degrade DNA[13,14] and to have a cross-resistance relationship with UV irradiation[18,19,20]. MC is known to cause the formation of cross-linked DNA[21,22], whereas UV irradiation causes the formation of thymine dimers in DNA[23,24]. From studies on UV-sensitive mutants of bacteria, it was assumed that DNA damaged by exposure to MC or UV was repaired by a similar mechanism[20]. Our experiments show that TM also brought about DNA degradation in intact cells and this reaction is possibly catalyzed by a nuclease. We presume that TM, as well as MC, causes a local distortion in the DNA molecule which is then excised by the nuclease which removes pyrimidine-dimers formed by UV irradiation.

Ackowledgement

The authors are grateful to Dr. H. SAITO, Institute of Applied Microbiology, Tokyo for supplying *B. subtilis* 168 and W 23 and to Dr. T. UOZUMI of this department for nuclease O. We also wish to thank Dr. H. SAKAI, Research Laboratories of Fujisawa Pharmaceutical Co., Ltd., Osaka for tomaymycin and kind advices.

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