MICROBIAL METABOLISM OF ACTINOMYCINS AND OTHER HETERODETIC ANTIBIOTIC PEPTIDES

D. Perlman,* A. B. Mauger, and Herbert Weissbach

Squibb Institute for Medical Research, New Brunswick, N.J.; Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland; and Research Foundation of the Washington Hospital Center, Washington, D. C.

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The microbial metabolism of actinomycin and other cyclic peptide antibiotics has received comparatively scant attention. No microbial degradations were reported until Katz and Pienta (1957) described a process in which growing cells of an Achromobacter species converted actinomycin D to antibacterially inactive red pigments. We have examined the degradation of actinomycins and other heterodetic antibiotic peptides by resting cells and a cell-free preparation of Actinoplanes (IMRU #F3-15) and have identified actinomycinic acid and actinomycin monolactone as major degradation products. (Figure 1).

The Actinoplanes culture was grown under submerged conditions used for various Streptomycetes in a medium containing Staley Special Nutrient 4-S and glycerol (Perlman et al., to be published). Twelve hours prior to harvest of the cultures 10 μg/ml of actinomycins were added to induce the degrading enzyme system.

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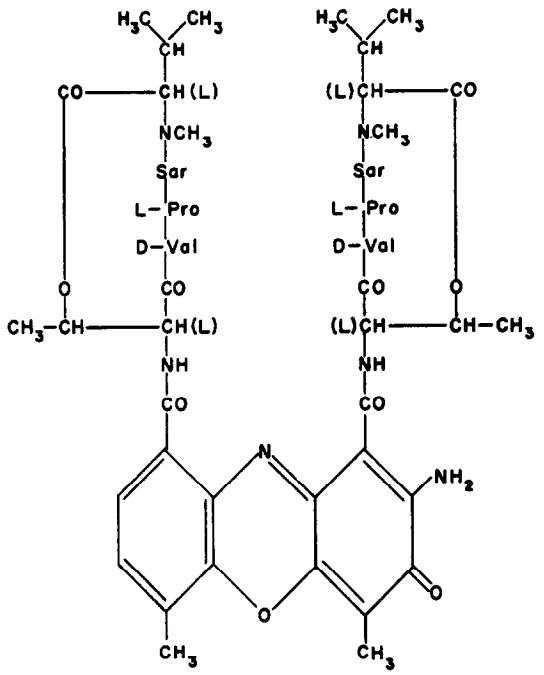

Fig. 1. Structure of actinomycin D. Actinomycin monolactone contains only 1 lactone ring while both lactone rings are hydrolyzed in actinomycinic acid.

The cells from three- to five-day-old fermentations were harvested by centrifugation and washed with 0.2 M phosphate buffer (pH 7.0). The cells were resuspended in sufficient phosphate buffer to give the original volume and 5 ml of this suspension were added to 20 ml beakers placed in a Dubnoff metabolic incubator maintained at 37°. After a short equilibration period, 250 μg of actinomycin D-^{3}H (3×10^{5} cpm)* were added to each beaker. The metabolism of the added actinomycin was determined by both bio-

*We are indebted to Professor E. Katz for the actinomycin D and to Dr. A. D. Azalos for actinomycin C (mixture) used in this series of experiments. Actinomycin D-^{3}H was prepared as previously described (Katz et al., 1965).

assays using growth inhibition of Staphylococcus aureus 209P (agar diffusion method) and chemical assays. In the latter, the cultures were centrifuged and the supernatant liquid extracted with 2 volumes of ethyl acetate (this removed the actinomycin). An aliquot of the ethyl acetate extract was assayed for radioactivity using a Packard Tri-Carb Liquid Scintillation counting system (model 314 EX) with a naphthalene-dioxane counting fluid (Bray, 1960). The aqueous phase was acidified to 0.4 N HCl and extracted with two volumes of ethyl acetate (under these conditions both the actinomycinic acid and actinomycin monolactone were extracted by the solvent). An aliquot of the ethyl acetate after acid extraction was also assayed for radioactivity.

Table I

Metabolism of Actinomycin by Washed Cells of Actinoplanes sp.

	Radioactivity in cell-free medium (percent of original)			
Incubation time	Actinomycin (neutral extractable)	Acidic metabolites (acid extractable)	Other metabolites (nonextractable)	Total recovered
hrs				
0	100	0	0	100
2	18	21	10	49
7.5	23	35	12	70

Typical analyses of experiments in which washed cells were incubated with actinomycin are presented in Table I. It can be seen that there is a rapid disappearance of the antibiotic from the medium. By 2 hours a significant amount of radioactivity originally added was in

the form of acid extractable metabolites or metabolites not extractable from acidic or neutral aqueous solution by ethyl acetate. Radioactivity determinations showed that 8 hours after addition of the antibiotic more than 70% of the actinomycin had been metabolized to compounds present in the medium.

The acidic metabolites were characterized by paper chromatography using a n-butanol:pyridine:water (4:1:5, upper phase) system, and by paper ionophoresis at pH 6.5 (pyridine-acetate buffer). The colored metabolites had the mobilities of actinomycinic acid and actinomycin monolactone prepared by chemical degradation of actinomycin D (Perlman et al, to be published). The products separated by paper ionophoresis were degraded by acid hydrolysis and shown by paper chromatography to contain the amino acids found in actinomycin.

Degradation of actinomycin was also accomplished in vitro: Cells from an actinomycin supplemented fermentation were collected by centrifugation and washed with 0.2 M phosphate buffer, pH 7.0. The washed cells were sonicated using a Bronwill sonifier and the suspension passed through a French pressure cell. The suspension was centrifuged at 8000 x g and solid ammonium sulfate was added to the supernatant fraction. The protein precipitating between 30 and 60% saturation contained the enzymatic activity. This fraction after dialysis against 0.01 M phosphate buffer (pH 7.0) contained 15 mg protein/ml.

The enzymatic degradations were carried out at 37° in 12 ml glass stoppered centrifuge tubes. The standard incubation contained enzyme, 10 μmoles of Tris buffer pH 7.8, 9 mμmoles actinomycin D-^{3}H

(8000 cpm/mμmole), and water to a final volume of 0.20 ml. The reactions were stopped by addition of 0.8 ml of 0.4 N HCl. Four ml of ethyl acetate were then added and the tubes shaken to extract the actinomycin and acidic metabolites into the organic phase. Three ml of the ethyl acetate layer were transferred to another tube containing 1.5 ml of 0.02 M phosphate buffer (pH 7.0), and after thorough mixing the solvent layer discarded. One ml of the aqueous phase (containing the actinomycinic acid and actinomycin monolactone) was assayed for radioactivity. Essentially no degradation of actinomycin occurred if enzyme was omitted, or if boiled enzyme was used.

Table II

In vitro Metabolism of Actinomycin

Protein	Acid extractable products
mg	mμmoles/60 min
0	0
0.15	0.28
0.30	0.50
0.75	1.20
0.75 (boiled)	<0.03

The effect of protein concentration on the rate of degradation is summarized in Table II. The reaction was linear for periods of time up to 90 minutes and the optimum pH range was between pH 7.0 and 8.0. The acidic metabolites were examined by paper ionophoresis

and shown to be a mixture of actinomycinic acid and actinomycin monolactone. Under these conditions only 1/7 as much actinomycinic acid was formed as actinomycin monolactone.

This enzyme preparation also inactivated other heterodetic antibiotic peptides including echinomycin, etamycin, and vernamycin B. (as shown by loss of antibacterial activity). However, since the enzyme system has not been purified it is not possible to determine whether one or more enzymes are involved in the degradation of this group of peptide antibiotics.

The lactone ring in actinomycin and the other heterodetic peptide antibiotics is a specific site at which enzymatic attack can take place. Our experiments show that Actinoplanes species (IMRU #F3-15) possess an esterase which can catalyze the hydrolysis of a lactone ring. Although preliminary experiments with washed cells showed that actinomycinic acid was the predominant product found, the enzymatic reaction resulted in the formation of the monolactone with little formation of the dicarboxylic acid. This may indicate that two enzyme systems are involved, or possibly that the first lactone ring is more readily hydrolyzed by the enzyme and the monolactone is a less satisfactory substrate for the enzyme. Experiments are in progress to determine whether the monolactone is an intermediate in the conversion to the dicarboxylic acid, and which of the lactone rings is preferentially attacked.

References

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