STUDIES ON A NEW ANTIBIOTIC M-92 PRODUCED BY *MICROMONOSPORA*

V. MECHANISM OF ACTION OF THE COMPONENT VA-2

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Effects of VA-2, a component of quinoid antibiotic M-92, on the incorporation of radioisotope-labeled compounds into the cells of *Staphylococcus aureus* were studied. Deoxyribonucleic acid (DNA) synthesis was immediately inhibited by the addition of VA-2. Significant inhibitions of ribonucleic acid and protein syntheses and minor reduction of peptidoglycan synthesis were observed after a short delay. VA-2 immediately induced the degradation of DNA prelabelled with [^{14}C]thymidine in the cells of *S. aureus*. In the examinations using *E. coli* enzyme and calf thymus DNA as a template, VA-2 prevented DNA-dependent DNA polymerase reaction. The inhibition of DNA polymerase I reaction was fairly reversed by increasing the concentration of template DNA, but slightly by that of the enzyme. Agarose gel electrophoresis showed that VA-2 elicited an extensive cleavage of PM2 cccDNA. VA-2 caused a primary conversion of the cccDNA to ocDNA at a low concentration (0.2 μg/ml), while at high concentrations (2.0 and 20 μg/ml) it cleaved the cccDNA to ocDNA and linear DNA progres sively. These cleavages were observed even at 0°C as well as at 37°C, and were enhanced with the addition of a reducing agent such as 2-mercaptoethanol or sodium borohydride.

VA-2, the most active component of antibiotic M-92, was isolated from culture broth and mycelia of *Micromonospora verruculosa* MCRL 0404[1,2]. VA-2 is an antibiotic having a quinoid nature[2] and affects many procaryotic and eucaryotic cells[3]. Because VA-2 somewhat resembles mitomycin C in the structural and biological properties, several experiments relating to the bactericidal action of VA-2 were carried out in parallel with mitomycin C. However, as already reported[4], VA-2 differs from mitomycin C in the following points: bactericidal action of VA-2 is biphasic kinetics, affected by cell concentration in culture and blocked by 2,4-dinitrophenol.

The unique bactericidal properties of VA-2 raise many interesting questions as to the mechanism of action of this antibiotic. This report concerns the mode of action of VA-2 in bacteria and also the interaction of VA-2 with PM2 phage DNA.

Materials and Methods

Antibiotics and Chemicals

VA-2 was separated and purified from the crude antibiotic M-92 preparation as described previously[2]. Other antibiotics were obtained from commercial sources. [^{14}C]Thymidine (56 mCi/mmole), [^{14}C]uridine (56 mCi/mmole), L-[U-^{14}C]phenylalanine (513 mCi/mmole) and D-[1-^{14}C]glucosamine (60.8 mCi/mmole) were purchased from The Radiochemical Centre, Amersham, Buckinghamshire. [Methyl-^{3}H]deoxythymidine 5′-triphosphate (83.2 Ci/mmole) was obtained from New England Nuclear, Boston, Mass. Deoxyribonucleoside triphosphates, *Escherichia coli* MRE600 DNA polymerase I and PM2 phage DNA were products of Boehringer Mannheim, Germany. Calf thymus DNA was purchased from Sigma Chemical Co., St. Louis. All other chemicals used were of reagent grade.

Microorganisms and Culture Conditions

Staphylococcus aureus 209P JC-1 maintained in our laboratory was grown in double strength nutrient broth (2NB, Difco) on a reciprocating shaker at 37°C. In these experiments bacterial cells in an early exponential stage were used, when absorbance at 600 nm showed approximately 0.2.

Incorporation of Radioactive Precursors into the Acid-insoluble Fraction of Growing Cells

DNA and RNA Syntheses: An exponentially growing culture was labelled with either [^{14}C]thymidine (0.1 μCi/ml) or [^{14}C]uridine (0.05 μCi/ml). After 15 minutes, the cultures were treated with various concentrations of VA-2. The incorporation of [^{14}C]thymidine and [^{14}C]uridine into trichloroacetic acid (TCA) precipitable material was measured, as described by HUGHES and MELLOWS[5]. The acid-insoluble precipitates were collected on a glass filter (Whatman, GF/C), washed with 5% TCA (2×6 ml) and ethanol (2×6 ml), dried and counted in 10 ml toluene containing *p*-bis-[2-(5-phenyloxazoyl)]benzene (0.3 g/liter) and 2,5-diphenyloxazole (4 g/liter) with an Aloca LSC-625 liquid scintillation spectrometer.

Protein Synthesis: Culture samples (0.5 ml) labelled with [^{14}C]phenylalanine (0.5 μCi/ml) were added to 10% TCA (6 ml), placed on ice for 30 minutes and finally heated at 90~95°C for 20 minutes. Then, the acid-insoluble precipitate was collected as described above for counting the radioactivity.

Peptidoglycan (cell wall) Synthesis: The uptake of D-[^{14}C]glycosamine into TCA precipitable material was measured, by a method similar to that described by HUGHES and MELLOWS[5]. Culture samples (0.5 ml) labelled with [^{14}C]glucosamine (0.1 μCi/ml) were added to 10% TCA (5 ml), whirled, placed on ice for 30 minutes and finally heated to 90~95°C for 20 minutes. On cooling, each sample was collected on a Whatman GF/C filter, washed successively with 5% TCA (3×5 ml) and ethanol (2×10 ml), dried and counted radioactivities.

Degradation of Intracellular DNA

Degradation of intracellular DNA prelabeled with [^{14}C]thymidine was examined as described by DAS, *et al*[6]. The exponentially grown cells in 2NB which was supplemented with [^{14}C]thymidine (0.1 μCi/ml) were washed with 0.9% physiological saline and resuspended in the original volume of non-radioactive medium. The suspension was incubated for 30 minutes at 37°C to ensure that the radioactive nucleotides were incorporated into cellular DNA. Then, the cell cultures were treated with different concentrations of VA-2. Culture samples (0.5 ml) were added to 20% chilled TCA (0.5 ml) and placed on ice for 30 minutes. The precipitate which contained radioactivity retained by cells was collected on a glass filter, washed successively with 25 ml of 10% TCA at 0°C, with 10 ml ethanol containing 2% potassium acetate at 0°C and diethyl ether at 0°C and counted. The filtrate (0.1 ml) containing the acid-soluble material was dried on a disc of filter paper (Whatman No. 1) and counted.

DNA-dependent DNA Polymerase Reaction

The reaction was assayed by conversion of [^{3}H]deoxythymidine 5′-triphosphate (TTP) into TCA insoluble material by the methods described by SETLOW[7]. *E. coli* DNA polymerase I was diluted with buffer comprising 67 mM potassium phosphate buffer (pH 7.4), 100 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol and 1 mg/ml bovine serum albumin and kept frozen in small portions at −20°C. Calf thymus DNA was dissolved in 10 mM tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 20 mM KCl, and kept frozen in small portions at −20°C. The polymerase reaction was examined in 0.3 ml of solution containing 67 mM potassium phosphate buffer (pH 7.4), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 33 μM each dATP, dCTP and dGTP, 33 μM (0.27 μCi/ml) [^{3}H]TTP, 50 μg/ml DNA and 2.5 units/ml enzyme. After addition of the enzyme, the reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by chilling and then 0.2 ml of 0.2 M $Na_4P_2O_7$ and 0.5 ml of 1 M $HClO_4$ were successively added to the reaction mixture. After the mixture had stood for 5 minutes at 4°C, the acid-insoluble precipitates were collected on a glass filter and washed with 0.1 M $Na_4P_2O_7$ in 1 M HCl (5×5 ml) and ethanol (3×5 ml) successively.

DNA Cleavage in Agarose Gel Electrophoresis

The covalently closed circular (ccc) DNA fraction of PM2 phage was separated from a commercial preparation of PM2 phage DNA (Boehringer Mannheim) by ethidium bromide - cesium chloride

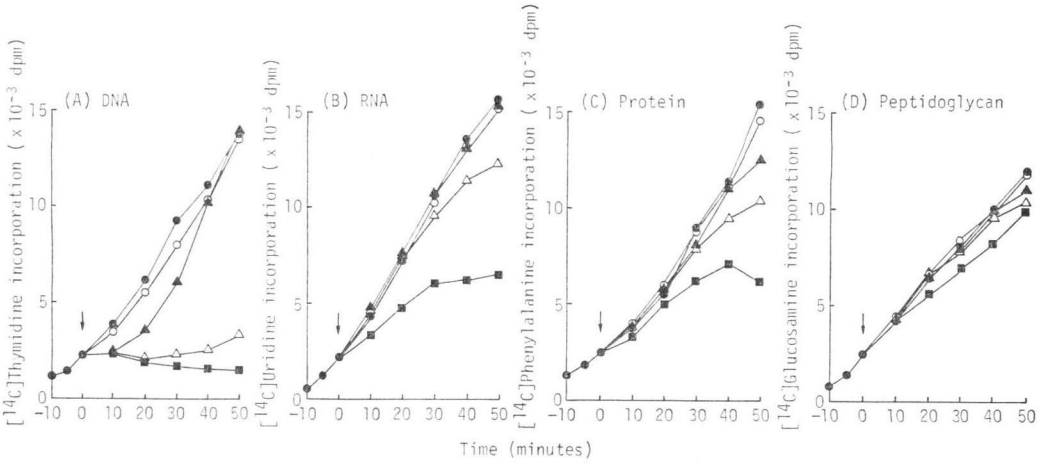
equilibrium density centrifugation and was dialyzed in 50 mM tris-HCl buffer (pH 7.6). The cleaving reaction of VA-2 against the cccDNA was carried out by the methods described by SUZUKI *et al.*[8] The reaction mixture containing 0.28 μg of PM2 phage cccDNA fraction in 25 μl of 50 mM tris-HCl buffer (pH 7.6) was incubated at 37°C or 0°C for 30 minutes in the presence or absence of an antibiotic and/or a reducing agent. Five microliters of a dye solution consisting of 0.025% bromophenol blue, 50% glycerol and 5% sodium dodecyl sulfate were added to the reaction mixture and 20 μl of the resultant mixture was applied into the well of 1% agarose (Seachem ME) gel slab. Electrophoresis was carried out at 20 volts for 16 hours in TEAS buffer (pH 8.0) comprising 50 mM tris-acetate, 20 mM sodium acetate, 2 mM disodium EDTA and 18 mM NaCl at room temperature. After electrophoresis, the gels were stained with TEAS buffer containing 0.5 μg/ml ethidium bromide and stained bands were photographed over UV light.

Results

Effect of VA-2 on Macromolecular Synthesis in the Cells of *S. aureus*

The overall syntheses of DNA, RNA, protein and peptidoglycan were measured in the presence of increasing concentrations of VA-2. As shown in Fig. 1 (A), addition of 0.1 μg/ml (final concentration) of VA-2 to growing *S. aureus* cells resulted in immediate and complete inhibition of DNA synthesis. At 0.01 μg/ml, the incorporation of [^{14}C]thymidine in the cells did not increase for about 30 to 40 minutes, and then seemed to increase. Addition of 0.001 μg/ml induced temporary and partial inhibition, while the incorporation of radioactivity was almost normal at 0.0001 μg/ml. It was interesting that concentrations exerting influences upon the DNA synthesis corresponded to those of VA-2 affecting the viability and growth of the cells which were reflected by turbidity, as observed when VA-2 was added to *S. aureus* growing cells[4]. Significant inhibition of RNA and protein syntheses [Fig. 1 (B) and (C), respectively] and a slight reduction of cell wall peptidoglycan synthesis [Fig. 1 (D)] were observed 20 to 30 minutes after exposure to VA-2 at the concentrations showing complete inhibition of DNA synthesis.

Fig. 1. Effect of VA-2 on DNA (A), RNA (B), protein (C) and peptidoglycan (D) syntheses in *S. aureus*.
An exponentially growing culture (25 ml, OD_{600nm} approximately 0.2) was labelled with either [^{14}C]-thymidine (0.1 μCi/ml), [^{14}C]uridine (0.05 μCi/ml), [^{14}C]phenylalanine (0.5 μCi/ml) or D-[^{14}C]glucosamine (0.1 μCi/ml). After 15 minutes, the culture was split into 5 ml aliquotes. One subculture was used as a control, whilst the others were treated with different concentrations of VA-2 (as indicated by the arrows). Samples (0.5 ml) were withdrawn and the acid (TCA)-precipitable radioactivity was determined.
● Control, ○ 0.0001 μg/ml, ▲ 0.001 μg/ml, △ 0.01 μg/ml, ■ 0.1 μg/ml.



DNA Degradation by VA-2 in the Cell of *S. aureus*

To elucidate the mechanism of the above inhibition of DNA synthesis, degradation of cellular DNA by VA-2 was studied with the cells of *S. aureus*. As shown in Fig. 2, VA-2 immediately induced marked degradation of DNA prelabelled with [^{14}C]thymidine. At 30 minutes after exposure to 0.1 μg/ml of VA-2, the amount of radioactivity remaining in TCA-precipitable fraction was about 70% of that of the control. The results suggested that the apparent decrease of [^{14}C]thymidine incorporation by VA-2 was not only due to the inhibition of DNA synthesis but also by the degradation of DNA.

Effect of VA-2 on DNA-dependent DNA Polymerase

Reaction with Isolated Enzyme

Inhibitory effect of VA-2 on DNA polymerase reaction examined with *E. coli* enzyme and calf thymus DNA as a template is illustrated in Fig. 3 together with those of reference antibiotics. VA-2 was more markedly active than mitomycin C and actinomycin D, but less than daunomycin. At 50 μg/ml (final concentration) of these antibiotics, inhibitory per cent in the reaction were: daunomycin 100%, VA-2 83%, actinomycin D 66%, mitomycin C 24%.

Fig. 2. Degradation of cellular DNA by VA-2.

S. aureus 209P JC-1 cells were grown in 2NB supplemented by [^{14}C]thymidine (0.1 μCi/ml). The exponentially grown cells were washed with 0.9% physiological saline and incubated for 30 minutes at 37°C in non-radioactive medium to ensure that the radioactive nucleotides were incorporated into cellular DNA. Then, the cells were treated with different concentration of VA-2. At indicated intervals, samples (0.5 ml) were withdrawn and the radioactivity retained by the cells was measured as the acid (TCA)-precipitable material. The filtrate (0.1 ml) containing the acid-soluble material was dried on a disc of filter paper (Whatman No. 1) and the radioactivity was measured.

[^{14}C]Thymidine in acid-precipitable fraction of cells: ● Control, ▲ 0.01 μg/ml, ■ 0.1 μg/ml.

[^{14}C]Thymidine in acid-soluble fraction of cells: ○ Control, △ 0.01 μg/ml, □ 0.1 μg/ml.

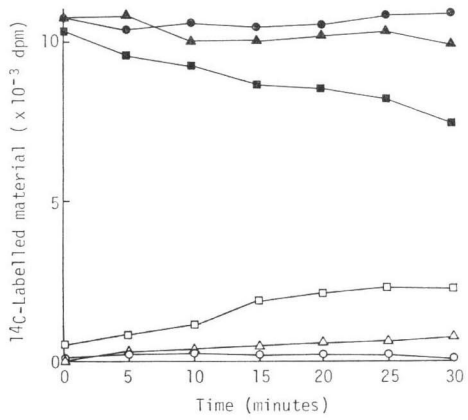
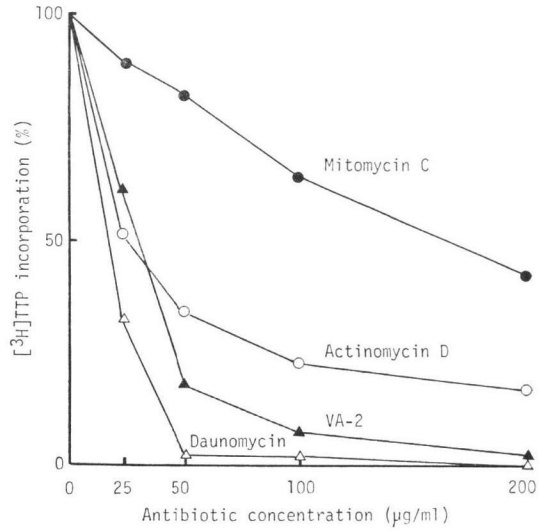


Fig. 3. Effects of VA-2 and reference antibiotics on DNA-dependent DNA polymerase reaction.

The reaction mixtures (0.3 ml) comprising 67 mM potassium phosphate buffer (pH 7.4), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 33 μM [^{3}H]TTP (0.27 μCi/ml), 33 μM each of three dNTPs, 50 μg/ml calf thymus DNA, 2.5 units/ml *E. coli* DNA polymerase I and various concentrations of antibiotics were incubated for 30 minutes at 37°C.



The inhibitory activities in the DNA polymerase I reaction were determined at various concentrations of either the template DNA or the enzyme. As shown in Fig. 4, the inhibition of DNA polymerase I activity by VA-2 was reversed by increasing the amount of DNA. The inhibition of DNA polymerase

Fig. 4. Inhibition of DNA polymerase reaction by VA-2 at different concentrations of primer DNA.

The reaction mixtures (0.3 ml) comprising 67 mM potassium phosphate buffer (pH 7.4), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 33 μM [^{3}H]TTP (0.27 μCi/ml), 33 μM each of other three dNTPs, 2.5 units/ml *E. coli* DNA polymerase I, different concentrations of calf thymus DNA together with 50 μg/ml of or without (control) VA-2 were incubated for 30 minutes at 37°C.

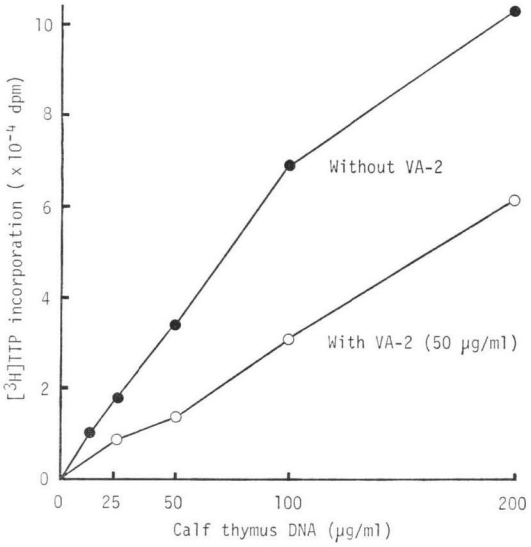
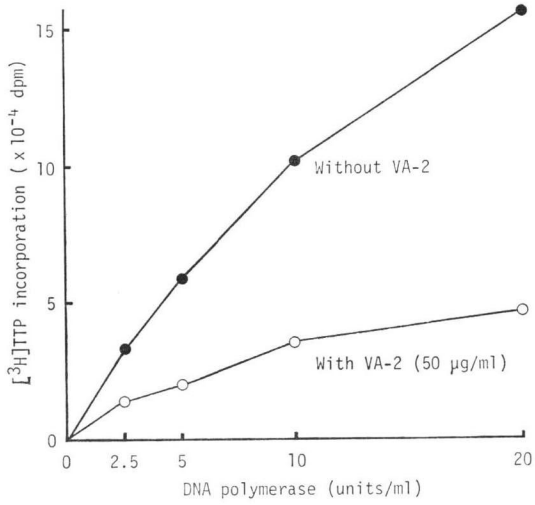


Fig. 5. Inhibition of DNA polymerase reaction by VA-2 at different concentrations of DNA polymerase.

The reaction mixtures (0.3 ml) comprising 67 mM potassium phosphate buffer (pH 7.4), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 33 μM [^{3}H]TTP (0.27 μCi/ml), 33 μM each of other three dNTPs, 50 μg/ml calf thymus DNA, different concentrations of *E. coli* DNA polymerase I together with 50 μg/ml of or without (control) VA-2 were incubated for 30 minutes at 37°C.



I reaction was also reversed by elevating concentrations of the enzyme (Fig. 5), but the reversible effect was not so strong as that of DNA concentration shown above. The results suggested that VA-2 directly interacted with DNA, resulting in the inhibition of DNA polymerase reaction.

Interaction of VA-2 with PM2 Phage DNA

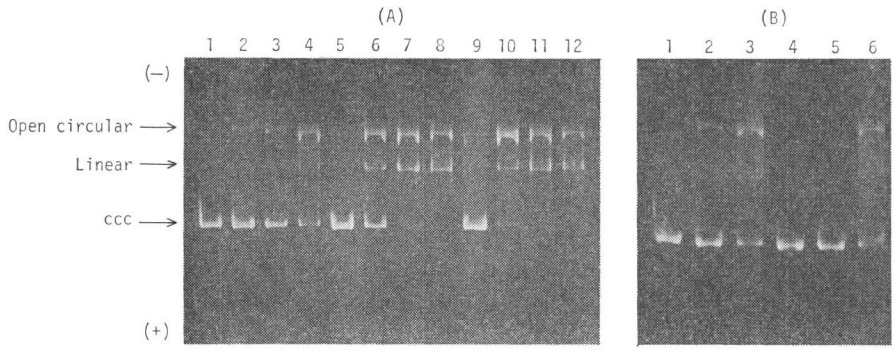
DNA-cleaving activity of VA-2 on PM2 phage cccDNA was examined by agarose gel electrophoresis. Cleavage patterns of the cccDNA with different concentrations of VA-2 at 37°C in the presence or absence of 2-mercaptoethanol were illustrated in Fig. 6 (A). The primary conversion of the cccDNA to open circular(oc) DNA was induced with the low concentration VA-2 (0.2 μg/ml: channel 2), whereas with high concentrations (2 and 20 μg/ml), more progress cleavages of the cccDNA to ocDNA and linear DNA occurred (channels 3 and 4). The DNA-cleaving activity of VA-2 was enhanced by the addition of 2-mercaptoethanol (channels 5~8), which was further increased with increasing amounts of the reducing agent (channels 10~12). As can be seen in channels 4 and 6, 0.2 μg/ml of VA-2 with 1 mM of 2-mercaptoethanol was as active as 20 μg/ml of VA-2 without the reducing agent. Sodium borohydride was found to be also effective for the enhancement of the activity of VA-2 (data are not shown). As shown in Fig. 6 (B), VA-2 gave a similar level of the DNA-cleaving activity at 0°C to that of 37°C.

Fig. 6. Agarose gel electrophoresis of PM2 phage DNA treated with VA-2 and effect of 2-mercaptoethanol on the activity of VA-2.

Electrophoresis in TEAS buffer (pH 8.0) through 1% agarose gel was conducted at 20 volts for 16 hours. PM2 phage cccDNA was incubated with VA-2 for 30 minutes at 37°C except for Nos. 4, 5 and 6 of plate (B) in the presence or absence of a reducing agent.

(A) 1: Control DNA, 2: +0.2 μg/ml VA-2, 3: +2 μg/ml VA-2, 4: +20 μg/ml VA-2, 5: +1 mM 2-mercaptoethanol, 6: +1 mM 2-mercaptoethanol and 0.2 μg/ml VA-2, 7: +1 mM 2-mercaptoethanol and 2 μg/ml VA-2, 8: +1 mM 2-mercaptoethanol and 20 μg/ml VA-2, 9: +10 mM 2-mercaptoethanol, 10: +10 mM 2-mercaptoethanol and 0.2 μg/ml VA-2, 11: +10 mM 2-mercaptoethanol and 2 μg/ml VA-2, 12: +10 mM 2-mercaptoethanol and 20 μg/ml VA-2.

(B) 1: Control DNA, 2: +2 μg/ml VA-2, 3: +20 μg/ml VA-2, 4: Control DNA, at 0°C, 5: +2 μg/ml VA-2, at 0°C, 6: +20 μg/ml VA-2, at 0°C.



Discussion

It was demonstrated that VA-2 immediately and completely blocked the incorporation of [^{14}C]-thymidine into the acid-precipitable fraction in the cells of *S. aureus*. At the same concentration, this antibiotic markedly prevented the incorporation of [^{14}C]uridine and [^{14}C]phenylalanine 20 to 30 minutes after treatment, but the inhibition seemed to occur as the result of stopping the DNA synthesis in the cells. VA-2 induced degradation of cellular DNA in *S. aureus*, which initiated immediately after addition of VA-2. These results suggest that the inhibition of [^{14}C]thymidine uptake by VA-2 attributed to the degradation of cellular DNA which acts as the chemoreceptor of VA-2. The inhibition of VA-2 on DNA polymerase I activity was reversed by increasing amounts of calf thymus DNA as a template in the reaction mixture. The results observed in the experiments suggest also that DNA polymerase reactions may be prevented as the result of the degradation of template DNA.

The results obtained by agarose gel electrophoresis revealed that the treatment of PM2 phage cccDNA with VA-2 (2~20 μg/ml) resulted in a cleavage of the cccDNA to ocDNA and linear DNA at 37°C. Under the experimental conditions employed, the degree of the cleavage was increased with increasing concentrations of VA-2 but was independent of the incubation temperature. Further, the activity of VA-2 was enhanced by the addition of a reducing agent such as 2-mercaptoethanol or sodium borohydride.

The chromophores obtained by methanol extraction from the proteinous antibiotics, auromomycin, macromomycin and neocarzinostatin have been reported to cause a similar type of DNA-cleaving activity to that of VA-2[9,10]. Among them, the chromophores of auromomycin and macromomycin were shown to exhibit PM2 DNA-cleaving activity without the presence of a reducing agent and the activity was stimulated by the addition of a reducing agent[9,10]. Furthermore, it was reported that the activity shown by auromomycin is independent of incubation temperature[8]. In contrast to these, the chromophore from neocarzinostatin was reported to give its PM2 DNA-cleaving activity only when a reducing agent such as 2-mercaptoethanol is present. The results described above suggested that the characteristics of DNA-cleaving activity of VA-2 is similar to that of the chromophore from auromomycin or macromomycin, but the conclusion must be await further experiments including direct com-

parisons with these compounds. The mechanism involved in the DNA-cleaving reaction of their chromophores has not yet been established.

On the other hand, the interaction of mitomycin C having a typical quinoid structure with PM2 phage cccDNA was examined in parallel with VA-2 in this study. Mitomycin C, however, did not exhibit any cleaving activity over the concentration range between 0.2 and 200 μg/ml and even with the presence of 10 mM 2-mercaptoethanol.

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