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Production, Isolation, and Properties of Azetomycins

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Streptomyces antibioticus synthesizes five actinomycins that differ in the "proline site" of the molecule. When cultured in the presence of azetidine-2-carboxylic acid (AzC), antibiotic synthesis was stimulated 40 to 50%, synthesis of actinomycin IV was inhibited, and one or both prolines were replaced by AzC. AzC incorporation could not be reversed by concomitant supplementation with proline or sarcosine, and only pipecolic acid affected a minor reversal of AzC incorporation. AzC-containing actinomycins were isolated and designated azet-I and azet-II; a third unresolved component or mixture was called azet-III. The molar ratio of AzC to proline was: azet-I, 1:1; azet-II, 2:0. Azet-III was equivocal. These azetidine actinomycins (azetomycins) were found to be potently inhibitory to the growth of selected gram-positive but not as potent to the growth of gram-negative organisms. The relative inhibitory affect against growth and ribonucleic acid synthesis in *Bacillus subtilis* was: actinomycin IV ≥ azet-I > azet-II >>> azet-III. Protein synthesis was affected similarly; however, kinetic studies with *B. subtilis* revealed that ribonucleic acid synthesis was inhibited rapidly followed by an inhibition of protein synthesis. At concentrations less than 1 μg/ml, deoxyribonucleic acid synthesis was stimulated by these actinomycins.

The controlled biogenesis of actinomycins by *Streptomyces* species has been investigated to determine (i) the essential structural features required of an amino acid for incorporation into the antibiotic molecule, and (ii) how such changes modify biological activity of actinomycin. Controlled biogenesis studies not only lend insight into the biosynthesis of polypeptide antibiotics but, in addition, generate new actinomycins that can be evaluated for carcinostatic activity and relative toxicity. Toxicity is the major drawback to the widespread clinical use of actinomycin IV (D) in the treatment of certain human malignancies.

Streptomyces antibioticus, when cultivated in a galactose-glutamic acid-mineral salts medium, produces five actinomycin components that differ solely at the 3' position ("proline site") of the molecule (Fig. 1). The composition of this mixture can be modified by supplementing cultures either with natural precursors of the antibiotic or with proline analogues. For instance, sarcosine supplementation will increase the synthesis of the minor components (actinomycins II and III) from 5 to 60% of the total mixture (10). These increases occur at the expense of actinomycin IV synthesis. Supplementation with proline analogues may result in the synthesis of new families of actinomycins at the expense of the five components synthesized normally (4, 9). Modification of biological activity of actinomycin by sarcosine, hydroxyproline, or pipecolate substitution in the molecule has been reported (5, 15).

The study presented here describes the biogenesis and some biological properties of actinomycins containing azetidine-2-carboxylic acid (AzC). It was demonstrated that not only does AzC supplementation stimulate antibiotic production, but AzC is also preferentially incorporated into the actinomycin molecule. Neither proline nor sarcosine, when added concomitantly, could reverse AzC incorporation. By contrast, pipecolic acid did compete to a small extent with AzC for incorporation. As with other actinomycins investigated, the primary inhibitory effect of the azetidine actinomycins (azetomycins) appears to be on ribonucleic acid (RNA) synthesis. Substituting one or both prolyl residues with sarcosyl, hydroxyprolyl, or pipecolyl residues resulted in reduced biological activity of actinomycin. By contrast, an equivalent substitution with AzC produced highly potent antibiotics.

MATERIALS AND METHODS

Chemicals and radiochemicals. [G-^{3}H]uridine (5 Ci/mmol), [*methyl*-^{3}H]thymidine (2 Ci/mmol), and ^{3}H-labeled amino acid hydrolysate (16 Ci/g) were purchased from New England Nuclear Corp., Boston, Mass. AzC was purchased from Calbiochem,

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San Diego, Calif. L-Proline and sarcosine were purchased from Sigma Chemical Co., St., Louis, Mo. L-Pipecolate was synthesized by the PtO_2 reduction of picolinate and resolved as the tartrate. It shows an $[\alpha]_{20}^{D}$ of $-23°$. Other chemicals and reagents were of the highest quality commercially available.

Production and isolation of actinomycin. *S. antibioticus* strain 3820 was first cultivated in NZ amine medium (Sheffield Chemical Co., Norwich, N.Y.) for 48 h at 28 C, harvested, and washed by centrifugation. The sediment, resuspended in 0.9% saline, was used to inoculate the production medium composed of glutamic acid, galactose, and mineral salts (13). After growth for 24 h at 28 C, AzC, sarcosine, pipecolic acid, or proline was added as indicated. Antibiotic production was monitored spectrophotometrically at 442 nm (12).

Actinomycin mixtures were extracted with ethyl acetate from culture filtrates, and the individual components were resolved by either circular or descending paper chromatography. The colored bands were subsequently eluted from the paper and the concentration was determined spectrophotometrically (12).

Chromatography. Separation and identification of actinomycin components was accomplished by circular paper chromatography using Whatman no. 1 filter paper and the solvent system 10% aqueous sodium *o*-cresotinate–ethyl acetate–butyl ether (4:3:1, vol/vol/vol). For preparative purposes, descending paper chromatography was employed using Whatman 3MM paper and the above solvent system. Recovery of the resolved components was achieved by cutting out the colored zones and eluting the material with 90% methanol. After evaporating to dryness, the actinomycin was partitioned into chloroform. Homogeneity of the components was verified by circular paper chromatography in two additional solvent systems: 10% aqueous sodium *o*-cresotinate–butyl acetate–butyl ether (4:3:1) and 10% aqueous sodium *o*-cresotinate–butanol–butyl ether (5:2:3).

Identification of amino acids. Individual actinomycin components were hydrolyzed for 20 h in 5 N barium hydroxide at 121 C in screw-capped (Teflon lined) test tubes (7). The hydrolysates were neutralized with sulfuric acid. Individual components were also hydrolyzed in 6 N HCl for 3 h under similar conditions and processed as described previously (12). These hydrolysates were subjected to two-dimensional thin-layer chromatography on cellulose-coated plates. The plates were developed with butanol–ethanol–3% aqueous ammonia (35:35:30) in one direction and with butanol-pyridine-water (1:1:1) in the other direction. The amino acids were visualized with ninhydrin or isatin reagent sprays and identified by comparison with authentic amino acid samples. In addition, samples of the alkaline hydrolysates were examined in a Beckman automatic amino acid analyzer, model 118. Amino acids were identified from previously established retention times and quantitated by integrating to determine the area under the curve of the tracings.

Test organisms and conditions of cultivation. The following organisms were obtained from the culture collection of the Department of Microbiology, University of California Medical Center, San Francisco: *Bacillus subtilis, Staphylococcus aureus, S. epidermidis, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella paratyphi* A. The bacteria were grown on Trypticase soy agar slants (BBL) at 37 C and stored at 4 C. For the determination of the minimum inhibitory concentration (MIC) of the actinomycins, the organisms were cultivated in tryptose phosphate broth (TPB) and diluted 1:1,000 with TPB before use. Exponentially growing cultures of *B. subtilis* were prepared from cultures incubated overnight in TPB and subsequently harvested by centrifugation at 25 C. The sediment was suspended to the original volume with warmed TPB and, after being diluted 1:10 with warmed medium, the culture was incubated at 37 C in a reciprocal shaking water bath. Growth was monitored turbidometrically in a Klett-Summerson colorimeter (filter no. 66).

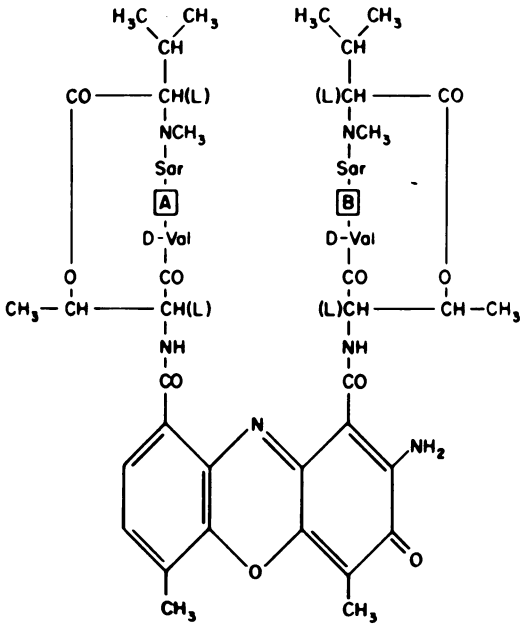

FIG. 1. *Structure of actinomycin. The sequence of amino acids is; L-threonine, D-valine (D-Val), A and B, sarcosine (Sar), N-methyl-L-valine. Actinomycin I: A = L-proline, B = 4-hydroxy-L-proline; actinomycin II: A = B = sarcosine; actinomycin III: A = sarcosine, B = L-proline; actinomycin IV: A = B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

Assays. The broth dilution method (1) was used to determine the MIC of the various actinomycins.

For the measurement of biopolymer synthesis, a TPB culture of *B. subtilis* in the exponential phase of growth was incubated with an actinomycin preparation and the appropriate ^3H percursor as described in Results. The reaction was terminated by the addition of cold 10% perchloric acid, and the total nucleic acids were fractionated according to the procedure of Schmidt and Thannhauser (19). Total deoxyribonu-

cleic acid (DNA) and RNA content was determined by the diphenylamine (3) and orcinol assays (17), respectively. Protein content was determined by the method of Lowry et al. (14). Radioactive content of nascent DNA, RNA, and protein as acid-precipitable polymers was determined in samples dissolved in Scintiverse (Fisher) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Production of the azetomycins. AzC was added at various concentrations (10 to 250 μg/ml) to cultures of *S. antibioticus* 24 h after incubation in production medium. All levels of AzC used stimulated antibiotic production (Fig. 2); however, at the 100- and 250-μg/ml concentrations, production was delayed for 2 days and then went on to exceed the control values. At the end of the 7-day incubation period the antibiotic titer exceeded 100 μg/ml as compared with 70 μg/ml in the control flasks.

Time course analysis and composition of actinomycin mixtures. The delay in production and the subsequent stimulation of production above control values prompted an investigation of the time course of synthesis of the components in the mixture. Actinomycin mixtures extracted from AzC-supplemented and unsupplemented cultures were examined by circular paper chromatography, and the components were quantitated as described. Table 1 lists the percent composition of the actinomycins harvested on days 3, 5, and 7. In the absence of AzC the amount of actinomycin IV produced constituted approximately 70% of the yield. The remainder was composed of actinomycins I, II, and III (data not shown) (9). Even at low concentrations of AzC, the production of actinomycin IV was greatly reduced whereas actinomycin V was barely detectable. The three novel components, which have been designated azetomycin I (azet-I), azetomycin II (azet-II), and azetomycin III (azet-III), made up the bulk of the yield. An examination of the 3rd- and 5th-day harvests revealed that the percentage of azet-I in the mixture of the 5th-day harvest exceeded the 3rd-day harvest, whereas the reverse was true of azet-II, at the three concentrations of AzC supplementation used. By contrast, the percentage of azet-III in the mixture was relatively constant. By day 7 of incubation the percent composition stabilized, showing a slight increase in actinomycin IV synthesis.

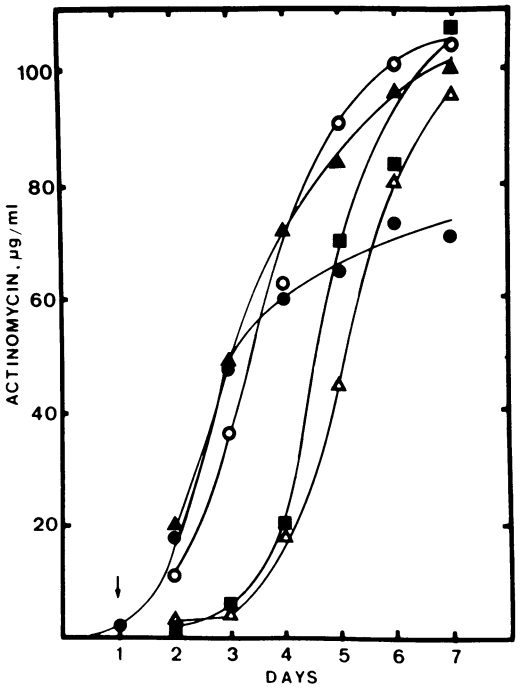

FIG. 2. *Effect of azetidine-2-carboxylic acid on actinomycin production by S. antibioticus. The organism was cultivated as described. After 24 h of growth the following concentrations of azetidine-2-carboxylic acid were added: 0 (●); 10 μg/ml (○); 50 μg/ml (▲); 100 μ/ml (■); 250 μg/ml (△). Cultures were harvested on the days indicated.*

Analogue competition. The inhibition of production of actinomycin IV indicated that proline incorporation into the antibiotic molecule was being inhibited. It seemed reasonable to determine whether this inhibition could be relieved by adding proline to cultures supplemented with AzC (10, 11). Even a fivefold excess of proline was incapable of preventing azetomycin synthesis (Table 2). In fact, there appeared to be an additional stimulation of azetomycin synthesis.

Sarcosine and pipecolic acid, two other amino acids known to compete with proline for incorporation, were added individually to AzC-supplemented cultures. Only pipecolate at the higher concentration was able to affect azetomycin synthesis to a slight extent (Table 2). Sarcosine supplementation inhibited actinomycin IV production and stimulated actinomycin II and III synthesis (data not shown) as expected (11); however, sarcosine was without effect on azetomycin synthesis when added to AzC-supplemented cultures.

Amino acid composition. Approximately 500 mg of crude azetomycin was prepared from 4 liters of culture filtrate. The mixture was readily resolved into four components (actinomycin IV, azet-I, azet-II, and azet-III) by descending paper chromatography, and the components were isolated as described. Since AzC

TABLE 1. *Composition of actinomycins produced by Streptomyces antibioticus in the presence of AzC*

AzC (μg/ml)	Percent composition of actinomycin components														
	Day 3					Day 5					Day 7				
	Azet-I	Azet-II	Azet-III	Act IV	Act V	Azet-I	Azet-II	Azet-III	Act IV	Act V	Azet-I	Azet-II	Azet-III	Act IV	Act V
0[a]				69.4	19.8				70.0	10.0				72.0	8.0
50	12.8	78.0	6.4	2.8	tr[b]	32.0	57.8	5.7	4.4	tr	31.8	52.5	6.2	9.4	tr
100	19.4	72.5	4.1	4.1	tr	27.4	62.3	5.8	4.6	tr	30.9	57.0	5.8	6.4	tr
250	14.9	78.0	5.5	tr	tr	30.1	60.2	5.6	4.1	tr	30.4	58.3	5.6	5.8	tr

[a] For clarity of presentation, the percentages for actinomycins (Act) I, II, and III were not listed.
[b] tr, Trace.

TABLE 2. *Effect of proline analogue combinations on the composition of actinomycins*

Proline (μg/ml)	Sarcosine (μg/ml)	Pipecolate (μg/ml)	Azetidine carboxylate (μg/ml)	Family of actinomycin (%)[a]		
				Act IV	AzC-Act	PA-Act
0	0	0	0	77.0		
0	0	0	100	5.3	85.0	
100	0	0	100	3.0	97.0	
500	0	0	100	3.7	96.0	
0	0	100	0	29.0		69.0
0	0	100	100	9.0	86.0	4.0
0	0	500	100	9.0	72.0	19.0
0	100	0	0	46.0		
0	100	0	100	2.6	97.5	
0	500	0	100	2.2	97.8	

[a] Act, Actinomycin; PA, pipecolate.

is readily destroyed by acid hydrolysis (6) and hydroxyamino acids such as threonine are destroyed by alkaline hydrolysis (2), samples of the chromatographically homogeneous components were subjected to both alkaline and acid hydrolysis. Both kinds of hydrolysates were examined by two dimensional thin-layer chromatography as described. In addition to *N*-methylvaline, valine, sarcosine, and threonine, azet-I and -III hydrolysates were found to contain both proline and AzC, whereas azet-II contained AzC. Proline was not detected. In addition, samples of the alkaline hydrolysates were processed in an amino acid analyzer. This analysis verified the presence of proline and AzC, which were subsequently quantitated. Azet-I contained 0.5 mol each of proline and AzC per mol of valine (Table 3). Azet-II contain approximately 1 mol of AzC per mol of valine and no proline, whereas the azet-III mixture appeared to have an AzC-to-proline ratio of 4:1 per mol of valine. Actinomycin IV hydrolysate contained 1 mol of proline per mol of valine, as expected.

Biological properties of the azetomycins: determination of MIC. A study of the antimicrobial properties of these actinomycins revealed that azet-I and azet-II were 80 and 70%

TABLE 3. *Imino acid content of actinomycin hydrolysates*

Actinomycin	Mol of imino acid/mol of valine	
	Proline	Azetidine-2-carboxylate
IV	0.985	0
Azet-I	0.515	0.493
Azet-II	0	0.77
Azet-III	0.144	0.59

as active, respectively, as actinomycin IV against *B. subtilis*, whereas azet-III was 20-fold less active. The same relative ranking obtained for the other gram-positive organisms tested (Table 4). All four actinomycins were essentially without effect against the gram-negative organisms tested, although the MIC for both azet-I and actinomycin IV was approximately 200 μg/ml for *E. coli*.

Stability of azetomycin solutions. The stability of the azetidine actinomycins was of particular concern because of the strained ring structure of AzC. An instability could result in erratic biological actively data. To test this point, the following experiment was conducted.

Actinomycins were dissolved in 0.05 M potassium phosphate buffer prepared in 0.9% sodium chloride at pH values of 5 through 8. MIC determinations were performed on (i) the same day the solutions were prepared, (ii) after storage for 24 h at 4 C, and (iii) after storage for 1 week at 4 C. No differences in MIC values were found when the time of storage or the pH value was varied. The MICs obtained were the same as those listed in Table 4. It was concluded that these antibiotics were stable under the experimental conditions used and that the azetidine ring is stable when incorporated into the new antibiotic molecule.

Effect on exponentially growing cultures of B. subtilis. An exponentially growing culture of *B. subtilis* was prepared as described in Materials and Methods, and the actinomycins were added when the cultures entered the exponential phase of growth. Within minutes of this addition there was a diminution in the rate of growth, as evidenced by a less rapid increase in turbidity; however, none of the actinomycins completely inhibited growth even at concentrations of 5 μg/ml (Fig. 3). Probit analysis of these data revealed that actinomycins IV, azet-I, and azet-II caused a 50% inhibition of growth at concentrations of 0.1, 0.14, and 0.14 μg/ml, respectively, whereas azet-III had a similar effect at 0.84 μg/ml.

Effect on DNA, RNA, and protein synthesis. Exponentially growing cultures of *B. subtilis* were prepared as described, to which were added an actinomycin component and the appropriate radioactive biopolymer precursor. Probit analysis of the data listed in Fig. 4 showed that azet-I and actinomycin IV inhibited the synthesis of RNA and protein by 50% at a concentration of 0.2 μg/ml. Comparable affects with azet-II were seen at 0.3 μg/ml. At lower concentrations these actinomycins were stimulatory to DNA synthesis (Fig. 4). Azet-III was the least

TABLE 4. *Antimicrobial activity of the actinomycins*

Organism[a]	Minimal inhibitory concentration (μg/ml)			
	Azet-I	Azet-II	Azet-III	Act IV
Bacillus subtilis	0.030	0.035	0.64	0.025
Staphylococcus aureus	0.15	0.15	1.25	0.1
S. epidermidis	0.4	0.4	2.0	0.4
Escherichia coli	200	>200	>200	≥200

[a] *Salmonella paratyphi* A and *Pseudomonas aeruginosa* were not inhibited at a concentration of 200 μg/ml.

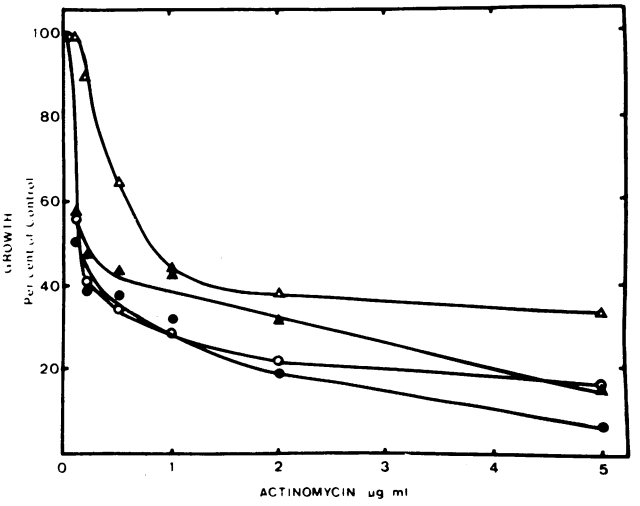

FIG. 3. *Effect of actinomycin IV, azet-I, azet-II, and azet-III on exponentially growing cultures of B. subtilis. Cultures were prepared as described and the drugs were added at the beginning of the exponential phase of growth. Turbidometric readings were taken every 15 min and plotted as a function of time. The slope of growth curve, at a given concentration of drug, was compared with control cultures (no drug) and expressed as a percentage. These data were then plotted as a function of drug concentration. Actinomycin IV (●); azet-I (○); azet-II (▲); azet-III (△).*

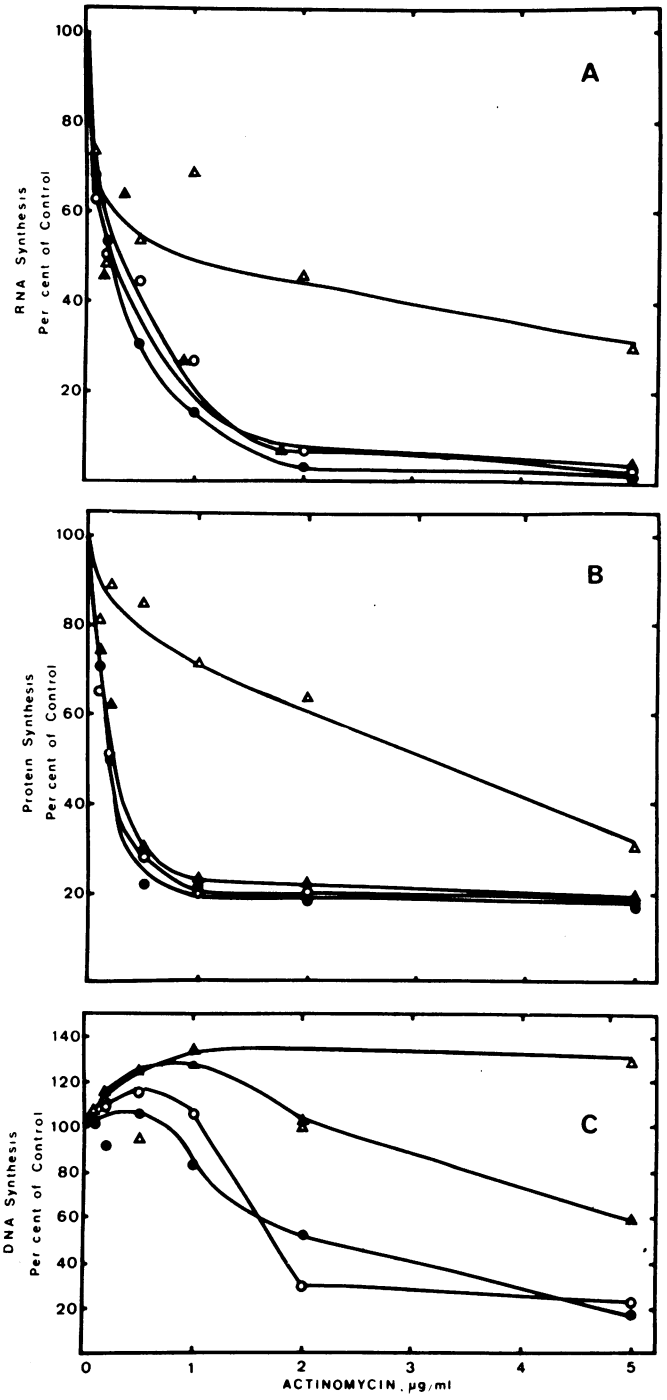

FIG. 4. *Effect of various concentrations of actinomycin IV, azet-I, azet-II, and azet-III on DNA, RNA, and protein synthesis. An exponentially growing culture of B. subtilis was added to flasks containing various concentrations of actinomycin IV (●), azet-I (○), azet-II (▲), or azet-III (△) and 1 µCi of [^{3}H]uridine (A), ^{3}H-labeled amino acid hydrolysate (B), or [methyl-^{3}H]thymidine (C) per ml. After 30 min of incubation, the reaction was terminated by the addition of an equal volume of cold 10% perchloric acid. RNA, DNA, and protein were extracted and assayed as described in the text.*

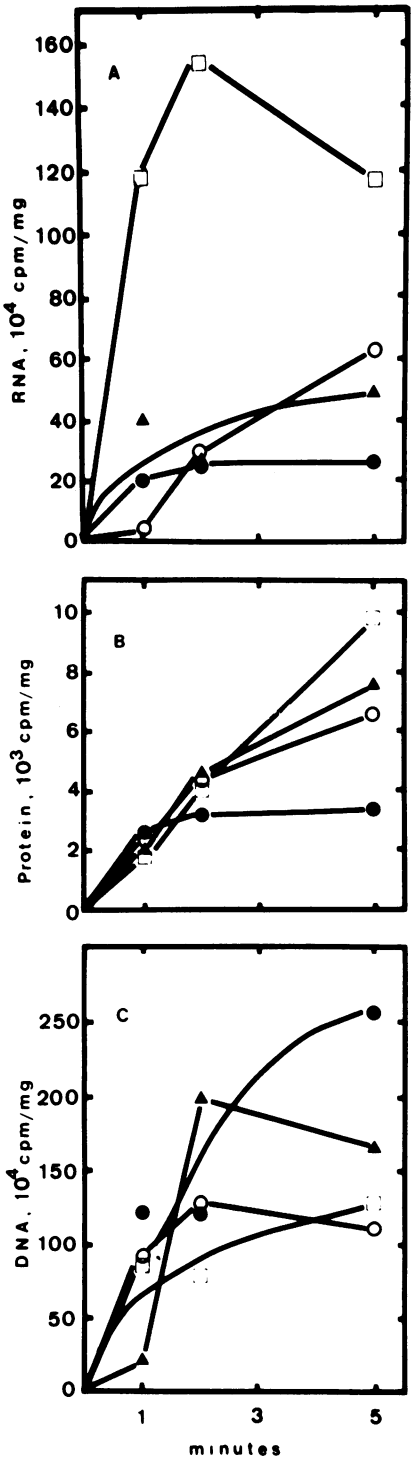

FIG. 5. *Time course of inhibition of DNA, RNA, and protein synthesis. An exponentially growing culture of B. subtilis was added to flasks containing 0.5 μg of actinomycin IV (●), azet-I (○), or azet-II (▲) per ml, or no drug (□), and 1 μCi of [^{3}H]uridine (A), ^{3}H-labeled amino acid hydrolysate (B), or [methyl-^{3}H]thymidine (C) per ml. The reaction was terminated as described after 1, 2, and 5 min of incubation.*

effective in inhibiting RNA and protein synthesis, requiring at least a fivefold greater amount to effect a 50% inhibition as compared with the other three actinomycins. In addition, azet-III was stimulatory to DNA synthesis at the concentrations tested.

Time course of biopolymer synthesis. The results listed in Fig. 4 could not be used to identify the primary effect of these actinomycins on biopolymer synthesis; consequently, a time course analysis was performed. Drug and biopolymer precursor were added to exponentially growing cultures of *B. subtilis* as described. The reactions were stopped at the intervals indicated in Fig. 5. The results obtained indicate that RNA synthesis is inhibited within 1 min of incubation in the presence of azet-I, azet-II, or actinomycin IV, whereas the inhibition of protein synthesis requires an additional minute of incubation before an inhibitory effect is seen (Fig. 5). The effect on DNA was erratic under these experimental conditions; however, the data did indicate that the primary effect of the azetomycins was on RNA synthesis.

DISCUSSION

The actinomycins produced by *S. antibioticus* contain 2 mol of L-valine per mol of antibiotic (8, 18). An interpretation of the data listed in Table 3 would indicate that azet-I contains 1 mol each of AzC and proline per mol of antibiotic. Similarly, azet-II contains 2 mol of AzC per mol of actinomycin. In light of the inhibition of actinomycin IV synthesis by AzC, these data could also indicate that one proline has been replaced by AzC in azet-I and that both prolines were replaced in azet-II. This analysis could explain the initial majority product as azet-II, assuming that the excess amount of AzC initially forced the incorporation of 2 mol of AzC. As the concentration of AzC diminished, only one proline was replaced in the antibiotic, resulting in the synthesis of azet-I. The overall stimulation of antibiotic synthesis seen in the presence of AzC could be due to the inhibition by this proline analogue of protein synthesis (7), thus making the endogenous amino acid pool more available for antibiotic synthesis.

The analytical results for azet-III do not lend themselves to this kind of explanation since they do not fall into a pattern reminiscent of other actinomycins. We have concluded that, in spite of demonstrating chromatographic homogenicity in three solvent systems, the azet-III

component is a mixture of actinomycins.

AzC, sarcosine, or pipecolate supplementation of cultures resulted in the inhibition of actinomycin IV synthesis with the concomitant synthesis of actinomycins in which one or both prolines were replaced. Proline or sarcosine could not prevent AzC incorporation. By contrast, pipecolate supplementation demonstrated a small reversal in AzC incorporation; however, the amount of actinomycin IV produced under both conditions was about the same, indicating that AzC was being replaced by pipecolate and not proline. These relative inhibitory effects on incorporation suggest an affinity of the antibiotic-synthesizing system for these amino acids. The affinity ranking for incorporation appears to be: AzC > pipecolate > sarcosine > proline.

As can be seen from the data presented here and elsewhere (5, 15), the biological activities of actinomycin IV, azet-I, azet-II, and Pip 1β are quite similar in terms of MIC and inhibition of RNA synthesis. There is a profound loss of activity when both prolines are replaced by pipecolate (Pip 2) (5) or sarcosine (actinomycin II) or when one proline is replaced by hydroxyproline (actinomycin I) or sarcosine (actinomycin III) (see review, 15). By contrast, the loss in activity due to replacing one or both prolines with AzC as in azet-I and -II is negligible.

Although it is generally agreed that the primary effect of actinomycin is expressed through its binding to the DNA molecule and thus its prevention of RNA synthesis, the precise mechanism involved remains elusive. Sobell (20) has proposed a model of binding that involves both intercalation of the actinocin portion of the molecule with DNA and hydrogen binding of the pentapeptide moieties. Since even biologically inactive actinomycins intercalate (17), it is reasonable to assume that the pentapeptide moiety is responsible for the differences seen in biological activity among the actinomycins. The small differences in amino acid composition of different actinomycins result perhaps in minor conformational changes of the cyclic peptides that are expressed as a change in affinity or avidity of binding due to a change in the diameter of the cyclic pentapeptides. The magnitude of the conformational change caused by AzC incorporation should be established. The availability of several actinomycins substituted with proline analogues could lead to the establishment of dimensional limits of the cyclic pentapeptide required for retention of biological activity.

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