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Effect of Structural and Stereochemical Methylproline Isomers on Actinomycin Biosynthesis

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The inhibitory effect of methylprolines on actinomycin biosynthesis by *Streptomyces antibioticus* was studied; the order of effectiveness was 3- >4- >5-methyl-DL-proline. *Cis*-3-methyl-DL-proline was 14 times more active than the *trans* isomer. It was also found that 4- and, possibly, 5-methylproline were incorporated into the actinomycin molecule. When 4-methylproline was present, three new actinomycins, representing 50 to 60% of the antibiotic mixture, were synthesized. Growth of the organism may be stimulated at concentrations (0.1 to 1.0 μg per ml) of 3-methylproline that inhibit antibiotic formation, thus providing additional evidence for a different mechanism of actinomycin synthesis from that of protein synthesis. Azetidine-2-carboxylic acid, piperdine-2-carboxylic acid, and hydroxyproline (but not sarcosine) reversed the inhibition due to 3-methylproline.

The actinomycins synthesized by *Streptomyces antibioticus* (Fig. 1) differ solely in the imino acid residues which are present in the peptide portion of the molecule (7). Previous investigations showed that certain proline analogues, such as hydroxyproline, piperidine-2-carboxylic acid, and azetidine-2-carboxylic acid, as well as sarcosine, influence actinomycin synthesis by competing with and replacing endogenously formed proline in the actinomycin peptide (7–9). Consequently, the formation of certain trace or minor components is greatly enhanced, or new actinomycins are synthesized.

Because of our continued interest in the influence of proline analogues upon actinomycin synthesis, we examined the effect of 3-, 4-, and 5-methyl-DL-proline upon antibiotic formation by *S. antibioticus*.

MATERIALS AND METHODS

Organism and conditions of cultivation. S. antibioticus strain 3720 was used throughout this investigation. To produce actinomycin mixtures, the organism was first grown in NZ-amine medium (9) for 48 hr at 28 C on a rotary shaking machine. After the mycelium had been washed in saline, the organism was inoculated into a glutamic acid-galactose-mineral salts medium (9, 21).

Compounds. 3-Methyl-DL-proline and 4-methyl-DL-proline (isomeric mixtures) were obtained by chemical synthesis (4). Separation of 3-methyl-DL-proline into *cis* and *trans* racemates was carried out as described elsewhere (15). The 5-methyl-DL-proline was a gift from Herman Gershon. L-Proline, L-threonine, sarcosine, D-valine and other amino acids were obtained from commercial sources.

Mycelium dry weight. The mycelium from 200 ml of culture medium was harvested by filtration on a tared filter paper and washed twice with 100 ml of deionized water. After drying in an oven (100 to 105 C) overnight, the papers were cooled in a desiccator and were reweighed.

Isolation of actinomycin. An actinomycin mixture was extracted from the medium (*p*H 8.0) into ethyl acetate (1:1); after drying with sodium sulfate, the organic layer was evaporated to dryness under reduced pressure. Further purification of the actinomycin mixture produced with 4-methylproline was achieved by silicic acid chromatography (21).

For recovery of actinomycin-like compounds that are extractable from the medium into ethyl acetate at *p*H 2.5, the following procedure was employed. After extraction of actinomycin, an equal volume of ethyl acetate was added, and the medium was re-extracted. The organic layer was discarded, and the medium was acidified to *p*H 2.5 with hydrochloric acid and then extracted once with ethyl acetate (1:1). The ethyl acetate fraction was washed once with water (5:1) and was then evaporated to dryness.

Hydrolysis of actinomycin mixtures. An actinomycin preparation in methanol was added to a Teflon-lined screw-capped test tube, and the sample was evaporated to dryness in a stream of nitrogen. Then, 5 ml of 6 N HCl was added, the tube was tightly

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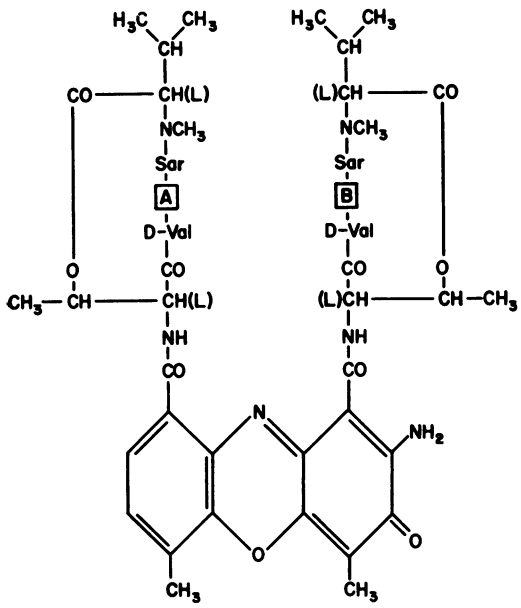

FIG. 1. *Structure of an actinomycin molecule. Actinomycin I: A,* L*-proline; B, 4-hydroxy-*L*-proline. Actinomycin II: A and B, sarcosine. Actinomycin III: A,* L*-proline; B, sarcosine. Actinomycin IV: A and B,* L*-proline. Actinomycin V: A,* L*-proline; B, 4-oxo-*L*-proline.*

capped, and the actinomycin preparation was hydrolyzed for 3 hr at 121 C and 15 lb of pressure.

After hydrolysis, a small amount of Norit A charcoal was added to remove humin-like material. The charcoal was removed by filtration over glass wool or by centrifugation and was then washed successively with 6 N HCl and water. The washings were combined with the hydrolysate, and the mixture was evaporated to dryness under reduced pressure. Water was added, and the sample was re-evaporated twice to remove excess acid.

Paper chromatography and high-voltage electrophoresis. Actinomycin mixtures were separated by circular paper chromatography by use of the solvent system: 10% aqueous sodium-*o*-cresotinate–dibutyl ether–*sym*-tetrachloroethane (3:2:1), as presented in an earlier publication (10).

For the separation of amino acids in actinomycin hydrolysates, we employed ascending paper chromatography on Whatman no. 1 paper in the following solvent systems: (a) *n*-butanol–acetic acid–phenol–water (30:10:10 g:50), (b) 77% ethanol, (c) *n*-butyl alcohol–water–formic acid (10:2:1), and (d) *t*-butyl alcohol–water–formic acid (5:1:1).

Separation of amino acids was achieved also by electrophoresis for 3 hr (Gilson Medical Electronics, Middleton, Wis.) at 4,600 v on Whatman no. 3MM paper impregnated with 4% formate buffer at H 1.9 (12).

Amino acids were observed by use of 0.2% ninhydrin in acetone; the imino acids were also detected with 0.2% isatin in acetone.

Assays. The concentration of actinomycin was determined spectrophotometrically at 443 mμ (12). We determined the relative amount of a component in an actinomycin mixture, separated by circular paper chromatography, as previously described (9).

Proline in hydrolysates was measured by the method of Troll and Lindsley (22). Valine was determined by the paper chromatographic technique of Naftalin (17).

RESULTS

*Effect of 3-, 4-, and 5-methyl-*DL*-proline upon actinomycin synthesis.* As shown in Fig. 2, synthesis of actinomycin was markedly inhibited by 3-, 4-, and 5-methyl-DL-proline. The order of the inhibitory effect of these compounds was 3- > 4- > 5-methylproline. A comparison of the activity of pure *cis*- and *trans*-3-methyl-DL-proline revealed that the *cis* isomer is approximately 14-fold more effective than the *trans* compound (Fig. 3).

The composition of an actinomycin mixture synthesized with one of the analogues present in the medium is given in Table 1. We observed quantitative and qualitative differences in the mixture that was formed. The actinomycin mixture produced in control flasks consisted primarily of actinomycin IV (79%), but when 3-, 4-, or 5-methylproline was employed synthesis of actinomycin IV was reduced. Moreover, synthesis of two or three new actinomycins occurred in the presence of 4-methylproline, whereas one new component, representing 12 to 15% of the mixture, was produced with 5-methylproline.

After purification, the actinomycin mixture (4-methylproline) was subjected to vigorous acid hydrolysis. In addition to sarcosine, valine, N-methylvaline, threonine, and proline, hydrolysates contained an imino acid. This compound possessed the identical electrophoretic mobility (28 cm) as authentic 4-methylproline, and its R_F value by paper chromatography (solvent a, 0.46; solvent b, 0.63; solvent c, 0.34; solvent d, 0.58) was also similar to that of 4-methylproline. The extent of incorporation of 4-methylproline into the actinomycins varied with the concentration of the analogue added to the medium. For example, when 4-methylproline was supplied at a concentration of 10 μg per ml, the actinomycin mixture contained a proline: 4-methylproline ratio of 1.0:0.8; however, when the analogue was present at 20 μg per ml, the ratio was 1.0:7.0.

*Effect of 3-methyl-*DL*-proline added prior to or during actinomycin synthesis.* The data presented in Fig. 4 reveal that 3-methylproline inhibited actinomycin synthesis when supplied prior to or during antibiotic synthesis. The decrease in anti-

biotic titer observed, after addition of the analogue, is probably caused by the destruction of the antibiotic previously synthesized. Because antibiotic synthesis is not resumed even after a prolonged period of incubation, it appears that the analogue is not metabolized by the organism.

Influence of 3-methyl-DL-proline upon growth of S. antibioticus. It was of interest to establish whether 3-methylproline directly inhibits some stage in actinomycin synthesis or exerts an indirect effect, following an initial inhibition of growth of the organism. The results of a number of experiments indicated that the inhibition of antibiotic production is a direct one. An inverse relationship exists between actinomycin formation and cell growth in the presence of various concentrations of 3-methylproline (24). For example, when 3-methyproline (1.0 μg per ml)

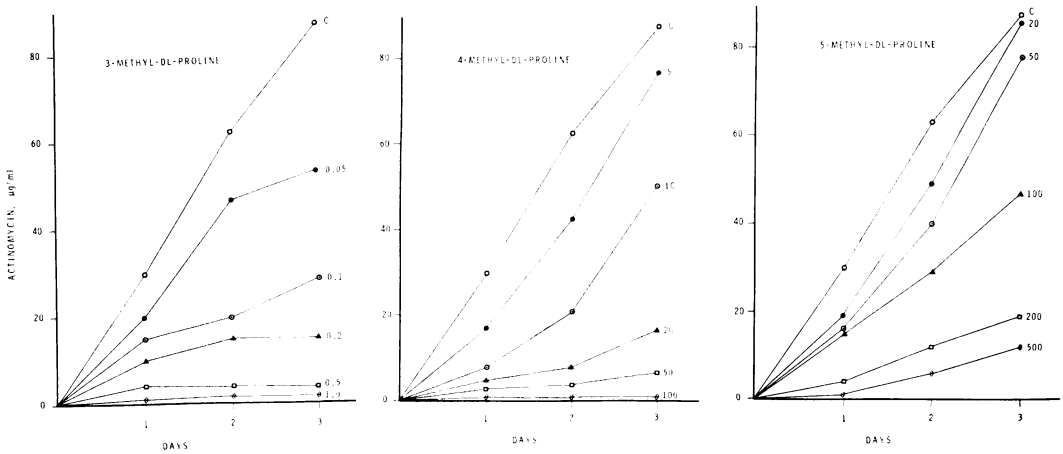

FIG. 2. *Effect of 3-, 4-, and 5-methyl-DL-proline on actinomycin biosynthesis. S. antibioticus was grown in a galactose-glutamic acid-mineral salts medium for 24 hr, and then the methylproline analogue was added to the medium. Concentrations of 3-methyl-DL-proline (μg/ml): ○, control; ●, 0.05; ⊙, 0.1; ▲, 0.2; □, 0.5; ⊖, 1.0. Concentrations of 4-methyl-DL-proline (μg/ml): ○, control; ●, 5; ⊙, 10; ▲, 20; □, 50; ⊖, 100. Concentrations of 5-methyl-DL-proline (μg/ml): ○, control; ●, 20; ⊙, 50; ▲, 100; □, 200; ⊖, 500.*

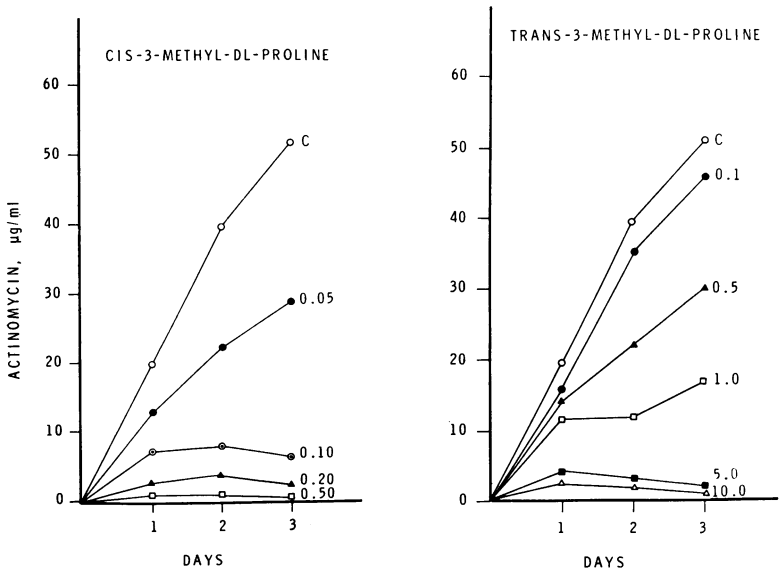

FIG. 3. *Effect of cis- and trans-3-methyl-DL-proline on actinomycin biosynthesis. Conditions as described for Fig. 2. Concentrations of the cis isomer (μg/ml): ○, control; ●, 0.05; ⊙, 0.1; ▲, 0.20; □, 0.50. Concentrations of the trans isomer (μg/ml): ○, control; ●, 0.1; ▲, 0.50; □, 1.0; ■, 5.0; △, 10.0.*

TABLE 1. *Influence of 3-, 4-, and 5-methyl-DL-proline on the composition of the actinomycin mixture synthesized*[a]

Compound	Concn (μg/ml)	Actinomycin titer (μg/ml)	Actinomycin component (%)				New components
			I	II and III	IV	V	
None	0	81	7.6	7.6	79.0	5.8	0
3-Methyl-DL-proline	0.1	29	16.3	13.5	62.5	7.7	0
	0.2	14	17.8	19.0	45.5	17.7	0
4-Methyl-DL-proline	5.0	77	7.6	7.5	65.6	0	19.3
	10.0	51	9.2	13.7	16.4	0	60.7
	20.0	17	22.8	18.4	8.9	0	50.0
5-Methyl-DL-proline	100.0	47	7.1	5.5	77.4	5.4	4.6
	200.0	19	15.0	4.2	63.4	5.7	11.7
	500.0	12	15.4	4.9	56.5	9.2	14.0

[a] *S. antibioticus* was grown as described in Materials and Methods. After 24 hr of incubation the appropriate methylproline analogue was added, and cultures were reincubated for an additional 72 hr. Actinomycin mixtures were extracted from the medium and chromatographed (*see* Materials and Methods).

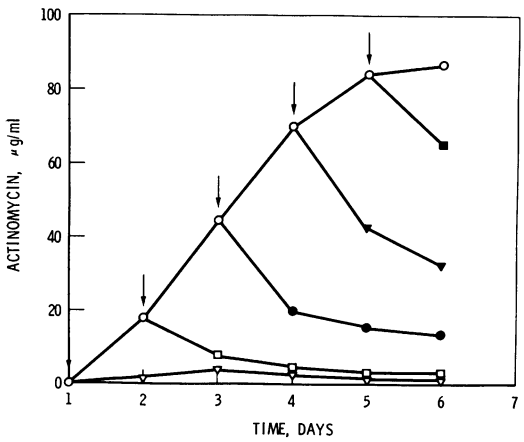

FIG. 4. *Effect of 3-methyl-DL-proline on actinomycin synthesis when added at different times. S. antibioticus was grown in glutamic acid-galactose-mineral salts medium for 1 day. 3-Methyl-DL-proline (1.0 μg per ml, final concentration) was added (↓) to each of two flasks at 1, 2, 3, 4, and 5 days. Incubation was resumed, and actinomycin titer was determined daily spectrophotometrically.*

was added at the onset of antibiotic formation, actinomycin production subsequently was inhibited by 95%; however, there was a fourfold increase in mycelial mass.

The enhanced growth obtained may be related to the absence of actinomycin rather than to the presence of the analogue. If 3-methylproline (0.05 to 1.0 μg per ml) was furnished at the time the organism was inoculated into the glutamic acid-galactose medium and the mycelium was harvested 24 hr later (prior to antibiotic synthesis), there was no appreciable difference in the amount of mycelium synthesized with or without the analogue. Stimulation of growth by 3-methylproline was noted only during the period when antibiotic formation normally occurs. 3-Methylproline, at concentrations of up to 100 μg per ml, did not affect the organism adversely; at 500 to 1,000 μg per ml, however, a 30 to 40% inhibition of mycelial formation was apparent. In contrast, a 50% inhibition of growth was achieved when actinomycin D (4 μg per ml) was added to freshly inoculated cultures.

Inhibition of synthesis of ethyl acetate-extractable, peptide-bound proline and valine. Further evidence that 3-methylproline blocks the synthesis of actinomycin or actinomycin-like peptides was obtained by determining the amount of peptide-bound proline and valine. Actinomycin was harvested from the culture medium by extraction with ethyl acetate at *p*H 8.0, whereas certain degradation products of actinomycin, such as actinomycinic acid, could be removed at *p*H 2.5. Analysis of hydrolysates of both the neutral and acid-extractable fractions revealed that there is little peptide-bound proline or valine in 3-methylproline-treated cultures (Table 2).

Reversal of 3-methyl-DL-proline inhibition with proline analogues. Exogenously supplied hydroxyproline, azetidine-2-carboxylic acid, and piperidine-2-carboxylic acid compete with endogenously formed proline and replace it in the antibiotic peptide (7–9). We carried out studies to establish

whether these compounds could reverse the inhibition of actinomycin synthesis obtained with 3-methylproline. The results given in Table 3 reveal that hydroxyproline and azetidine-2- and piperidine-2-carboxylic acids, at concentrations approximately 25 to 1,000 times greater than 3-methylproline, will significantly reverse the inhibition of antibiotic synthesis. Growth of the organism was not enhanced by the compounds used to reverse this inhibition. At 10,000-fold greater concentrations, sarcosine, which also can replace proline in the antibiotic peptide, was unable to prevent the inhibition.

Proline can also reverse the inhibition due to 3-methylproline. However, it is not fully clear whether the reversal is a direct one, since mycelial growth and antibiotic production are enhanced at high concentrations (1,000 to 2,500 μg per ml) of proline in the absence of 3-methylproline. Moreover, proline is rapidly metabolized by *S. antibioticus* so that concentrations effective for reversal of inhibition apparently are not maintained.

TABLE 2. *Peptide-bound proline and valine formed during antibiotic synthesis with 3-methylproline (3-MP)*[a]

Ethyl acetate fraction	Hr	Proline (μmoles/100 ml)		Valine (μmoles/100 ml)	
		−3-MP	+3-MP	−3-MP	+3-MP
Neutral	48	1.77	0.10	2.4	0.38
	72	4.10	0.05	4.5	0.30
	96	7.40	0.08	8.5	0.20
Acid	48	0.10	0.16	0.22	0.29
	72	0.75	0.26	0.7	0.18
	96	1.75	0.11	1.0	0.28

[a] 3-MP (1 μg per ml, final concentration) was added at 24 hr to cultures growing in glutamic acid-galactose-mineral salts medium. Actinomycin was harvested subsequently at the times indicated. Valine and proline were determined in hydrolysates as described in Materials and Methods.

TABLE 3. *Concentration of proline analogue required to reverse (50%) 3-methylproline inhibition*[a]

Amino acid	Concn (μg/ml)
L-Proline	1,000–2,500
DL-Piperidine-2-carboxylic acid	500–1,000
4-Hydroxy-L-proline	500
Azetidine-2-carboxylic acid	25

[a] The organism was grown in glutamic acid medium for 24 hr, and then 3-methyl-DL-proline and the appropriate imino acid were added. Actinomycin titer was determined after an additional 24 hr of incubation.

The actinomycins produced with hydroxyproline, sarcosine, piperidine-2-carboxylic acid, or azetidine-2-carboxylic acid in the medium, as well as those mixtures synthesized in the presence of 3-methylproline, were examined chromatographically to determine whether 3-methylproline affected the biosynthetic process. We observed no significant quantitative or qualitative differences in the composition of the antibiotic mixture synthesized when 3-methylproline was present.

DISCUSSION

Data obtained in several laboratories have provided evidence that the mechanism for synthesis of polypeptide antibiotics [actinomycin (11–13), tyrocidine (14), polymyxin (19), bacitracin (3), gramicidin S (1), and edeine (2)] differs from the one described for protein synthesis.

In the case of actinomycin, studies with certain inhibitors of protein synthesis showed that a two- to threefold stimulation of the rate and extent of antibiotic synthesis occurred when protein synthesis was blocked (11–13). The experiments with 3-methyl-DL-proline provided additional data that protein and actinomycin syntheses proceed by different biochemical mechanisms. Actinomycin synthesis is virtually stopped by 3-methylproline (1.0 μg per ml), whether supplied at the onset or during actinomycin synthesis (23). However, the amount of mycelium produced in the presence of the proline analogue may be enhanced. Inhibition of cellular growth has not been observed with concentrations of 3-methylproline 50 to 100 times that required to inhibit antibiotic synthesis.

From previous findings (20), as well as from our results, it is apparent that small steric or structural differences in a molecule (such as changes in the location of a substituent group) can affect the ability of a compound to influence actinomycin synthesis both qualitatively and quantitatively. Actinomycin synthesis was inhibited by 3-, 4-, and 5-methylproline; 3-methylproline was the most inhibitory compound, and *cis*-3-methyl-DL-proline was approximately 14 times more active than the *trans* isomer. Although the site and nature of the inhibition remain unknown, it is noteworthy that the compound is the most potent inhibitor of actinomycin synthesis discovered thus far. At this point, one can only speculate on the role of 3-methylproline as an inhibitor of actinomycin synthesis. It is unlikely that 3-methylproline blocks proline synthesis, because cellular growth is not inhibited when the organism is grown in the presence of 3-methyl-

proline. It is more likely that 3-methylproline may act by combining reversibly with an enzyme responsible for the incorporation of proline into the antibiotic peptide. 4-Methylproline and, probably, 5-methylproline are incorporated readily into the antibiotic molecule in lieu of proline. The presence of 3-, 4-, or 5-methylproline in the medium reduced the amount of actinomycin IV (two prolines) synthesized, with 4-methylproline profoundly depressing its synthesis. Quantitative data of actinomycin hydrolysates (4-methylproline) and paper chromatography have provided further evidence for the replacement of proline by 4-methylproline.

The low specificity of the proline site in the antibiotic peptide has been recognized previously (7–9). Thus, sarcosine, hydroxyproline, piperidine-2-carboxylic acid, azetidine-2-carboxylic acid, and L-thiazolidine-4-carboxylate (18*a*) can all replace proline in actinomycin. In addition to 4- and 5-methylproline, mentioned here, recent studies revealed that the haloprolines (4-fluoro-, 4-chloro-, and 4-bromoproline) may also substitute for proline (*unpublished data*). In light of the mechanism of action of actinomycin IV, namely, its effect on deoxyribonucleic acid-dependent ribonucleic acid synthesis, it would be interesting to compare the biological activity of certain of these actinomycin analogues.

Knowledge of the effect of these proline analogues on protein synthesis by *S. antibioticus* is still fragmentary. Azetidine-2-carboxylic acid is incorporated into the protein of *Escherichia coli* and mung bean seedlings (5), but no evidence has been presented for the incorporation of the higher analogue of proline, piperidine-2-carboxylic acid, into cellular constituents. A slight incorporation of hydroxyproline into the protein of the chick embryo was noted, but the process was considered to be a nonphysiological one (16). Recent investigations showed that both *trans*- and *cis*-fluoroproline are incorporated into protein in a number of cellular systems, both in vivo and in vitro (6). In light of these findings, it would be desirable to establish whether any of these analogues of proline are incorporated into cellular proteins by *S. antibioticus*.

In conclusion, we would like to point out that a normal amino acid constituent of bottromycin B is *cis*-3-methyl-L-proline (18).

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LITERATURE CITED

1. BERG, T. L., L. O. FROHOLM, AND S. G. LALAND. 1965. The biosynthesis of gramicidin S in a cell-free system. Biochem. J. **96**:43–52.
2. BOROWSKA, Z. K., AND E. L. TATUM. 1966. Biosynthesis of edeine by *Bacillus brevis* Vm_4 *in vivo* and *in vitro*. Biochim. Biophys. Acta **114**:206–209.
3. CORNELL, N., AND J. E. SNOKE. 1964. Biosynthesis of bacitracin and protein. Biochim. Biophys. Acta **91**:533–536.
4. COX, D. A., A. W. JOHNSON, AND A. B. MAUGER. 1964. A modified proline synthesis. J. Chem. Soc., p. 5024–5029.
5. FOWDEN, L., D. LEWIS, AND H. TRISTAM. 1967. Toxic amino acids: their action as antimetabolites. Advan. Enzymol. **29**:89–163.
6. GOTTLIEB, A. A., Y. FUJITA, S. UDENFRIEND, AND B. WITKOP. 1965. Incorporation of *cis*- and *trans*-4-fluoro-L-prolines into proteins and hydroxylation of the trans isomer during collagen biosynthesis. Biochemistry **4**:2507–2513.
7. KATZ, E. 1960. Biogenesis of the actinomycins. Ann. N.Y. Acad. Sci. **89**:304–322.
8. KATZ, E., AND W. A. GOSS. 1958. Influence of amino-acids on actinomycin biosynthesis. Nature **182**:1668–1669.
9. KATZ, E., AND W. A. GOSS. 1959. Controlled biosynthesis of actinomycin with sarcosine. Biochem. J. **73**:458–465.
10. KATZ, E., P. PIENTA, AND A. SIVAK. 1958. The role of nutrition in the synthesis of actinomycin. Appl. Microbiol. **6**:236–241.
11. KATZ, E., AND H. WEISSBACH. 1962. Effect of chloromycetin and penicillin on the incorporation of amino acids into actinomycin and protein by *Streptomyces antibioticus*. Biochem. Biophys. Res. Commun. **8**:186–190.
12. KATZ, E., AND H. WEISSBACH. 1963. Incorporation of C^{14}-labeled amino acids into actinomycin and protein by *Streptomyces antibioticus*. J. Biol. Chem. **238**:666–675.
13. KATZ, E., M. WISE, AND H. WEISSBACH. 1965. Actinomycin biosynthesis. Differential effect of chloramphenicol on protein and peptide antibiotic synthesis. J. Biol. Chem. **240**:3071–3078.
14. MACH, B., E. REICH, AND E. L. TATUM. 1963. Separation of the biosynthesis of the antibiotic polypeptide tyrocidine from protein synthesis. Proc. Natl. Acad. Sci. U.S. **50**:175–181.
15. MAUGER, A. B., F. IRREVERRE, AND B. WITKOP. 1966. The stereochemistry of 3-methylproline. J. Am. Chem. Soc. **88**:2009–2015.
16. MITOMA, C., T. E. SMITH, F. FRIEDBERG, AND C. R. RAYFORD. 1959. Incorporation of hydroxyproline into tissue proteins by chick embryos. J. Biol. Chem. **234**:78–80.
17. NAFTALIN, L. 1948. Quantitative chromatographic estimation of α-amino acids. Nature **161**:763.
18. NAKAMURA, S., N. TANAKA, AND H. UMEZAWA. 1966. Bottromycin A_1, A_2 and their structures. J. Antibiotics (Tokyo) Ser. A **19**:10–12.

18*a*. NISHIMURA, J. S., AND W. F. BOWERS. 1967. Evidence for the incorporation of L-thiazolidine-4-carboxylate into actinomycins by *Streptomyces antibioticus*. Biochem. Biophys. Res. Commun. **28**:665–670.

19. PAULUS, H., AND E. GRAY. 1964. The biosynthesis of polymyxin B by growing cultures of *Bacillus polymyxa*. J. Biol. Chem. **239**:865–871.

20. SIVAK, A., AND E. KATZ. 1962. Biosynthesis of the actinomycin chromophore. Influence of α-, 4-, 5- and 6-methyl-DL-tryptophan on actinomycin synthesis. Biochim. Biophys. Acta **62**:80–90.

21. SIVAK, A., M. L. MELONI, F. NOBILI, AND E. KATZ. 1962. Biosynthesis of the actinomycin chromophore. Studies with DL-(7α-^{14}C) tryptophan and L-(Me-^{14}C) methionine. Biochim. Biophys. Acta **57**:283–289.

22. TROLL, W., AND J. LINDSLEY. 1955. A photometric method for the determination of proline. J. Biol. Chem. **215**:655–660.

23. YOSHIDA, T., A. B. MAUGER, B. WITKOP, AND E. KATZ. 1966. Influence of methylproline isomers upon actinomycin biosynthesis. Biochem. Biophys. Res. Commun. **25**:66–72.

24. YOSHIDA, T., H. WEISSBACH. AND E, KATZ. 1966. Inhibitory effect of actinomycin upon the producing organism. Arch. Biochem. Biophys. **114**:252–255.