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Inhibition of Multiplication of Coliphage-T2 by Actinomycin

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SUMMARY

The effect of actinomycin, a polypeptide antibiotic, on the multiplication of bacteriophage T2 was studied. At the concentrations tested, actinomycin neither prevented the growth of *Escherichia coli* B, the host bacterium of the phage, nor inactivated free phage particles. The antibiotic inhibited the multiplication of the phage without interfering with lysis or synthesis of deoxyribonucleic acid and protein. The properties of the deoxyribonucleic acid liberated into the actinomycin-lysate were studied. The facts that it was non-sedimentable on high-speed centrifugation, non-precipitable by antiphage serum and sensitive to deoxyribonuclease, indicate that the phage deoxyribonucleic acid produced in the presence of actinomycin is not assembled in a coat of protein. The serum-blocking power test was performed to ascertain whether phage-related protein was produced by phage-infected bacteria incubated with actinomycin. Evidence was obtained that a lysate exposed to the antibiotic contained as much phage-related material with serum-blocking power as a control lysate. It was, therefore, concluded that actinomycin exerted its inhibitory action on phage multiplication at the stage of their maturation.

A complex formed by actinomycin and deoxyribonucleic acid isolated from phage particles, or from phage-infected cells, was demonstrated by zone-electrophoresis. The inhibition of maturation caused by actinomycin is thought to be due to the formation of an antibiotic-phage deoxyribonucleic acid complex.

INTRODUCTION

It is well known that actinomycin is one of a series of polypeptide antibiotics and possesses cytostatic effects on various organisms. Recently, evidence has been obtained by biochemical or biological means that it forms a complex with deoxyribonucleic acid (DNA) but not with ribonucleic acid (RNA) or protein. Kirk (1960) has shown that DNA isolated from various sources has not only an antagonistic effect on the inhibitory action of actinomycin on a biochemical reaction, but also changes the absorption spectrum of the antibiotic, and that the latter inhibits the transformation of *Haemophilus influenzae* Graciae from sensitivity to resistance to streptomycin, when it is added to transforming DNA of a resistant strain. Studing the action of nucleic acids on the inhibition of growth of *Neurospore crassa* caused by actinomycin, Kernsten *et al.* (1960) suggested that actinomycin reacted directly with DNA, oligonucleotides of DNA and to a much lesser extent with RNA, perhaps by formation of dye-polymer complexes. Kawamata and Imanishi (1960, 1961) were able to

isolate a DNA-actinomycin complex by zone-electrophoresis, which was formed *in vitro* or within bacterial cells whose growth was inhibited by the antibiotic. They assumed that the abnormal metabolism of DNA in a wide variety of organisms as a result of its interaction with actinomycin caused the overall effects of the antibiotic.

In view of the role of DNA in organisms, it seemed of interest to study the action of actinomycin on the reproduction of bacteriophages, because the bacteria-bacteriophage system offers many advantages for study of the interaction of various agents with nucleic acids.

The present paper, part of which has been reported briefly by Nakata *et al.* (1961), describes in detail studies on the effect of actinomycin on the multiplication of bacteriophages.

MATERIALS AND METHODS

1. *Strains of bacteriophage and bacteria*

The bacteriophage strain studied was T2r, rapid-lysing mutant of T2, and *Escherichia coli* strain B(H) was used as the host bacterium. Both were obtained from Dr. M. Sekiguchi of Kanazawa University.

2. *Actinomycin*

The actinomycin used in this study was crystalline actinomycin S (Kawamata and Fujita, 1960) supplied by Daiichi Seiyaku Pharmaceutical Co. Ltd. Because of the water insolubility of this antibiotic, the stock solution (1 mg/ml) was prepared by adding 0.2 ml of acetone to 10 mg of the antibiotic and then 9.8 ml of distilled water to this solution. It was then stored in a refrigerator.

3. *Experimental conditions*

Most of the experiments were performed as follows; the bacteria inoculated into a glucose-salts synthetic medium containing (per liter) 2.5 g of $(NH_4)_2HPO_4$, 1.5 g of KH_2PO_4, 5 g of NaCl, 3 g of sodium glutamate, 3 g of glucose, and 0.1 g of $MgSO_4.7H_2O$ at pH 7.0, was incubated overnight at 37°C. After diluting the culture in fresh medium, the cells were further incubated at 37°C with mechanical shaking in a water bath. Growth was followed by measuring the change in optical density at 660 mμ. When the bacteria, growing logarithmically, had attained an optical density of 0.3, corresponding to 5×10^8 cells/ml, they were harvested and washed once with glucose-free salts synthetic medium and then suspended in a similar medium. T2 bacteriophage was added to the cell suspension (5×10^8 cells/ml) at various multiplicities of infection, as described in each experiment. After 5 minutes incubation to allow adsorption, the mixture was diluted into medium containing glucose and antibiotic and then cultured with shaking at 37°C for the desired time.

The other techniques employed were standard phage methods (Adams, 1950).

4. *Analytical methods for nucleic acids and protein*

For the determination of nucleic acids and protein, the following procedures wer carried out; an aliquot of the bacterial suspension or phage lysate was chilled in an ice water bath and rapidly acidified by adding 10 N perchloric acid to give a final concentration of 0.5 N. After standing for 30 minutes in the cold, the insoluble residue was collected by centrifugation and then heated with 0.5 N perchloric acid at 90°C for 15 minutes in order to extract nucleic acids. The amount of DNA and RNA in the extracts was determined according to the methods of Burton (1956) and Mejbaum (1939), respectively. The residue remained was dissolved in a small volume of 1 N NaOH, and protein was determined by the method of Lowry *et al.* (1951).

5. *Serum-blocking power test*

The serum-blocking power of the lysates was tested by a modification of De Mars' method (1953), details of which are described in the text.

6. *Method of zone-electrophoresis*

For zone-electrophoresis, a plastic cell (30 mm × 10 mm × 400 mm) of horizontal type was used. The electrophoresis was performed in borate buffer, pH 8.3, ionic strength 0.1 with potato starch as the supporting substance. After running for approximately 17 hours at a constant current of 3 mA/cm^2 at 5°C, the starch was cut into 1 cm sections. Then each section was eluted with 0.1 M NaCl. The resulting eluates were analysed for nucleic acids and actinomycin.

RESULTS

1. *Effect of antibiotic on growth of the host bacteria*

The growth of *E. coli* B was followed by parallel observations of the increase in optical density at 660 mμ and of colony forming viable cells after addition of the antibiotic. Some 2 to 3 divisions of the bacteria occurred without any delay in the division time at concentrations of antibiotic up to 100 μg/ml. Exponentially growing cells exposed to a concentration of 30 μg/ml of actinomycin for 3 hours had the same ability to produce active phage particles on infection as cells grown in the absence of the antibiotic.

The synthesis of nucleic acids and protein in the bacteria also occurred normally in the presence of actinomycin (Table 2).

2. *Effect of antibiotic on the multiplication of the bacteriophage*

Addition of actinomycin to T2 phage-infected cells resulted in the inhibition of phage multiplication, which was progressively impaired by increasing concen-

Table 1. Effect of Actinomycin on the Multiplication of T2r Bacteriophage

The bacteria suspended in a glucose-free salts synthetic medium at a concentration of 5× 10^8 cells/ml were infected with T2r phage at a multiplicity of infection of 0.2. After 5 minutes adsorption, a sample was diluted two thousandfold and put into growth tubes containing a glucose-salts synthetic medium with various concentrations of actinomycin, as shown in the table. The mixtures were then incubated at 37° C for 60 minutes with shaking. The infective centers in each tube were assayed.

Time of incubation (min.)	Concentration of actinomycin (μg/ml)	Titer of phage (/ml)	Increase in infectivity (% of control)
0	0	7.4×10^4	——
60	0	5.7×10^6	100
60	1	2.6×10^6	45
60	3	1.7×10^6	29
60	10	6.8×10^5	12
60	30	5.8×10^5	10
60	100	2.5×10^5	4

trations of the antibiotic. One step growth experiments were performed in a glucose-salts synthetic medium containing various concentrations of actinomycin. Table 1 shows the results of one of the experiments, in which re-adsorption of the first growth progeny on uninfected bacteria or bacterial debris was avoided by dilution. Below a level of 30 μg/ml of the antibiotic, about 90 % of the multiplication of the phage was prevented as judged from the antibiotic-free control. At a concentration of 100 μg/ml, the antibiotic almost completely inhibited the reproduction of bacteriophage. The data from another type of experiment of this kind are plotted in Fig. 1. Little difference in the latent periods was observed, whereas

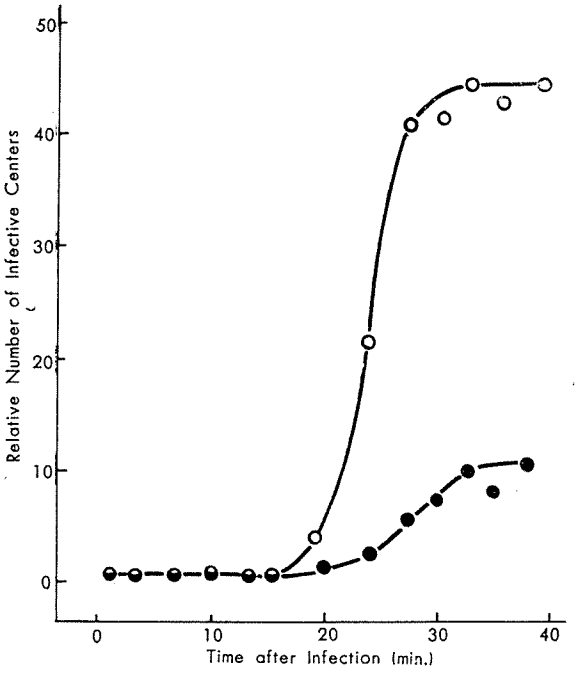

Fig. 1. One-step Growth Curve of T2r Phage in a Glucose-salts Synthetic Medium with and without Actinomycin

The bacteria were infected with T2r phage at a multiplicity of 0.1. After incubation at 37° C for 5 minutes, the culture was diluted and transferred to two duplicate growth tubes, containing glucose-salts synthetic medium with (●) and without (○) actinomycin at a concentration of 30 μg/ml. All tubes were incubated at 37° C with shaking. Samples from the duplicate cultures were plated at various times during incubation, and the results were plotted on a linear scale. The control culture gave an average burst size of 43, and the antibiotic culture one of 11.

the rate of increase in the number of infective centers differed greatly in the presence and absence of the antibiotic. The average burst size in the culture containing antibiotic was about one-fourth of that of the control. It must be noted that

exposure of free phage particles to a concentration of actinomycin sufficient to prevent phage multiplication (100 μg/ml) for 60 minutes at 37°C did not cause any inactivation of the phage.

A single burst experiment at a concentration of 30 μg/ml of actinomycin was made in order to determine the actual yields of phage from individual cells. Less than ten infective phage particles developed in individual cells. In another type of experiment, lysis of the infected cells was followed by measuring the optical density. Clearing occurred at the same time in cultures with or without actinomycin. Therefore it may be concluded that, in the presence of actinomycin, individual cells infected with the phage produce only one-tenth or one-fourth as much active phage as the controls.

3. *Effect of antibiotic on the synthesis of nucleic acids and protein in phage-infected bacteria*

Since actinomycin inhibited multiplication of the phage but did not prevent growth of the host bacteria or inactivate free phage particles, the effect of the antibiotic is thought to be primarily on phage reproduction. So its effect on the synthesis of nucleic acids and protein in phage-infected cells was studied. The results are illustrated in Table 2. The net increase of DNA in cells infected with the phage was about four times that of uninfected cells after 30 minutes incubation. It should be noted that phage-infected bacteria synthesized DNA at the same rate in the presence and absence of actinomycin. Moreover, little difference was observed in the synthesis of other components of infected cells in the presence and absence of antibiotic. That is, in both cases, there was immediate cessation of net synthesis of RNA and a slight increase in protein in phage-infected bacteria even at a concentration of 100 μg/ml of actinomycin. An additional experiment showed that the infected bacteria produced DNA in the same way whether the antibiotic was added

Table 2. Effect of Actinomycin on the Synthesis of Nucleic Acids and Protein in T2r Phage-infected and Uninfected *E. coli* B.

The bacterial suspension containing 5×10^8 cells/ml was divided into two portions, one of which was infected with T2r phage at a multiplicity of 7. The other was uninfected. Tubes containing 10 ml of suspensions of infected or uninfected cells were incubated with various concentrations of actinomycin, as described in the table at 37° C for 30 minutes, and analysed for nucleic acids and protein. The values illustrated in the table represent relative amounts of each component.

Time of incubation (min.)	Conc. of actinomycin (μg/ml)	Uninfected cells			Phage-infected cells		
		DNA	RNA	Protein	DNA	RNA	Protein
0	0	100	100	100	100	100	100
30	0	130	135	126	220	106	110
30	10	137	152	123	217	107	111
30	30	134	161	122	213	106	110
30	100	134	148	125	204	98	110

5 minutes before or 5 minutes after infection. Paradoxically, this suggests that actinomycin does not prevent the synthesis of protein in phage-infected bacteria, because it is known that there is no synthesis of phage DNA in infected cell in which protein synthesis has been suppressed from the beginning by the presence of chloramphenicol (Tomizawa and Sunakawa, 1956).

4. *Properties of DNA in a lysate made with actinomycin*

The above data indicate that, in the presence of actinomycin, the phage-infected bacteria on lysis liberate a large number of non-infective phage particles. T-even bacteriophages generally have the following characteristics; they can be readily separated from the general debris present in a lysate by differential centrifugation, and they can be precipitated specifically with antiphage serum by virture of their antigen-antibody reaction. It was, therefore, expected that analysis of the properties of the DNA in the lysate prepared in the presence of antibiotic might indicate the nature of the non-infective phage particles. The results are shown in Table 3. The lysate made in the presence of 30 μg/ml of the antibiotic contained nearly

Table 3. Properties of DNA in a Lysate Prepared with Actinomycin

Lysates with and without actinomycin were prepared as follows; the cells were suspended in a glucose-free salts synthetic medium at a concentration of 5×10^8 cells/ml and then infected with T2r phage at a multiplicity of 1.9. After 5 minutes adsorption, the mixture was mixed with glucose and divided into two portions, a control, and one with actinomycin at a concentration of 30 μg/ml. The cultures were incubated at 37° C for 120 minutes with shaking. After storage overnight at 5° C, the lysates were analysed for plaque production and DNA. The antibiotic-free control lysate contained active phage at a concentration of 1.9×10^{10} particles/ml, whereas the actinomycin-lysate contained only 1.4×10^9/ml. The values in parentheses represent the percentages of the amount of DNA in the initial lysate.

Fraction	DNA found (μg-P/10 ml)	
	control lysate	Lysate with actinomycin
Initial lysate	21.0 (100)	24.9 (100)
Low-speed centrifugation supernatant	18.8 (90)	17.8 (73)
Sediment at 20,000 g	7.1 (34)	0.5 (2)
Precipitate with antiserum	11.2 (53)	0.5 (2)
Deoxyribonuclease-resistant	8.1 (39)	2.5 (10)

the same amount of DNA as that of an antibiotic-free control lysate, but the infectivity of the former lysate was less than one-tenth of that of the latter. When the two lysates were centrifuged at 3,000 g for 15 minutes, the majority of the DNA remained in the supernatant fluid, both in the presence and in the absence of the antibiotic. However there was a significant difference between the control and antibiotic-treated lysates in the amount of DNA in the sediment obtained by centrifugation at 20,000 g. At this gravity, normal T2 bacteriophage but not free nucleic acids were sedimentable. Furthermore, the DNA which had been pro-

duced by infected cells in the presence of the antibiotic was not precipitable by anti-T2 phage serum and was sensitive to deoxyribonuclease. These results indicate that this DNA has no protein coat which reacts with the antiserum and protects the viral DNA from deoxyribonuclease.

5. *Detection of phage-related protein in the actinomycin-lysate*

It had been found that viral DNA is synthesized by T2 phage infected bacteria exposed to actinomycin but liberated without assembled in a protein coat. Hence, in the next experiments, the serum-blocking power and bactericidal activity of a lysate made in the presence of actinomycin were tested in order to determine whether the viral protein was produced in the presence of the antibiotic.

a) The serum-blocking power (combining ability of phage or phage-related materials for phage-neutralizing antibody) of the lysate prepared in the presence of actinomycin was assayed in connection with the number of infective phage particles. Aliquots of 0.7 ml of each lysate and threefold (or twofold) dilutions of these were mixed with 0.9 ml aliquots of anti-T2 phage serum diluted 1: 1,500 and then incubated at 37°C for 14 hours to allow completion of the reaction. The amount of phage-nuetralizing activity remaining in the mixture was assayed by adding 0.4 ml of T2h phage (10^6 particles/ml), the host range mutant of T2, as test phage. After further incubation at 48° C for 2 hours, its activity was assayed on *E. coli* B/2 in order that the results should not be complicated by plaque forming ability of the blocking phage (T2), if this remained in the mixuture. In the data assembled in Table 4, a difference is seen between the lysates produced in the presence and absence of actinomycin. The antibiotic-treated lysate (in experiment I) had only one-third the infective phage concentration of the control lysate, but the tube with the former lysate contained more surviving test phage than did the latter. In experiment II, in which the actinomycin-lysate was made at a level of 100 μg/ml of actinomycin, the same relation was clearly seen. The actinomycin-lysate contained only one-twentieth of the infective phage particles of the control, but there was much less inactivation of test phage in the mixture containing the former lysate. Since the survival of test phage must vary with the amount of blocking agent present, in both cases, the results indicate that a lysate made in the presence of actinomycin contains not only infective phage particles but also other materials with serum-blocking power. Parallel studies were made on the supernatants of the two lysates obtained by high-speed centrifugation. The amount of blocking agents and of infective phage particles in these showed more clearly the properties of the materials with serum-blocking power. The supernatant of the lysate made in the presence of actinomycin contained most of the blocking agents from the original lysate (before centrifugation), although less infectivity. On the other hand, the supernatant of the control lysate contained much lower amounts of serum-blocking agents than the initial lysate. The fact that the antibiotic-treated lysate

contained more non-sedimentable serum-blocking agents, suggests that phage-infected bacteria incubated with actinomycin synthesize a normal amount of materials with serum-blocking power, most of which is non-infective and non-sedimentable by high-speed centrifugation.

Table 4. Comparison of the Serum-Blocking Power of Lysates made with or without Actinomycin

The lysates with or without actimomycin were prepared as follows; a bacterial suspension containing 5×10^8 cells/ml was divided into two portions, to one of which actinomycin was added 2 minutes before infection. The cultures were infected with T2r phage at a multiplicity of 0.11 and shaken at 37° C for 120 minutes. The concentration of actinomycin was 30 μg/ml in experiment I, and 100 μg/ml in experiment II. All lysates were stored overnight at 5° C and then assayed for their serum-blocking power and plaque forming ability. In experiment I, the control lysate contained 2.4×10^{10} particles/ml of active phage and the actinomycin-lysate cotained 8.1×10^9/ml. In experiment II, the former lysate contained 1.4×10^{10} particles/ml, whereas the latter contained 1.4×10^8/ml. Supernatant fluids from each lysate were obtained by centrifugation at 36,000 g for 60 minutes, and their infective titers both showed unsedimented phage particles in the supernatants. The details of the assay method for the serum-blocking power are described in the text.

Experiment	Mixture No.	Lysate	Infective T2r titer (/ml)	Titer of test phage (/ml)
	1	——	——	3.4×10^6
	2	control-lysate	1.1×10^{10}	9.4×10^4
	3	1 / 3 dilution of No. 2	3.6×10^9	4.8×10^2
	4	actinomycin-lysate	4.7×10^9	1.9×10^5
	5	1 / 3 dilution of No. 4	1.6×10^9	1.4×10^4
	6	supernatant of control-lysate	2.3×10^9	1.6×10^3
	7	1 / 3 dilution of No. 6	7.6×10^8	4.4×10^1
	8	supernatant of actinomycin-lysate	1.2×10^9	6.6×10^5
	9	1 / 3 dilution of No. 8	4.0×10^8	6.4×10^3
	1	——	——	5.1×10^6
	2	control-lysate	2.8×10^9	3.9×10^5
	3	1 / 2 dilution of No. 2	1.4×10^9	7.1×10^4
	4	actinomycin-lysate	1.5×10^8	4.9×10^5
II	5	1 / 2 dilution of No. 4	7.3×10^7	2.0×10^3
	6	supernatant of control-lysate	1.0×10^9	2.4×10^3
	7	1 / 2 dilution of No. 6	5.2×10^8	2.0×10^2
	8	supernatant of actinomycin-lysate	9.2×10^7	1.6×10^5
	9	1 / 2 dilution of No. 8	4.6×10^7	1.7×10^3

b) The lysates prepared in the presence and absence of actinomycin were also tested for plaque production and bactericidal activity. Constant amount of lysates and successive twofold dilutions of them were put into tubes containing an equal volume of bacterial suspension at the logarithmic phase of growth (5×10^7 cells/ml). After incubation at 37°C for 10 minutes, the survival of bacteria were assayed by plating aliquots from each tube. As shown in Fig. 2, all the

bactericidal activity could be accounted for by the active phage present. There was not evidence of materials with host-killing activity, but not plaque forming

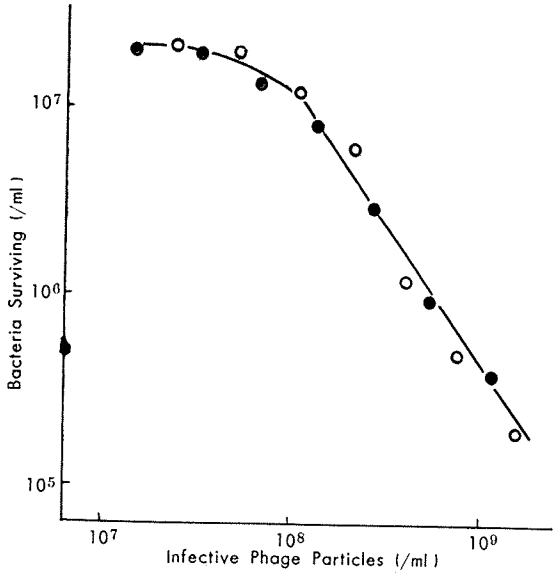

Fig. 2. Comparison of Bactericidal Activity of Lysates made with and without Actinomycin

Lysates with or without actinomycin were prepared as for Table 4. The actinomycin-lysate contained a concentration of 30 μg/ml of actinomycin. After storage overnight at 5° C, the lysates were assayed for plaque forming particles and bactericidal activity; ○—○, control lysate; ●—●, actinomycin-lysate. The control lysate contained 1.7×10^{10} particles of infective phge/ml, the antibiotic-treated lysate 1.2×10^{9}/ml. The details of the procedure for assaying bactericidal activity are described in the text.

ability, in the actinomycin-lysate. However, this does not necessarily imply that phage protein is not produced normally by infected cells in the presence of the antibiotic, because the host-killing ability may arise only from integration of some protein components, if not all, to an organized form.

6. *The relation between the rate of inhibition and the time of addition of the antibiotic*

From the above evidence, it is tentatively concluded that the inhibitory action of actinomycin is on the maturation of phage units. According to this interpretation, the addition of the antibiotic at various intervals after infection suppresses phage multiplication only to the extent that the reactions inhibited by the antibiotic have not yet taken place. Fig. 3 shows a representative experiment on this, in which the antibiotic was added at various intervals after infection and the tubes were assayed when the increase in infective centers in the control tube

was complete. Actinomycin inhibited the multiplication of the phage most when added within 6 minutes of infection. Introduction of the antibiotic within 9 to

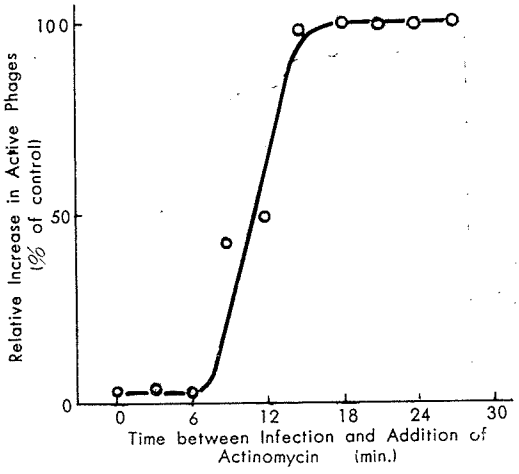

Fig. 3. Relation between the Rate of Inhibition and the Time of Addition of Actinomycin

A culture of bacteria was infected with T2r phage of a multiplicity of 0.1 and incubated at 37° C for 5 minutes. It was diluted and transferred into a glucose-salts synthetic medium. The mixture was incubated at 37° C with shaking. At various intervals during incubation, aliquots of the mixture were transferred to fresh medium containing actinomycin (30 μg/ml), and shaking continued. After total of 40 minutes incubation, all tubes were assayed for active phage particles. The yield of active phage (measured after a total of 40 minutes incubation) when the culture was transferred to the medium containing antibiotic was plotted. The antibiotic-free control culture gave a burst size of 40.

12 minutes after infection resulted in a linear increase in phage that progressively aproached the yield obtained in the absence of inhibitor. When added 15 minutes after infection, the growth of the phage could no longer be prevented by the antibiotic. This seems to show that at this time the process of phage synthesis had reached a stage when it was no longer inhibited by actinomycin, and suggests that the inhibitory action of the antibiotic is exerted on some early steps in the maturation process.

7. *Zone-electrophoretic pattern of the complex formed between actinomycin and phage-related DNA*

Attempts were made to detect the complex of actinomycin with DNA isolated from T2 bacteriophage or from phage-infected cells by zone-electrophoresis. DNA isolated from the phage particles was mixed with actinomycin *in vitro* and subjected to zone-electrophoresis. Figure 4 shows the electrophoretic pattern of the complex of actinomycin with viral DNA. The amount of each substance

was plotted against the distance travelled from the origin toward either electrode. The DNA of the bacteriophage was found in the same fractions as actinomycin,

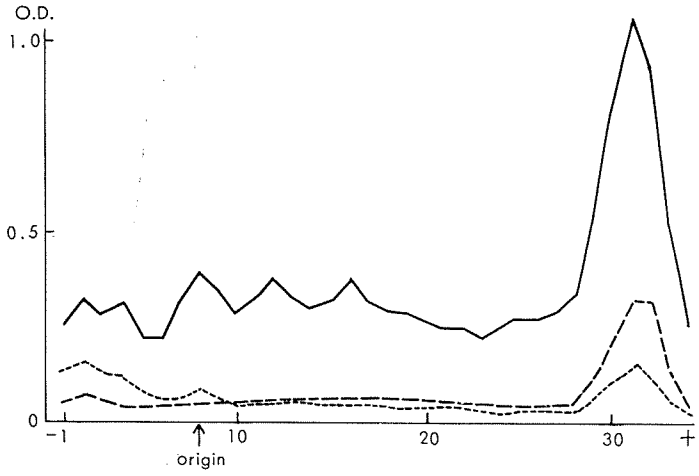

Fig. 4. Zone-electrophoretic Pattern of T2r Phage DNA Treated with Actinomycin

1.8 mg of DNA (dissolved in distilled water) isolated from T2r bacteriophage was mixed with 1.2 ml of actinomycin solution (in distilled water at a concentration of 1 mg/ml), and incubated at 37° C for 30 minutes. Then, the mixture was subjected to zone-electrophoresis for 18 hours. Each section was eluted with 9 ml of 0.1 M NaCl. The absorbances at 260 mμ (—— representing total nucleic acids), at 440 mμ (- - - - actinomycin) and at 600mμ (– – –) after the diphenylamine reaction of each eluate were plotted. In fractions of Nos. 31 to 33, there are found DNA and actinomycin. The actinomycin found in Nos. 1 to 3 is thought to be the excess that did not reacted with DNA, because it alone travelled to these fractions.

which alone travelled to the cathode. Studies were also made on whether a complex was formed in phage-infected cells in which viral DNA was synthesized. 200 ml of cell suspension containing 5×10^8 cells/ml was infected with T2 phage at a multiplicity of infection of 6. After 5 minutes incubation for adsorption, actinomycin was added to the mixture to give a final concentration of 100 μg/ml. The culture was incubated for a further 15 minutes. During this period, the DNA content of the culture increased about one and half time. The infected cells were chilled and harvested and ground with alumina in the cold. Material was eluted from the alumina with 6 ml of 0.1 M NaCl. After low-speed centrifugation to remove alumina and bacterial debris, the resulting eluate was centrifuged in the cold for 30 minutes at 12,000 g. 2 ml of the resulting supernatant was subjected to electrophoresis. As shown in Fig. 5, most of the cell constituents travelled toward the anode. A similar interaction was observed between actinomycin and DNA in phage-infected bacteria. However, it was not possible to find actinomycin in the RNA and protein fractions.

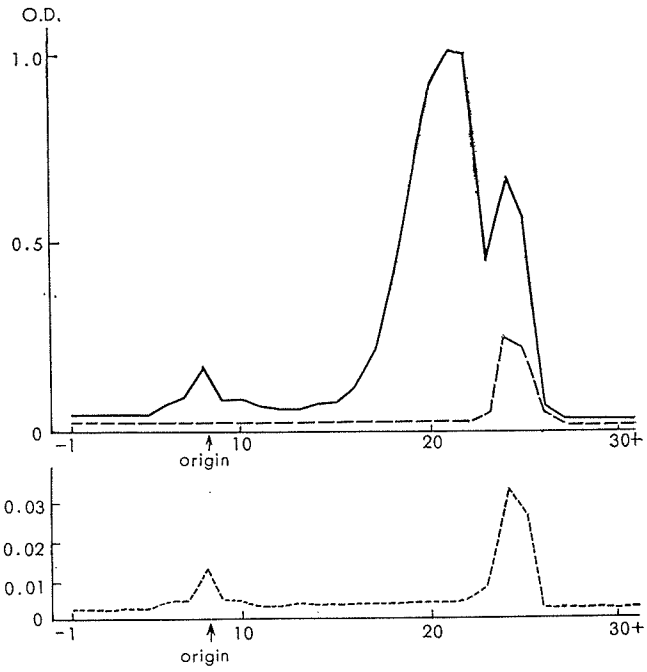

Fig. 5. Zone-electrophoretic Pattern of Extract of Phge-infected *E. coli* B Incubated with Actinomycin

Electrophoresis was performed for 14 hours. Each section of starch was eluted with 5 ml of 0.1 M NaCl. The absorbances at 260 mμ (—— representing total nucleic acids), at 440 mμ (- - - - actinomycin) and at 600 mμ (– – –) after the diphenylamine reaction of each eluate were plotted. Fractions Nos. 19 to 22 contained a large amount of RNA, Nos. 24 and 25 contained DNA. Actinomycin was found only in Nos. 24 and 25, and not in Nos. 19 to 22. The details of the procedure for extraction are described in the text.

DISCUSSION

Actinomycin is known to inhibit the growth of a wide variety of Gram-positive bacteria strongly and that of Gram-negative bacteria to a limited extent. Papers on the mode of action of actinomycin D report that the synthesis of RNA and protein by *Bacillus subtilis* (Slotnick, 1960) and *Staphylococcus aureus* (Kirk, 1960) is inhibited when the antibiotic is added at growth-inhibiting concentrations (0.25-0.75 μg/ml) to cultures of these bacteia in a logarithmic stage of growth. However, the growth of *E. coli* B, a Gram-negative bacterium, was unaffected by this antibiotic. The net synthesis of nucleic acids and protein also occurred normally at concentrations of up to 100 μg/ml.

The present observations appear to provide the first information about the effect of actinomycin on the bacteria-bacteriophage system. The inhibitory action

of various chemical agents on the multiplication of bacteriophage has usually been investigated in relation to their action on the growth of uninfected bacteria. The results to be expected may be classified as follows: (1) the growth of both bacteria and phage may be inhibited, (2) bacterial growth may be inhibited without any effect on phage reproduction, (3) phage multiplication may be inhibited without affecting bacterial growth. As already mentioned, the action of actinomycin on the T2 phage-*E. coli* B system falls into the last category. This antibiotic neither inactivated free phage particles nor prevented adsorption of the phage by the host bacterium. When bacteria were infected with T2 bacteriophage in the presence of actinomycin, they synthesized phage protein and nucleic acid and were lysed normally, but liberated few infective particles. Actinomycin is thus thought to inhibit some final step of phage reproduction, namely maturation. Only one class of substances, the acridine dyes, are known to prevent the maturation of phage units. It is supposed that actinomycin may act on the process of phage growth at the same stage as these substances, so that below considerations of the mechanism of the inhibitory action of actinomycin will be made in comparison with that of proflavine, an acridine dye, which has been the subject of reports by many investigators. Scince the first observation of Foster (1948) that proflavine inhibits phage multiplication while permitting cellular lysis after a normal latent period, a series of experiments relevant to the problem of maturation have been performed to find the step at which proflavine is inhibitory. De Mars *et al.* (1953) found incomplete phage particles in a lysate produced in the presence of proflavine, which they named "doughnuts", and later phage-related materials with serum-blocking power (De Mars, 1955). Therefore, it seems clear that proflavine inhibits phage production without interfering with lysis or synthesis of phage DNA and protein. In the presence of proflavine, phage-infected bacteria on lysis liberate viral DNA and protein but not infective phage particles. These observations, together with another line of evidence obtained genetically by phenotypic mixing (Novick and Szilard, 1951; Streisinger, 1956), led to the general belief that the enclosure of the phage precursor DNA in its protein coat and its endowment with a complex tail structure occurs during the maturation process of phage synthesis. Proflavine is known to interact with DNA (Lerman, 1961). Therefore it may influence the utilization of phage DNA for the process of maturation. Kay (1959) studied the inhibition of bacteriophage multiplication by proflavine and its reversal by certain polyamines, and thought that the proflavine block was due to an interference in the incorporation of polyamines into the newly synthesized phage DNA, which then could not be orientated to form the core of the phage head.

As already mentioned, actinomycin tends to form a complex specifically with DNA. This property seems to be unaffected by the existence of an unusual pyrimidine residue (5-hydroxymethylcytosine) in T-even phage DNA. There-

fore, the complex formation of actinomycin with precursor DNA synthesized in phage-infected bacteria may result in the abortion of the latter to form the core of phage head.

There is, however, no convincing evidence for the direct precursor nature of phage-related materials with serum-blocking power (De Mars, 1955) and of "doughnuts" (Kellenberger and Séchaud, 1957), much less for the participation of nucleic acids and protein in the process of maturation. The possibilities could not be excluded that these compounds inhibit the synthesis of an unknown phage constituent or of an enzyme required for the assembly of each precursor component, or that they alter the biological activity of the nucleic acid produced. The interfering action of actinomycin on the biological activity of DNA was shown by the observation that the infectivity of ϕX174 bacteriophage DNA on spheroplasts declined after treatment with the antibiotic (Nakata and Kawamata, unpublished). However, because of technical difficulties, it could not be decided whether the DNA which was synthesized in the presence of actinomycin, but which did not interacted with the antibiotic, had normal biological activity. Therefore the problem of the mechanism of maturation appears to involve many complicated problems.

A few differences were found between the effects of proflavine and actinomycin on phage multiplication. At an appropriate concentration of proflavine, not only did phage-infected bacteria produce no active phage particles but also even the original infective particles were lost (Foster, 1948). However, there was no loss of original infective phage even at the highest concentration of actinomycin tested. Moreover, removal of proflavine by dilution of the culture of growing phage resulted, without any latent period, in the liberation of active phage whereas, in the case of actinomycin, removal of the antibiotic by dilution did not cause an increase in infective phage particles. These differences may be caused by differences in the interaction of these substances or by the number of molecules of these substances which interact with one functional DNA molecule.

Although more extensive studies on the mode of action of actinomycin are desirable, and comparative studies on the effect of actinomycin and proflavine on various species of bacteriophage may give valuable information on the mechanism of multiplication of the phage. The modes of action of these two substances differ in several ways, but in particular, it should be noted that actinomycin specifically interacts with DNA but not with RNA.

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REFERENCES

Adams, M. H. (1950). Methods of study of bacterial viruses. *Methods in Medical Research* **2,** The Year Book Publishers, Chicago

Burton, K. (1956). A study of the condition and mechanism of diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315-323.

De Mars, R. I., Luria, S. E., Fisher, H. and Levinthal, C. (1953). The production of incomplete bacteriophage particles by the action of proflavine and the properties of the incomplete particles (1). *Ann. Inst. Pasteur* **84,** 113-128.

De Mars, R. I. (1955). The production of phage-related materials when bacteriophage development is interrupted by proflavine. *Virology* **1,** 83-99.

Foster, R. A. C. (1948). An analysis of the action of proflavine on bacteriophage growth. *J. Bacteriol.* **56,** 795-809.

Kawamata, J. and Fujita, H. (1960). Chemical studies on actinomycin S. I. Amino acid content. *J. Antib.* Ser. A **13,** 295-297.

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

Kawamata, J. and Imanishi, M. (1961). Mechanism of action of actinomycin, with special reference to its interaction with deoxyribonucleic acid. *Biken's J.* **4,** 13-24.

Kay, D. (1959). The inhibition of bacteriophage multiplication by proflavine and its reversal by certain polyamines. *Biochem. J.* **73,** 149-154.

Kellenberger, E. and Séchaud, J. (1957). Electron microscopical studies of phage multiplication II. Production of phage-related structures during multiplication of phages T2 and T4. *Virology* **3,** 256-274.

Kernsten, W., Kernsten, H. and Rauen, H. M. (1960). Action of nucleic acids on the inhibition of growth by actinomycin of *Neurospora crassa*. *Nature* **187,** 60-61.

Kirk, J. M. (1960). The mode of action of actinomycin D. *Biochim. Biophys. Acta* **42,** 167-169.

Lerman, L. S. (1960). Structural considerations in the interaction of DNA and acridines. *J. Mol. Biol.* **3,** 18-30.

Lowry, O. L., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Mejbaum, W. (1939). Über die Bestimmung kleiner Pentosemengen, Insbesondere in Derivaten der Adenylsäure. *Hoppe-Seyler's Z. physiol. Chem.* **258,** 117-120.

Nakata, A., Sekiguchi, M. and Kawamata, J. (1961). Inhibition of multiplication of bacteriophage by actinomycin. *Nature* **189,** 246-247.

Nakata, A. and Kawamata, J. (unpublished).

Novick, A. and Szilard, L. (1951). Virus strains of identical phenotype but different genotype. *Science* **113,** 34-35.

Slotnick, J. J. (1960). Mechanism of action of actinomycin D in microbiological systems. *Ann. N. Y. Acad. Sci.* **89,** 342-347.

Streisinger, G. (1956). Phenotypic mixing of host range and serological specificities in bacteriophages T2 and T4. *Virology* **2,** 388-398.

Tomizawa, J. and Sunakawa, S. (1956). The effect of chloramphenicol on deoxyribonucleic acid synthesis and the development of resistance to ultraviolet irradiation in *E. coli* infected with bacteriophage T2. *J. Gen. Physiol.* **39,** 553-565.