Actinomycin Analogues Containing Pipecolic Acid: Relationship of Structure to Biological Activity

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Received for publication 19 February 1968

Streptomyces antibioticus synthesizes a mixture of actinomycins which differ at the "imino acid" site of the peptide chains. In the presence of exogenous pipecolic acid, several new actinomycins were synthesized and 70% of the proline in the antibiotic mixture was replaced by the analogue. Three new antibiotics (designated Pip 1 α , Pip 1 β , and Pip 2) were isolated from culture filtrates, purified, and crystallized. The molar ratio of pipecolic acid to proline was: Pip 1 α , 1:0; Pip 1 β , 1:1; Pip 2, 2:0. These compounds inhibited the growth and cell division of gram-positive, but not gram-negative, bacteria. The relative inhibitory activity against bacteria, *Escherichia coli* deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase in vitro, and RNA synthesis in *Bacillus subtilis* and mouse L-929 cells was: actinomycin IV = Pip 1 β > Pip 2 > Pip 1 α . Protein synthesis in *B. subtilis* was less affected, and DNA synthesis was inhibited only at higher concentrations of antibiotic tested. In L cells, DNA formation was reduced less than RNA synthesis, whereas protein synthesis was not blocked under the experimental conditions employed. Kinetic studies with *B. subtilis* revealed that RNA synthesis was inhibited rapidly followed by an inhibition of protein synthesis. All four antibiotics markedly inhibited the replication of vaccinia virus and reovirus in tissue culture cells, but the production of poliovirus was resistant to the antibiotics. These actinomycins bind to DNA, resulting in an elevation of its T_m and a decrease in the peak extinction of the actinomycins. The mode of action, as well as the structure-activity relationships among the actinomycins, are discussed relative to a previously proposed model of binding.

The actinomycins are highly inhibitory for gram-positive bacteria, but, owing to limited cellular permeability (24, 26), they exhibit limited or no activity against gram-negative microorganisms and fungi (30, 44). Moreover, actinomycin IV (D) has been employed as a carcinostatic agent in the treatment of certain human neoplasms, such as Wilms' tumor and chorio-carcinoma (9, 35), although excessive toxicity of the antibiotic has restricted its potential usefulness. Studies concerning the mode of action of actinomycin IV have revealed that actinomycin is a potent inhibitor of deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis (16, 33). Consequently, actinomycin has become a very useful tool for studies on the synthesis of messenger RNA and protein, as well as on the replication of viruses (32).

Streptomyces antibioticus synthesizes a number of actinomycins which differ solely at the imino acid site of the molecule (20; Fig. 1). The composition of an actinomycin mixture produced may vary, quantitatively and qualitatively, depending upon the conditions of cultivation (17, 40). Previous investigations have shown that analogues of proline can influence actinomycin synthesis by competing with, and replacing, endogenously synthesized proline in certain of the actinomycin components (18, 19). As a consequence, the formation of minor components may be greatly enhanced or the synthesis of new actinomycins may occur. In the case of pipecolic acid, the higher analogue of proline, as many as five new actinomycins are formed (19). Three of the actinomycins (designated Pip 1 α , Pip 1 β , and Pip 2) have been isolated from culture filtrates. In this report, certain biological properties of these components are described and are compared with those of actinomycin IV.

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FIG. 1. Structure of actinomycin. The sequence of amino acids is L-threonine, D-valine (D-val), A and B, sarcosine (Sar), N-methyl-L-valine. Actinomycin I, A = L-proline, B = 4-hydroxy-L-proline; actinomycin II, A = B = sarcosine; actinomycin III, A = sarcosine, B = L-proline; actinomycin IV, A = B = L-proline; actinomycin V, A = L-proline, B = 4-keto-L-proline.

MATERIALS AND METHODS

Radioisotopes. Uridine-2-¹⁴C (24 mc/mmole), thymidine-2-¹⁴C (30 mc/mmole), L-valine-1-¹⁴C (22 mc/mmole), and a uniformly labeled ¹⁴C-amino acid mixture (1.5 mc/mg) were purchased from New England Nuclear Corp., Boston, Mass. ³H-guanosine triphosphate (800 mc/mmole) was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y.

Amino acids, nucleic acids, and nucleotides. Ribonucleoside triphosphates were purchased from Sigma Chemical Co., St. Louis, Mo. DL-Pipecolic acid·HCl and other amino acid standards were obtained from commercial sources. Actinomycin IV was provided by D. Hendlin, Merck, Sharp and Dohme Research Laboratories. DNA preparations extracted from *Pseudomonas aeruginosa* and *Bacillus subtilis* strain 2564 were kindly supplied by Julius Marmur. The DNA derived from mouse fibroblast cells (L-929 cells) was prepared by the method of Marmur (27).

Test organisms and conditions of cultivation. The following microorganisms were obtained from the culture collection of the Department of Microbiology, Georgetown University Schools of Medicine and Dentistry: *B. subtilis*, *Sarcina lutea*, *Staphylococcus aureus* (penicillin-sensitive), *S. aureus* (penicillin-resistant), *S. albus*, *Escherichia coli*, *P. aeruginosa*, *Salmonella paratyphi*, *Aerobacter aerogenes*. *B. subtilis* (Marburg strain) ATCC 6051 was also employed.

The bacteria were grown on Trypticase Soy Agar slants (BBL) at 37 C and then stored at 4 C. *B. subtilis*

(Marburg) was cultivated also in Demain's medium (6) supplemented with 0.5% N-Z case (Sheffield Chemical Co., Norwich, N.Y.). Growth was measured turbidimetrically in a Klett nephelometer (filter #54).

HeLa S3-1 and mouse L-929 cells were grown in suspension culture in Eagle's medium (7) supplemented with 5% fetal bovine or horse serum. Cell suspensions were concentrated by centrifugation and infected with poliovirus Mahoney type 1 (23), the WR strain of vaccinia virus (36), or the Abney strain of type 3 reovirus (41). Virus yields were determined by plaque assay (13, 22, 36).

Assays. The agar-streak method was used to determine the sensitivity of bacteria to the actinomycins (43). For the measurement of biopolymer synthesis, a culture of L cells or *B. subtilis* (Marburg) in liquid medium was incubated with an actinomycin preparation and a ¹⁴C-labeled precursor as described in Results. The reaction was terminated by the addition of cold 5% perchloric acid (PCA). Nucleic acids were fractionated by the procedure of Schmidt and Thannhauser (39); DNA and RNA were determined by the diphenylamine test (3) and the orcinol method (28), respectively. Protein was assayed by the method of Lowry et al. (25). Samples were transferred to stainless-steel planchets, dried, and counted in a Nuclear-Chicago low-background gas-flow counter.

E. coli RNA polymerase (1,800 units/mg) was purchased from Biopolymers, Inc., General Biochemicals, Chagrin Falls, Ohio, and assayed with L-cell DNA as primer, as previously described (4).

Difference spectra and melting profiles. A Cary model 11 spectrophotometer was used to measure difference spectra of DNA; thermal denaturation profiles of L-cell DNA were measured in a Gilford model 2000 recording thermospectrophotometer.

RESULTS

Amino acid composition of actinomycins. The actinomycins, designated Pip 1α, Pip 1β, and Pip 2, were obtained from culture filtrates of *S. antibioticus* incubated previously in 50 liters of a medium containing pipecolic acid. The physical and chemical properties of the crystalline compounds will be described in a forthcoming publication. Threonine, valine, sarcosine, and N-methylvaline were present in each component. In contrast to actinomycin IV (Table 1), actino-

TABLE 1. Imino acid content of the crystalline actinomycins

Actinomycin	Moles of imino acid per mole of actinomycin	
	Proline	Pipecolic acid
IV	1.8	0
Pip 1α	0	0.9
Pip 1β	0.9	1.05
Pip 2	0	2.2

mycin Pip 2 contained two residues of pipecolic acid and no proline, actinomycin Pip 1 β contained one molecule each of proline and pipecolic acid, and actinomycin Pip 1 α contained one molecule of pipecolic acid and an unidentified imino acid.

Spectrum of activity of the actinomycins. In a study of the antimicrobial activity of the various actinomycins by the agar-streak assay method (Table 2), actinomycin Pip 1 β proved to be as active as actinomycin IV against *B. subtilis*, *S. lutea*, and *S. albus*. However, actinomycin Pip 1 β was only one-half to one-third as active as actinomycin IV against two strains of *S. aureus*. By comparison, actinomycin Pip 2 was one-fifth to one-twentieth and actinomycin Pip 1 α was one-tenth to one-seventieth as active as actinomycin IV. All four antibiotics were essentially without effect against gram-negative bacteria, although a slight reduction of growth of *P. aeruginosa* was noted with actinomycin IV and Pip 1 β at 100 μ g per ml.

Effect upon exponentially growing cultures of *B. subtilis*. The inhibition of an exponentially growing culture of *B. subtilis* (Marburg) by the various actinomycins is shown in Fig. 2. Growth of the organism was delayed for 1 to 2 hr by 0.1 μ g of actinomycin IV per ml and was completely inhibited by 0.5 μ g per ml. Similar results were obtained with actinomycin Pip 1 β . With actinomycin Pip 2 and Pip 1 α , 0.5 and 2.0 μ g per ml, respectively, were required for a partial inhibition of growth. For essentially complete inhibition, 2 to 5 μ g of actinomycin Pip 2 and more than 10 μ g of actinomycin Pip 1 α per ml were required.

Microscopic examination of slides prepared from cultures exposed to the different actino-

mycins for 2 hr revealed a marked elongation of the inhibited cells in many instances. Actinomycin Pip 1 α , which was the least active of the antibiotics studied, exhibited the most marked inhibition of cell division, as evidenced by the pronounced elongation and pseudobranched cells. In the case of actinomycin Pip 1 α (0.5 to 1.0 μ g per ml), continued cell elongation may contribute to the increase in turbidity over that of the control culture (Fig. 2).

Inhibition of RNA, DNA, and protein synthesis in *B. subtilis* and *L* cells. The effect of actinomycins IV, Pip 1 α , Pip 1 β , and Pip 2 on the synthesis of bacterial RNA, DNA, and protein is shown in Fig. 3. Although all three processes were inhibited, RNA synthesis was the most sensitive of the three systems tested. Approximately 50% inhibition of RNA synthesis was achieved with actinomycins IV and Pip 1 β at a concentration of 0.5 μ g per ml. The same level of inhibition of protein and DNA synthesis was achieved with 1 and 2 μ g of antibiotic per ml of medium, respectively. By comparison, three to four times as much actinomycin Pip 2 was needed to attain similar effects. Actinomycin Pip 1 α was the least active compound; the concentrations required for 50% inhibition were 3.5 to 4 μ g per ml for RNA and 8 μ g per ml for protein. Only a 10% reduction in DNA synthesis was noted with 10 μ g of the antibiotic per ml of medium.

RNA synthesis in *L* cells was considerably more sensitive to the actinomycins than the same process in *B. subtilis* (Fig. 4), possibly because tissue cells are more permeable than bacterial cells. It was found that approximately 0.05 μ g of actinomycin IV, Pip 1 β , or Pip 2 per ml was required for a 50% inhibition of RNA synthesis in *L* cells. DNA synthesis was less affected; the order of activity of the actinomycins was Pip 1 β > Pip 2 > IV > Pip 1 α . In agreement with the high order of stability of animal cell messenger RNA, the formation of protein was not inhibited after 1 hr of exposure to the drugs at a concentration of 2 μ g per ml.

Time course of inhibition of biopolymer synthesis in *B. subtilis*. The previous study with *B. subtilis* revealed that RNA synthesis was the most sensitive of the systems tested with the antibiotics. Since the formation of protein and DNA was less affected, it was of interest to ascertain the time course of the inhibitory process with actinomycin IV and Pip 1 β .

Exponentially dividing cells were incubated in the presence of 0.5 μ g of actinomycin IV or Pip 1 β per ml of medium and the appropriate radioactive precursor. As shown in Fig. 5, inhibition of RNA synthesis occurred within the first minute. Protein synthesis was not affected until after the

TABLE 2. Antimicrobial activity of the actinomycins

Organism ^a	Minimal inhibitory concn (μ g/ml)			
	IV	Pip 1 α	Pip 1 β	Pip 2
<i>Bacillus subtilis</i>	0.02	0.25	0.02	0.1
<i>Sarcina lutea</i>	0.04	0.45	0.04	0.17
<i>Staphylococcus aureus</i> (penicillin-sensitive).....	0.046	3.0	0.09	0.75
<i>S. aureus</i> (penicillin-resistant).....	0.1	5.0	0.3	1.5
<i>S. albus</i>	0.16	5.0	0.16	1.0

^a The following organisms were not inhibited at a concentration of 100 μ g/ml: *Escherichia coli*, *Salmonella paratyphi* A, and *Aerobacter aerogenes*. Actinomycins IV and Pip 1 β at 100 μ g/ml caused a slight reduction of growth of *Pseudomonas aeruginosa*.

FIG. 2. Effect of actinomycins IV, Pip 1 α , Pip 1 β , and Pip 2 on the growth of *Bacillus subtilis*. The cultures were incubated at 37 C; at the beginning of the exponential phase of growth (arrow), the actinomycins were added at the following concentrations: 0.1, 0.5, 1.0, 2.0, 5.0, and 10 μ g per ml of culture. Growth was measured turbidimetrically with a Klett nephelometer. A Klett reading of 50 corresponded to an optical density of 0.410 at 540 m μ in a Beckman DU spectrophotometer, a Klett reading of 100 was equivalent to an optical density of 0.840 at 540 m μ . In the latter case, the number of viable cells was 3.8×10^8 cells/ml.

FIG. 3. Effect of various concentrations of actinomycins IV, Pip 1 α , Pip 1 β , and Pip 2 on RNA, DNA, and protein synthesis in *Bacillus subtilis*. Exponentially growing cells were incubated for 15 min with the actinomycin indicated, and 0.1 μ per ml of (A) uridine-2- 14 C, (B) L-valine-1- 14 C, or (C) thymidine-2- 14 C. The incubation was terminated by the addition of an equal volume of cold 10% PCA. RNA, DNA, and protein were extracted and assayed as described in Materials and Methods.

fourth minute of incubation, presumably the time when messenger RNA became limiting. Under the experimental conditions employed, DNA synthesis was not inhibited.

Previous investigations have shown that actinomycin IV depresses the synthesis of all species of DNA-dependent RNA, with soluble RNA synthesis the least sensitive process (8, 10, 29). As reported above, RNA synthesis by L cells was inhibited 90 to 95% during exposure for 1 hr to 1 to 2 μ g of actinomycin IV, Pip 1 β or Pip 2 per ml. However, actinomycin Pip 1 α depressed RNA synthesis only 40% (Fig. 4). This finding suggested that certain species of RNA, possibly soluble RNA, were formed, whereas synthesis of other species of RNA was blocked under the conditions of antibiotic treatment. A subsequent experiment revealed that exposure of L cells to actinomycin Pip 1 α (1 μ g per ml) for a longer period of time (5 hr) inhibited RNA production by 85%, and that an additional 2-hr incubation with the antibiotic did not result in further inhibition.

The nature of the RNA species which continued to be synthesized in the presence of actinomycin Pip 1 α was investigated. L cells were exposed to actinomycin Pip 1 α (1 μ g per ml) for 5 hr, uridine-2- 14 C (0.06 μ Ci/ml) was then added, and the incubation was continued for 2.5 hr. The cells were harvested and the RNA was extracted as described previously (38). The RNA from the uninhibited and actinomycin-treated cultures was analyzed by sucrose gradient centrifugation (2). The density gradient profiles of the RNA fractions are shown in Fig. 6. The tracings of absorbance at 260 m μ and acid-precipitable radioactivity in the preparation from the uninhibited cells are similar, indicating the presence of newly formed species of 28S and 16S ribosomal RNA and 4S soluble RNA. Ribosomal precursor RNA (35 to 45S) was also present (38). The RNA from the actinomycin-treated culture consisted of small amounts of high molecular weight material and a peak of radioactivity in the 4S region comparable to that in the uninhibited culture, suggesting that

FIG. 4. Inhibition of RNA and DNA synthesis in L-cells with the actinomycins. Replicate 20-ml suspensions of 3.7×10^5 cells per ml were incubated with various concentrations of actinomycin IV, Pip 1 α , Pip 1 β , or Pip 2. After 1 hr, uridine-2- 14 C (0.06 μ C per ml) (A) or thymidine-2- 14 C (0.1 μ C per ml) (B) was added, and the incubation was continued for 1 hr. The cells were harvested by centrifugation (2,000 rev/min, 10 min), and the pellet was suspended in 10 ml of cold 5% PCA. RNA and DNA were extracted and assayed as described in Materials and Methods.

the inhibited cells synthesized almost as much 4 S RNA as the uninhibited culture.

Effect on virus replication. It has been found previously that the replication of DNA viruses in tissue culture cells is extremely sensitive to inhibition by actinomycin IV, presumably because the formation of virus-specific messenger RNA is blocked at low drug levels (31, 32). RNA virus production has been reported to be inhibited by or resistant to actinomycin IV, depending upon the virus and cell types used, the concentration and the time of addition of the antibiotic, and other experimental conditions. The basis for the inhibition of RNA virus synthesis by actinomycin is not well understood.

Experiments were carried out to test the effect

of the various actinomycins on the replication of vaccinia virus, which contains double-stranded DNA, and on the replication of two RNA viruses: poliovirus, which contains single-stranded RNA and reovirus type 3 which has a double-stranded RNA genome but also contains a single-stranded ribopolymer (1, 42). It was found that vaccinia virus replication in HeLa cells was reduced 90% by any one of the actinomycins at a level of 0.1 μ C per ml, and that 0.5 μ C per ml was completely inhibitory. Reovirus yields were suppressed by 1 μ C per ml but not by 0.1 μ C per ml as reported previously for actinomycin IV (41). Poliovirus replication in HeLa cells was unaffected by actinomycins Pip 1 α and Pip 1 β at a concentration of 2 μ C per ml, but actinomycins Pip 2 and IV reduced the yield of poliovirus 28 and 85%, respectively; however, the final titers increased 1,000- (Pip 2) and 200-fold (IV) as compared with the titer at 1 hr after infection (Table 3). Other investigators have noted a similar inhibition of poliovirus replication, and have attributed it to the strain of cells used (14), the strain of poliovirus employed (37), and the serum used to supplement the growth medium (5).

Effect on *E. coli* DNA-dependent RNA polymerase. As shown in Fig. 7, actinomycin IV and Pip 1 β are potent inhibitors of RNA polymerase. With *E. coli* RNA polymerase and L-cell DNA as template, both actinomycins, at 2 μ C per ml, inhibited 3 H-guanosine triphosphate incorporation into the acid-insoluble product of the enzymatic reaction by 85 to 90%. Actinomycins Pip 1 α and Pip 2 were far less inhibitory; at a concentration of 10 μ C per ml, incorporation of guanosine triphosphate label was 30% of that observed in the control.

Difference spectroscopy with actinomycin-DNA complexes. The effect of L-cell, *B. subtilis*, and *P. aeruginosa* DNA on the absorption spectrum of actinomycins IV, Pip 1 α , Pip 1 β , and Pip 2 is shown in Fig. 8. A depression of the optical density of all four actinomycins occurred in the presence of the DNA preparations. Moreover, in the presence of the DNA preparations, a spectral shift to longer wavelengths was observed with actinomycins Pip 1 β and IV but not with actinomycin Pip 1 α . Actinomycin Pip 2 displayed such a shift only in the presence of *Pseudomonas* DNA.

The requirement for guanine residues in DNA as binding sites for actinomycin IV has been demonstrated (21). Further, it has been pointed out that the binding of DNA to actinomycin correlates to some extent with the guanine content of the DNA used (11, 12). The difference spectra (Fig. 8) provide additional evidence in support of this view. The most pronounced

FIG. 5. Time-course of inhibition of RNA, DNA, and protein synthesis in *Bacillus subtilis*. Exponentially growing cells received $0.1 \mu\text{C}$ per ml of (A) uridine-2-¹⁴C, (B) L-valine-1-¹⁴C, or (C) thymidine-2-¹⁴C. Actinomycin IV or Pip 1β ($0.5 \mu\text{g}$ per ml) was added immediately to the appropriate flasks, and the reaction was terminated at the times indicated by the addition of an

effect upon the visible spectrum of an actinomycin preparation was obtained with *Pseudomonas* DNA [guanylic and cytidylic acids (G + C) molar ratio = 66%]; the spectral changes observed with L-cell DNA (G + C, 42%) and *B. subtilis* DNA (G + C, 42%) were less.

Thermal denaturation of the actinomycin-DNA complex. The thermal denaturation profiles of L-cell DNA with the antibiotics was studied in two buffer systems. Since the color of an actinomycin preparation faded when added to $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl in 0.015 M sodium citrate), the determination was conducted also in Tris-NaCl buffer [0.001 M tris(hydroxymethyl)-aminomethane-chloride, $\text{pH } 7.4$, in 0.005 M NaCl]. To compensate for the fading in SSC, an additional amount of actinomycin was added to cuvettes to achieve a final optical density comparable to that attained with 23 to $27 \mu\text{g}$ per ml in Tris-NaCl buffer. The T_m of 70°C for L-cell DNA increased in the presence of actinomycins IV, Pip 1β, Pip 1α, and Pip 2, to 82 , 75 , 75 , and 72°C , respectively (Fig. 9A). In $0.1 \times \text{SSC}$ (Fig. 9B), the T_m of L-cell DNA was slightly higher (71°C), and the addition of actinomycin IV, Pip 1β, Pip 1α, or Pip 2 increased the T_m to 85 , 76 , 72 , and 72°C , respectively. The difference in melting behavior seen in the two buffer systems may reflect the difference in the ionic strength of the buffers or the effect of a change in pH which occurs upon heating Tris buffer.

DISCUSSION

The actinomycins employed previously for studies on the mechanism of action of the antibiotic have differed chiefly at the imino acid site of the molecule (33). In particular, the variation has involved compounds of the pyrrolidine carboxylic acid series, e.g., proline, hydroxyproline, oxoproline. The actinomycins used in the present study differ in that proline is replaced by the higher analogue of proline, piperidine-2-carboxylic acid (pipecolic acid). These actinomycins were synthesized by *S. antibioticus* during growth in a medium containing pipecolic acid. As described in more detail elsewhere, actinomycin Pip 2 contains two residues of pipecolic acid and no proline, actinomycin Pip 1β contains one residue each of proline and pipecolic acid, and actinomycin Pip 1α possesses one molecule of pipecolic acid and a second imino acid, possibly a form of hydroxyproline.

equal volume of cold 10% PCA. RNA, DNA, and protein were extracted and assayed as described in Materials and Methods.

FIG. 6. Sedimentation of cellular RNA. Replicate 150-ml suspensions of *L* cells (4.2×10^6 cells/ml) were incubated in the presence (B) and absence (A) of actinomycin Pip 1 α (1 μ g/ml) and uridine-2- 14 C as described in Results. RNA was extracted with phenol, dissolved in 2 ml of acetate buffer, and layered onto a 15 to 30% sucrose density gradient prepared in acetate buffer (MgCl₂ omitted). After centrifugation at 25,000 rev/min for 18 hr in a Spinco SW-25.1 rotor, samples were collected through a flow cell and the optical density at 260 m μ was continuously recorded (solid line). A 2-mg amount of carrier RNA was added to each fraction, and the RNA was precipitated by the addition of an equal volume of cold 5% PCA. The precipitate was collected by centrifugation, dissolved in 0.3 N NH₄OH, and plated for radioactivity measurements.

TABLE 3. Effect of actinomycin on virus replication^a

Virus	Actino- mycin concn	Actinomycin Pip 1 α		Actinomycin Pip 1 β		Actinomycin Pip 2		Actinomycin IV	
		Titer	Percentage inhibition	Titer	Percentage inhibition	Titer	Percentage inhibition	Titer	Percentage inhibition
Vac- cinia	μ g/ml								
	0.0	360×10^5	—	360×10^5	—	360×10^5	—	360×10^5	—
	0.1	29×10^5	92	40×10^5	89	11×10^5	97	10×10^5	97
	0.5	3×10^5	99.2	1×10^5	99.7	1×10^5	99.7	3×10^5	99.2
Reo	1.0	7×10^5	98	5×10^5	98.6	7×10^5	98.2	2×10^5	99.4
	0.0	13×10^7	—	13×10^7	—	13×10^7	—	13×10^7	—
	0.1	13×10^7	0	19×10^7	0	9.6×10^7	26	13×10^7	0
	1.0	8.1×10^7	38	1.9×10^7	85	5.2×10^7	60	2.2×10^7	83
Polio	0.0	28×10^8	—	28×10^8	—	28×10^8	—	28×10^8	—
	2.0	30×10^8	0	32×10^8	0	20×10^8	28	4×10^8	85

^a The titers measured before the appearance of progeny virus were as follows. Vaccinia virus: 3 hr after infection, 9.7×10^5 . Reovirus: 4 hr after infection, 2.9×10^6 . Poliovirus: 1 hr after infection, 2×10^6 .

Actinomycin IV has been shown to inhibit DNA-directed RNA synthesis but is inhibitory to DNA synthesis only at much higher concentrations (16, 33). The biological activity of actinomycin is believed to be due to its ability to bind to helical DNA (11), its binding capacity paralleling the guanine content of the DNA preparation (11, 12). A model has been proposed to explain the mechanism of antibiotic action (15). According to this model, actinomycin

binds to the guanine in the minor groove of helical DNA so that it is unavailable as a template for RNA synthesis. It has been postulated that under these conditions actinomycin can form up to seven hydrogen bonds. These include the guanine amino group hydrogen-bonded to the quinoidal oxygen of the actinomycin chromophore and the amino group of the chromophore bonded to the ring nitrogen (N₃) of guanine and to the oxygen bridge of deoxyribose. It has been

FIG. 7. Inhibition of *Escherichia coli* DNA-dependent RNA polymerase. The reaction mixture (55 μ liters) contained: 20 μ moles each of adenosine triphosphate, cytidine triphosphate, uridine triphosphate, and 3 H-guanosine triphosphate (20 mc/mmole), 2,000 μ moles of tris(hydroxymethyl)aminomethane-chloride buffer, pH 7.9, 600 μ moles of 2-mercaptoethanol, 50 μ moles of $MnCl_2$, 200 μ moles of $MgCl_2$, 13.5 μ g of L-cell DNA, and 0.5 μ g of RNA polymerase (1,800 units/mg). The tubes were incubated at 37 C for 15 min, and the reaction was terminated by the addition of 1 ml of cold 5% trichloroacetic acid. The acid-insoluble material was collected on a 25-mm nitrocellulose membrane and washed several times with 5% trichloroacetic acid. The membranes were dried and counted in a liquid scintillation counter.

suggested that the lactone rings in actinomycin act to stabilize the peptide chains in a conformation which permits the formation of four additional H-bonds between the four peptide-NH groups and the phosphodiester oxygens of the DNA strand opposite the guanine which interacted with the chromophore. The amino acid composition of both peptide chains probably contributes to the biological activity of the actinomycin molecule; e.g., the replacement of proline by hydroxyproline can change the minimal inhibitory concentration in certain biological systems by a factor of 50 (33). The small differences in amino acid composition of different actinomycins is thought to result in minor conformation changes of the peptide, and these changes are considered responsible for the different biological activity of the various actinomycins (34).

The pipecolic acid-containing actinomycins (i) inhibit the growth of gram-positive bacteria but not gram-negative microorganisms (Table 2), (ii) preferentially block RNA synthesis in both *B. subtilis* and mouse fibroblast cells (Fig. 3 and 4), and (iii) inhibit *E. coli* DNA-dependent RNA polymerase in vitro (Fig. 7). Further, elongated,

FIG. 8. Difference spectra of the actinomycins. Solutions of an actinomycin preparation were prepared in Tris-NaCl buffer, and the absorbance between 350 and 600 $m\mu$ was measured in the presence or absence of a DNA preparation. Concentration of DNA (μ g per ml): L-cell, 37; *Bacillus subtilis*, 33; *Pseudomonas aeruginosa*, 34. Concentration of the actinomycins (μ g/ml): Pip 1 α , 36; Pip 1 β , 37; Pip 2, 42; IV, 42.

vacuolated, and beaded cells of *B. subtilis* were observed when subinhibitory concentrations of the antibiotics were employed. Similar findings have been reported with actinomycin IV (16). A comparison of the pipecolic acid-containing actinomycins with actinomycin IV reveals that significant quantitative differences in biological activity and complex formation with DNA were generally observed. The effect of replacing one

FIG. 9. Thermal denaturation of actinomycin-DNA complexes in Tris-NaCl buffer (A) and in $0.1 \times$ SSC (B). *L*-cell DNA, 22 μ g per ml. Concentration of the actinomycins, in μ g per ml: (A) Pip 1 α , 27; Pip 1 β , 23; Pip 2, 27; IV, 23. (B) Pip 1 α , 54; Pip 1 β , 46; Pip 2, 54; IV, 46.

proline residue with pipecolic acid (actinomycin Pip 1 β) is apparently minimal with regard to biological activity. With respect to the binding to DNA, such a substitution results in an actinomycin molecule which is not as effective as actinomycin IV in stabilizing the DNA helix to the denaturing effects of heat (Fig. 9). However, changes in the absorption spectrum of actinomycin Pip 1 β in the presence of DNA resemble those obtained with actinomycin IV (Fig. 8). Replacing both proline residues with pipecolic acid (actinomycin Pip 2) results in a 5- to 20-fold reduction of biological activity and a significantly reduced ability to bind to DNA, as judged by thermal denaturation and difference spectra. Although the chemical nature of actinomycin Pip 1 α has not been fully elucidated, it is evident that the presence of pipecolic acid (and possibly a hydroxyproline derivative) further decreases the biological activity of the actinomycin molecule. The nature of the unidentified imino acid in actinomycin Pip 1 α is presently under investigation. Although it behaved like 4-hydroxyproline on a Moore and Stein column, a preliminary investigation by two-dimensional paper chromatography revealed that the unknown compound was not one of several hydroxyprolines or hydroxypipecolic acids investigated. The presence of a hydroxy imino acid in the antibiotic molecule is consistent with a decrease in biological activity, as noted previously with actinomycin I (20).

The binding of actinomycin to DNA is prob-

ably responsible for the biological properties of an actinomycin; however, thermal denaturation changes and difference spectra alone do not permit one to predict the biological effectiveness of an actinomycin preparation. Reich et al. (33) observed that the peak extinction of actinomycin I, X₀₈, and X₀₈ acetate were depressed as efficiently as actinomycin IV; however, actinomycin IV was shown to possess a 5- to 10-fold greater biological activity. In the present studies, the relationship for biological activity of the actinomycins (IV = Pip 1 β > Pip 2 > Pip 1 α) does not appear to hold completely for either the elevation of T_m or the effect of DNA upon the antibiotic absorption spectrum. More sensitive methods of measuring binding (e.g., equilibrium dialysis) may prove useful in relating binding capacity to biological activity.

The actinomycin peptides may or may not be symmetrical (Fig. 1); consequently, the composition of each peptide chain may be of considerable importance with respect to biological activity and binding to DNA. In the case of actinomycin Pip 1 β , it is not known whether the pipecolic acid is located in the A or B chain. Determination of its position might provide a better understanding for the biological behavior noted with actinomycins IV and Pip 1 β .

ACKNOWLEDGMENTS

A portion of this investigation was taken from a thesis presented by the senior author in partial fulfill-

ment of the requirements for the Ph.D. degree in the Department of Microbiology, Georgetown University Schools of Medicine and Dentistry, Washington, D.C.

A preliminary report was presented at the 67th Annual Meeting of the American Society for Microbiology, New York, N.Y., 30 April–4 May, 1967.

This investigation was supported by Public Health Service research grant CA 06926 from the National Cancer Institute and training grant TI AI 298 from the National Institutes of Health.

This paper is dedicated to Professor Selman A. Waksman on the occasion of his eightieth birthday.

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