Inhibition of Protein Synthesis in Mammalian Cells by Actinobolin

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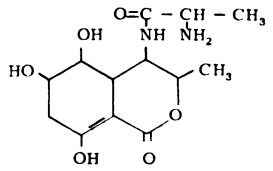
SUMMARY

Actinobolin, an antibiotic with a broad spectrum of biological activity, has been studied with respect to its metabolic effects in mammalian cells. In mouse Adenocarcinoma 755 cells, both *in vivo* and in cell culture, actinobolin inhibited the synthesis of protein and DNA, but exerted a much smaller effect on the synthesis of RNA and had no effect on the synthesis of soluble purine derivatives. The rate and extent of uptake of [^{3}H]actinobolin in cell cultures suggested passive diffusion across the cell membrane, with no subsequent covalent bonding to cellular constituents. In a cell-free system, containing washed ribosomes and 100,000 × *g*–pH 5 fractions from mouse Adenocarcinoma 755 cells grown in culture, actinobolin inhibited the incorporation of labeled amino acids in the presence of endogenous messenger or of polyuridylate. At higher concentrations actinobolin inhibited the incorporation of thymidylate into DNA in a crude DNA synthetase system. These results suggest that the primary metabolic effect of actinobolin in mammalian cells is on protein synthesis.

In the cell-free system actinobolin (*a*) did not inhibit the formation of aminoacyl-tRNAs, (*b*) did not cause the release of peptides from previously charged ribosomes or prevent such release by puromycin, and (*c*) inhibited the binding of [^{14}C]phenylalanyl-tRNA to the poly U–ribosome complex, but not the binding of poly U to ribosomes. These results show that actinobolin acts at a late stage of protein synthesis, perhaps by competing with aminoacyl-tRNAs. Such an action would be compatible with the fact that actinobolin contains an L-alanine moiety.

INTRODUCTION

Actinobolin, an antibiotic isolated by Haskell and Bartz (1), has a broad spectrum of antibacterial activity (2) and is also active against certain experimental tumors and leukemias (3, 4). Recently reported studies on actinobolin have shown it to be a derivative of L-alanine (5–7), and the structure shown below has been assigned (7). In spite of a number of investigations, the precise mode of action of this compound is still undefined. Growth-inhibitory effects of actinobolin against *Escherichia coli* were reversed by certain metal ions and by a variety of metabolites, among which phenyl-



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alanine was the most effective (8). Actinobolin is a good chelating agent (1), but results with both bacteria and leukemia-bearing animals suggest that its cytotoxicity cannot be attributed to removal of metal ions (4, 8). In *E. coli*, actinobolin inhibited the synthesis of protein and RNA and caused the production of filaments; inhibition of RNA synthesis appeared to be a consequence of inhibition of protein synthesis (9, 10). We report here studies of the effects of actinobolin in mammalian cells and in cell-free systems that indicate that the primary metabolic effect is on a late stage of protein synthesis and that the mechanism of action may be competition with aminoacyl-tRNA for binding to the polysomes. A preliminary account of some of these results has been presented (11).

MATERIALS AND METHODS

Compounds and materials. Actinobolin sulfate dihydrate was a gift from Dr. John Dice, Parke, Davis and Company, Detroit. The following were the sources of other compounds and materials: Miles Pharmaceutical Company, Elkhart, Indiana, poly U, [2-^{14}C]poly U, 70 S, 50 S, and 30 S ribosomes and ribosomal subunits from *E. coli*; New England Nuclear Corporation, Boston, sodium [^{14}C]formate, [2-^{14}C]glycine, [Me-^{3}H]thymidine, [4, 5-^{3}H]leucine, and the following uniformly labeled ^{14}C compounds: glutamine, phenylalanine, alanine, leucine, lysine, and phenylalanyl-tRNA; Schwarz Laboratories, Orangeburg, New York, [2-^{14}C]uridine and [2-^{14}C]thymidylate; Sigma Chemical Company, St. Louis, pyruvate kinase. Actinobolin was tritiated by catalytic gaseous exchange by New England Nuclear Corporation, and the product was purified in our laboratories by recrystallization to constant specific activity; the recrystallized sample was shown by thin layer chromatography to contain essentially all the ^{3}H label (90–97%) and by bioassay to be equivalent to unlabeled actinobolin in inhibiting *E. coli*. The final product had a specific activity of 150 μCi/mg and yielded only one radioactive spot (detectable by radioautography) upon paper chromatography in the following solvents: (*a*) equal volumes of 93.8% 1-butanol and 44% aqueous propionic acid; (*b*) isobutyric acid–concentrated NH_4OH–H_2O (57:4:39, v/v/v); (*c*) 2, 2, 3, 3-tetrafluoro-1-propanol–H_2O–90% formic acid (25:15:0.5, v/v/v); (*d*) 0.1 M sodium phosphate (pH 6.8)–solid $(NH_4)_2SO_4$–1-propanol (100:60:2, v/w/v); (*e*) methanol–concentrated HCl–H_2O (50:17:33, v/v/v); (*f*) 70% aqueous 2-propanol in an NH_3 atmosphere.

Animals and cell cultures. For studies of the effects of actinobolin *in vivo*, C57 black mice bearing subcutaneous implants of mouse Adenocarcinoma 755 were used. The principal cell culture used was a line of Adenocarcinoma 755 established in culture in our laboratories and grown either on glass or in suspension culture under the same conditions described for H. Ep. No. 2 cells (12). Procedures have been described elsewhere for the isolation and assay of crude protein, soluble purines, and polynucleotides from solid tumors (13) and for assay of incorporation of precursors into DNA, RNA, and protein of cell cultures (14); details are given in the tables and figures.

Protein synthesis in vitro. From adenocarcinoma cells grown in suspension culture, ribosomes and 100,000 $\times$ *g*–pH 5 fractions were prepared as described elsewhere (15) by methods that are essentially those of Littlefield and Keller (16) and O'Neal and Griffin (17). All compounds and cell fractions were made up in standard buffer of the following composition: 0.005 M $MgCl_2$, 0.025 M KCl, 0.25 M sucrose, 0.05 M Tris HCl (pH 7.6), and 0.006 M mercaptoethanol. The standard assay mixture contained the following in a final volume of 0.5 ml: phosphoenolpyruvate, 2.5 μmoles; pyruvate kinase, 5 μg; ATP, 0.25 μmole; GTP, 0.0075 μmole; radioactive amino acid, 0.005 μmole; 21 unlabeled amino acids, 0.025 μmole of each; ribosomal suspension, 0.1 ml; 100,000 $\times$ *g*–pH 5 fraction, 0.2 ml; and standard buffer to volume. In some experiments the 10,000 $\times$ *g* supernatant fraction was substituted for the ribosomes and the 100,000 $\times$ *g* supernatant fraction precipitated at pH 5. At the end of the incubation, samples were prepared and assayed for ^{14}C by a

modification (15) of the method of Mans and Novelli (18), which involved precipitation of protein on paper discs and assay of the discs after extraction with cold trichloracetic acid (for isolation of protein plus aminoacyl-tRNA) or with hot trichloracetic acid (for isolation of protein). Any deviations from the standard conditions are noted.

Sucrose density gradients. Analysis of fractions by density gradient separation involved the use of 10–30% or 10–40% gradients prepared from ribonuclease-free sucrose. The sample was placed on the top of a 30-ml gradient which was centrifuged at 4° for 2 hr at 25,000 rpm in a model L or model L-2 Spinco centrifuge. *E. coli* ribosomes and subunits with sedimentation coefficients of 70 S, 50 S, and 30 S were used as markers. Fractions of 1 ml each were collected and their ultraviolet absorption was determined. Radioactivity was determined by collection of the sample on a Millipore filter with pores of 0.45-μ diameter (19), after which the filter was placed in a vial (containing a standard scintillator mixture: 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)] benzene per liter of toluene), which was then assayed in a Packard liquid scintillation spectrometer.

Binding of poly U and tRNA to ribosomes. The Millipore filter technique of Nirenberg and Leder (19) was used to study the effects of actinobolin on the binding of [^{14}C]poly U to ribosomes and [^{14}C] phenylalanyl-tRNA to ribosomes in the presence of poly U; details are given in Tables 4 and 5.

DNA synthesis in vitro. A crude DNA synthetase system was prepared from H. Ep. No. 2 cells in culture as described elsewhere (14). The method of Bollum (20) was used for the preparation of DNA primer and for the assay of DNA polymerase activity. The assay involved determination of the incorporation of [2-^{14}C]dTMP into acid-insoluble material.

RESULTS

Uptake of actinobolin and effects on cell growth. Actinobolin was not highly cytotoxic to Adenocarcinoma 755 cells. The concentration required for 50% inhibition of cell proliferation after 72 hr of exposure was about 7×10^{-5} M (Fig. 1). In animals the daily dose for inhibition of tumor growth is about 1000 mg/kg (3). These levels of actinobolin are 7–20 times greater than the amounts of cycloheximide that produce toxicity to cultured cells or to animals (14, 21, 22). The amount of [^{3}H] actinobolin taken up by cells (0.08 μCi/g) was somewhat less than the concentration of actinobolin in the medium (0.15 μCi/ml) (Fig. 2). This result indicates that there was no extensive formation of covalent bonds or tightly bound complexes between actinobolin and cellular constitu-

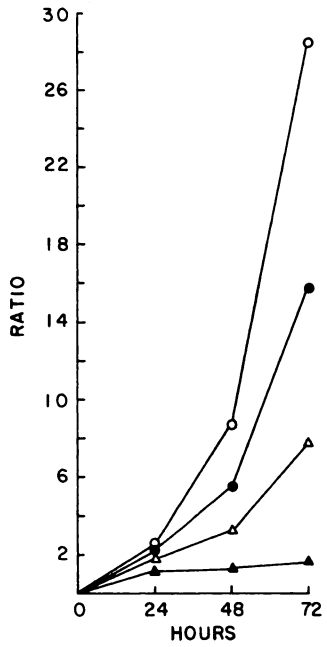

FIG. 1. *Inhibition of growth of Adenocarcinoma 755 cells in culture by actinobolin*

Cells in suspension culture (10^7 cells/ml at the initiation of the experiments) were allowed to grow for 72 hr in the presence or absence of actinobolin. Cell counts were made at the indicated times in a Coulter counter. The ordinates are the ratios of the number of cells at a given time to the number at the beginning of the experiment. See the text for references to medium and culture conditions. ○——○, Control; ●——●, actinobolin, 0.07 mM; △——△, actinobolin, 0.14 mM; ▲——▲, actinobolin, 0.56 mM.

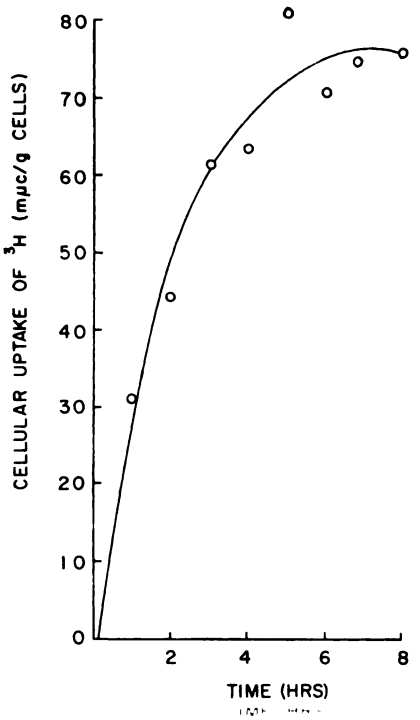

FIG. 2. *Uptake of ^3H from [^3H]actinobolin by Adenocarcinoma 755 cells in suspension culture*

To 1 liter of medium containing approximately 1.3 g (4×10^8) of cells, [^{3}H]actinobolin was added to give a final concentration of 0.15 μCi (1 μg)/ml. At each hour for 8 hr after the addition of actinobolin, two 40-ml samples of the culture were removed for assay. Each sample was centrifuged at $1000 \times g$ for 10 min, and the residual cells were washed three times by centrifugation with 0.9% NaCl solution. The cell pellet was suspended in 0.9% NaCl, and the suspension was assayed for ^{3}H in a liquid scintillation spectrometer. There was essentially no ^{3}H in the second and third washes.

ents, because such processes should promote concentration of actinobolin in the cells. The first wash from the harvested cells contained 0.06 μCi, which would correspond to contamination of the cells with 0.4 ml of medium; it is also possible that the wash removed some actinobolin from within the cell. The second and third washes contained essentially no radioactivity. Thus, the combined amounts of radioactivity in the cells and in the first wash were no greater than would result from equilibration of the cells with the medium. These results suggest, but do not prove, that actinobolin enters cells by passive diffusion.

Attempts to demonstrate binding of actinobolin. Actinobolin was incubated separately with tRNA, ribosomes, poly U, the $100{,}000 \times g$–pH 5 fraction, or the complete standard incubation mixture. After incubation for 20 min, the mixture was poured onto a Sephadex G-25 column and eluted with standard buffer, pH 7.6. The presence of these other constituents did not alter the elution pattern produced by actinobolin alone. This result shows that there was no formation of covalent bonds between actinobolin and the other constituents of the incubation mixture, and also suggests the absence of strong secondary bonding forces. Since these results were all negative, the elution profiles are not presented.

Metabolic effects in intact cells. In solid Adenocarcinoma 755 *in vivo*, actinobolin was without effect on the incorporation of [^{14}C]formate into soluble purine derivatives, but markedly inhibited the incorporation of [^{14}C]formate into DNA and protein; incorporation of formate into RNA was inhibited to a much smaller extent (Table 1). In cell cultures of Adenocarcinoma 755, actinobolin inhibited the synthesis of protein and DNA to a much greater extent than the synthesis of RNA, as indicated by its effects on the utilization of thymidine, leucine, and uridine (Fig. 3). These effects of actinobolin are qualitatively similar to those of cycloheximide, a known specific inhibitor of protein synthesis (14, 22).

Protein synthesis in vitro. In the protein-synthesizing system obtained from Adenocarcinoma 755 cells, actinobolin inhibited the incorporation of phenylalanine, leucine, glutamine, and lysine into protein in the presence of endogenous messenger (Table 2), and also the poly U-stimulated incorporation of phenylanine (Fig. 4). Since actinobolin was used as the sulfate, it was necessary to establish that sulfate itself did not inhibit protein synthesis; data showing that sulfate does not affect this system are shown in Fig. 4. The possibility that actinobolin might act by complexing poly U was excluded by the fact that increasing the con-

TABLE 1

Effects of actinobolin on synthesis of DNA, RNA, and protein by Adenocarcinoma 755 in vivo

C57 black mice bearing 12-day-old implants of Adenocarcinoma 755 were given an intraperitoneal injection of actinobolin sulfate (2.7 mmoles/kg). One hour later this treatment was repeated, and immediately thereafter each animal received an intraperitoneal injection of sodium [^{14}C]formate (10 μCi/25 g; specific activity, 1.95 mCi/mmole). Four hours after administration of formate, the animals were killed and the tumors were homogenized and extracted with cold trichloracetic acid. Individual purines were isolated from the soluble fraction and from the DNA and RNA fractions of the residue (see the text for references to methods). The crude protein fraction was the residue remaining from extraction of a portion of the homogenate three times with hot 5% trichloracetic acid.

	Specific activities						
		Acid-soluble		DNA		RNA	
	Protein	Adenine	Hypoxanthine	Adenine	Guanine	Adenine	Guanine
	cps/μg	*cps/μg*		*cps/μg*		*cps/μg*	
Control	0.033	3.2	3.2	0.38	0.35	0.72	0.47
Treated	0.005	3.4	3.1	0.09	0.06	0.59	0.24

centration of poly U did not affect the activity of actinobolin in inhibiting protein synthesis (Fig. 5). The concentration of actinobolin required to inhibit protein synthesis in the cell-free system was not much greater than the amounts required for inhibition of cell growth (Fig. 1) or inhibition of protein synthesis in intact cells (Fig. 3).

The presence of actinobolin did not affect the differences in [^{14}C]phenylalanine incorporation between the fraction insoluble in cold trichloracetic acid (protein plus aminoacyl-tRNA) and the fraction insoluble in hot trichloracetic acid (protein) (Table 3). These data indicate that actinobolin is without effect on the formation of aminoacyl-tRNAs.

Studies with sucrose density gradients. Figure 6 shows the results of experiments with the 10,000 $\times$ *g* fraction, in which poly-

TABLE 2

Inhibition by actinobolin of incorporation of amino acids into protein by a cell-free system

The conditions were those of the standard assay (see the text). The values given are derived from determinations of incorporation after a 40-min incubation period.

Actinobolin concentration	Substrate	Incorporation
mM		*% control*
1.4	[^{14}C]Phenylalanine	67
1.4	[^{14}C]Leucine	63
1.4	[^{14}C]Glutamine	47
2.8	[^{14}C]Lysine	48

TABLE 3

Differences in ^{14}C content between fractions insoluble in cold trichloracetic acid and those insoluble in hot trichloracetic acid following incubation of cell-free preparations with [^{14}C]phenylalanine and actinobolin

The conditions were those of the standard assay, with an incubation period of 40 min. The values given are the differences resulting from subtraction of the counts in the fraction insoluble in hot trichloracetic acid from the counts in the fraction insoluble in cold trichloracetic acid. Values for one experiment are given. The magnitude of the differences in ^{14}C content varied between experiments, but in no experiment did the presence of actinobolin cause a decrease in the difference.

Actinobolin concentration	Differences in ^{14}C content
mM	*cpm*
0.54	452
0.81	435
1.08	382
1.35	422
1.62	441
2.03	423

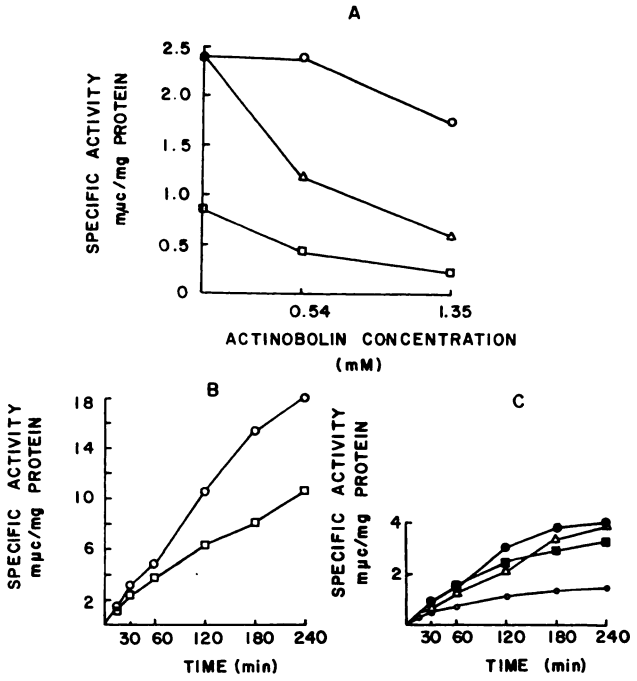

FIG. 3. *Effects of actinobolin on the utilization of [2-^{14}C]thymidine, [2-^{14}C]uridine, and L-[4,5-^{3}H]leucine by Adenocarcinoma 755 cells in culture*

Actinobolin was added to cells in suspension culture (10^7 cells/ml), followed 1 hr later by the labeled precursor [2-^{14}C]thymidine, 2.0 μCi; [2-^{14}C]uridine, 6.0 μCi; and L-[4,5-^{3}H]leucine, 8.0 μCi; control cultures received only the labeled compounds. Two hours after addition of the labeled compounds, the cells were harvested, washed free of medium with 0.9% NaCi solution, and extracted successively with boiling ethanol, cold 5% and 3% aqueous trichloracetic acid, ethanol, and ether. The residue, dissolved in Hyamine, was assayed for ^{14}C and ^{3}H in a liquid scintillation spectrometer.

A. Inhibition as a function of actinobolin concentration: O——O, [2-^{14}C]uridine; □——□, [2-^{14}C]thymidine; △——△, [4,5-^{3}H]leucine.

B and C. Inhibition as a function of time at concentrations of actinobolin (when present) of 0.54 mM. [2-^{14}C]Thymidine (B): O——O, control; □——□, treated. [2-^{14}C]Uridine (C): ●——●, control; ■——■, treated. [4,5-^{3}H]leucine (C): △——△, control; O——O, treated.

somes were labeled with [^{14}C]phenylalanine by incubation of the complete reaction mixture for 10 min before the addition of inhibitor. The 10-min incubation gave heavy labeling of the ribosomes (10-min control), which was increased by an additional 20-min incubation (30-min control). Actinobolin prevented the additional labeling during the last 20 min but did not cause loss of the ^{14}C already associated with the ribosomes at 10 min. Puromycin, on the other hand, produced the expected release (23) of label already associated with the ribosomes. Actinobolin and puromycin in combination were no more effective than puromycin alone. In experiments with purified ribosomes (Fig. 7), actinobolin, when present throughout the incubation period, produced a marked reduction of incorporation of [^{14}C]phenylalanine into ribosome-associated polypeptides. When purified ribosomes were first labeled with [^{14}C] phenylalanine, isolated by centrifugation, and then reincubated in the presence of puromycin, the expected marked loss (23) of the label from the heavier fractions occurred, with a concomitant increase in ^{14}C in the lighter fractions (Fig. 8). Actinobolin was without effect on the previously labeled ribosomes and did not prevent the action of puromycin in causing premature release of polypeptides.

TABLE 4

Effects of actinobolin, cycloheximide, and puromycin on binding of L-[^{14}C]*phenylalanyl-tRNA to ribosomes in the presence of polyuridylic acid*

The reaction mixture contained ribosomes (0.25 mg of RNA); poly U, 5.6 μg (50 μμmoles); and [^{14}C]phenylalanyl-tRNA, 10.3 μg (16 μμmoles of ^{14}C; 1.4×10^{-3} μCi), in 0.1 ml of buffer, pH 7.6. After incubation at 24° for 20 min, the entire mixture was poured onto a Millipore filter which had been washed with 3 ml of standard buffer. The incubation vessel was washed twice with 3-ml portions of the standard buffer used for protein synthesis, and finally the filter was washed with another 3-ml portion of buffer. The filter, after drying at room temperature or under a lamp, was assayed in a liquid scintillation spectrometer.

Compound added	Concentration	[^{14}C]Phenylalanyl-tRNA bound	Percentage of control
	μM	*μμmoles*	
None		2.18	100
Actinobolin sulfate	0.27	2.03	93
	1.4	1.83	84
	2.8	1.39	64
	8.4	0.96	44
Cycloheximide	1.8	2.01	92
	3.6	2.16	99
	7.2	2.07	95
Puromycin	1.0	1.92	88
	2.0	1.83	84
	4.0	1.69	78

The principal peak in the profiles of Figs. 6–8 corresponds to a value of about 80 S, as determined by the use of 70 S ribosomes from *E. coli* as a marker.

Binding of aminoacyl-tRNA and poly U. Actinobolin inhibited the binding of [^{14}C] phenylalanyl-tRNA to mammalian ribosomes, and the extent of inhibition increased with the concentration of actinobolin (Table 4). The specificity of its action is shown by the essential inactivity in this respect of both cycloheximide and puromycin, which inhibit protein synthesis *in vivo* and *in vitro* at much lower concentrations than those required for inhibition by actinobolin. Neither actinobolin, cycloheximide, nor puromycin inhibited the binding of poly U to ribosomes (Table 5).

DNA synthesis in vitro. Because actinobolin inhibited DNA synthesis in intact cells, it was desirable to study its effects in a system *in vitro*. As shown in Fig. 9, actinobolin did inhibit the incorporation of [^{14}C]dTMP into acid-insoluble material in a crude DNA polymerase system from H. Ep. No. 2 cells. Sulfate ions, at concentrations higher than those resulting from the amount of actinobolin sulfate used, were without effect. Because of the high concentration of actinobolin required for this inhibition, it was conceivable that any organic compound at this concentration

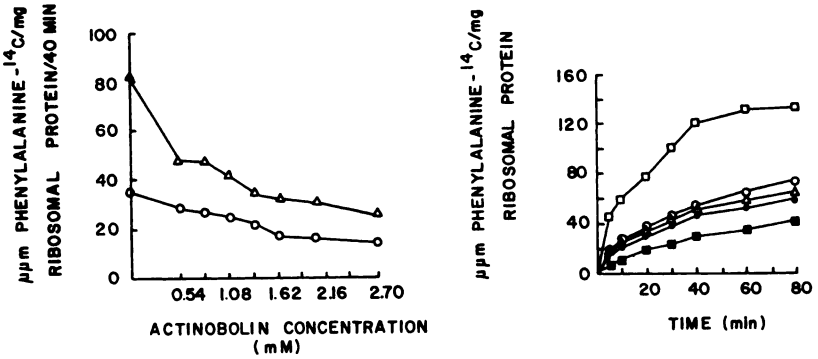

FIG. 4. *Inhibition by actinobolin of incorporation of* [^{14}C]*phenylalanine into acid-insoluble material in a cell-free system from Adenocarcinoma 755 cells*

The conditions were those of the standard assay described in the text. Left, inhibition in the presence and absence of poly U as a function of actinobolin concentration: O——O, actinobolin; △——△, actinobolin plus poly U (400 μg/ml). Right, inhibition as a function of time and the absence of effect of sulfate; actinobolin, when used, was present at a concentration of 1.35 mM. O——O, Control (no actinobolin or poly U); ■——■, actinobolin; □——□, poly U (400 μg/ml); ●——●, actinobolin plus poly U (400 μg/ml); △——△, Na_2SO_4 (2 mM). The ordinates are in units of picomoles of [^{14}C]phenylalanine.

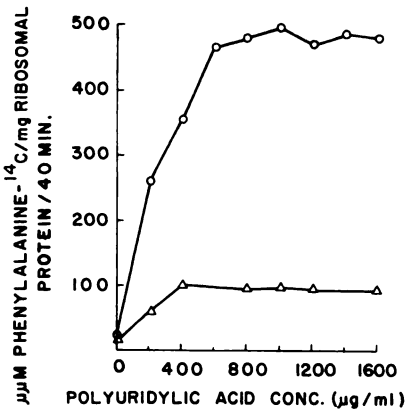

FIG. 5. *Failure of high concentrations of poly U to prevent actinobolin-induced inhibition of incorporation of [14C]phenylalanine into acid-insoluble material*

The conditions were those of the standard assay (see the text and Fig. 4), except for the addition of poly U and actinobolin. Actinobolin, when present was at a concentration of 1.35 mM, and the incubation time was 40 min. O——O, Control; △——△, actinobolin. The ordinate is in units of picomoles of [14C]phenylalanine.

might be inhibitory. That the inhibition is somewhat specific is shown by the fact that urethane, at even higher concentrations, did not inhibit. These results show that actinobolin does inhibit DNA synthesis *in vitro*, but that the concentration necessary is much higher than that required for inhibition of protein synthesis *in vitro*.

TABLE 5

Effects of actinobolin, cycloheximide, and puromycin on binding of [14C]polyuridylic acid to ribosomes

The incubation mixture consisted of ribosomes (0.25 mg of RNA), [14C]poly U (3.6×10^{-3} μCi; 1.4 μμmoles), and the inhibitor (when present) in a total volume of 0.1 ml of standard buffer, pH 7.6. The extent of binding was assayed by the Millipore filter technique described in Table 4. After incubation at 24° for 20 min, the entire mixture was poured onto a Millipore filter, which was then washed with standard buffer. The filter was assayed for 14C in a liquid scintillation spectrometer.

Compound added	Concentration	Poly U bound	Percentage of control
	mM	*μμmoles*	
None (control)		5.05	100
Actinobolin sulfate	0.27	5.27	104
	1.4	5.21	103
	5.6	5.16	102
Cycloheximide	0.35	5.08	101
	1.8	4.99	99
	7.2	5.03	100
Puromycin HCl	0.2	5.10	101
	1.0	4.57	90
	4.0	5.08	101

DISCUSSION

The results obtained indicate that the growth-inhibitory action of actinobolin is due to its capacity to inhibit protein synthesis. Although actinobolin also inhibited DNA synthesis both in intact cells and in cell-free systems, the relative concentrations required for inhibition of DNA and protein synthesis suggest that the latter process is the primary site of action. It is well known that in mammalian cells concomitant synthesis of protein is required for synthesis of DNA and that inhibition of protein synthesis therefore produces a marked and rapid inhibition of DNA synthesis (see ref. 24 for literature citations). In this connection it is also pertinent that gross effects of actinobolin on the synthesis of DNA, RNA, and protein in intact cells are similar to those produced by cycloheximide, a specific inhibitor of protein synthesis that is without effect on DNA synthesis in cell-free systems (14).

Actinobolin inhibits the synthesis of protein at a point past the formation of aminoacyl-tRNAs. The only specific effect of actinobolin noted in the cell-free system was interference with the binding of phenylalanyl-tRNA to the poly U–ribosome complex. This action could explain the inhibition of protein synthesis produced by actinobolin and is also an effect compatible with its structure as a derivative of alanine. Since actinobolin contains an amino acid moiety with a free amino group, it is conceivable that it could interfere with protein synthesis by direct covalent attachment to the developing polypeptide chain, as does puromycin (23). That actinobolin does not act similarly to puromycin is indicated by the observations (*a*) that [3H]actinobolin apparently was not itself incorporated into polypeptide chains in the cell-free protein-

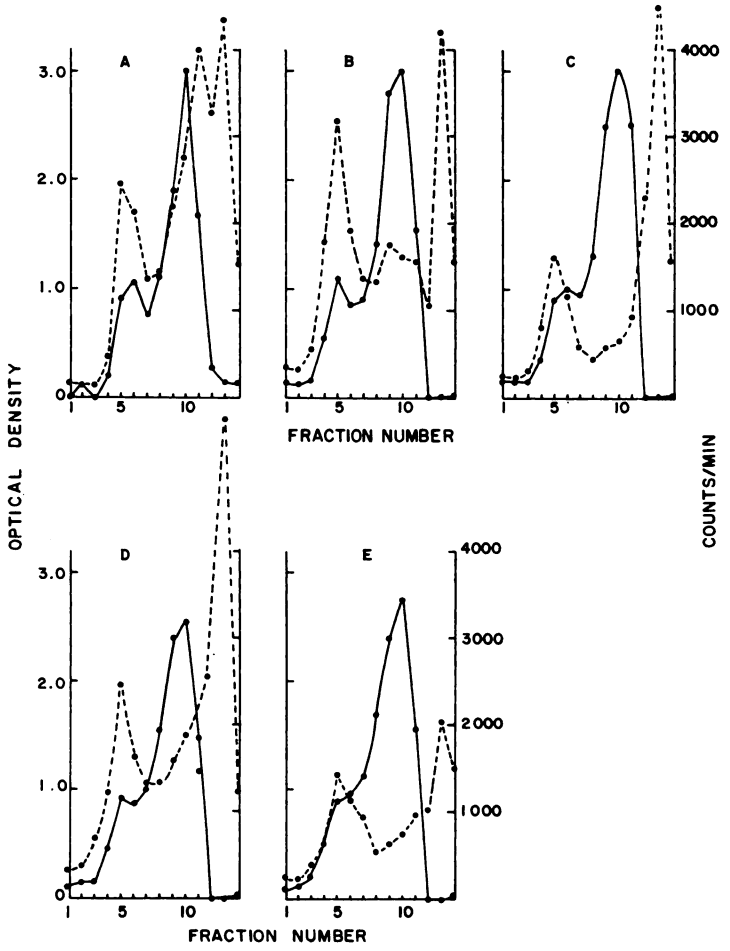

FIG. 6. *Effects of actinobolin and puromycin on labeling of ribosomes by [^{14}C]phenylalanine (10,000 × g fraction)*

The conditions were those of the standard assay, except that the 10,000 × *g* supernatant fraction replaced the ribosomes and 100,000 × *g* supernatant fraction precipitated at pH 5. The complete system was incubated for 10 min before addition of inhibitor; the inhibitor was added, and the incubation was continued for 20 min. A. Ten-minute control. B. Thirty-minute control. C. Puromycin HCl, 50 μg/mg of ribosomal RNA. D. Actinobolin sulfate, 500 μg/mg of ribosomal RNA. E. Actinobolin sulfate (500 μg/mg of ribosomal RNA) plus puromycin HCl (50 μg/mg of ribosomal RNA). The 10-min and 30-min controls show the profiles at the end of the 10-min and 30-min incubations. At the end of the incubation, aliquots of the mixture were layered on 30 ml of 10–40% sucrose, and separations and assays were performed as described in the text. ●- - -●, Counts per minute per fraction; ●——●, optical density at 260 mμ.

synthesizing system, (*b*) that actinobolin did not cause release of incomplete polypeptides, and (*c*) that actinobolin did not prevent the action of puromycin in causing such release.

Actinobolin is a potent chelating agent (1), and chelation must be taken into account as a possible explanation of some of its effects. Chelation, however, does not appear to be a satisfactory explanation of inhibition of protein synthesis in the cell-free system, because magnesium is the only metal ion known to be required for this sequence of reactions, and a concentration of actinobolin of 1.4 mM inhibits by 30–40% in the presence of Mg^{++} at a concentration of 5 mM. Thus, if all Mg^{++} ions were complexed with actinobolin in a 1:1 ratio,

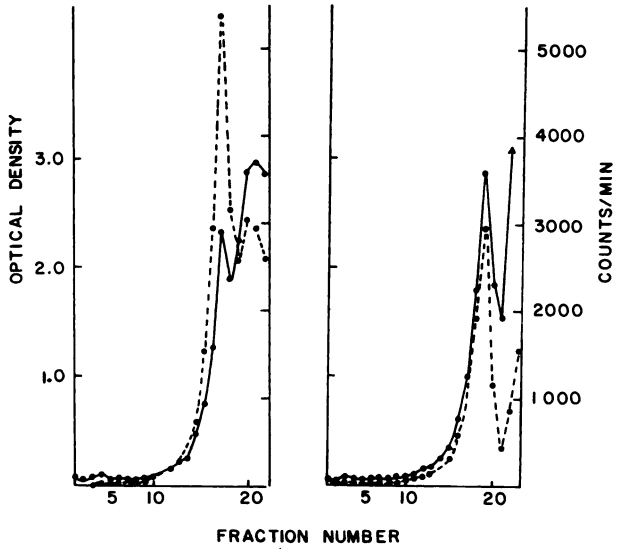

FIG. 7. *Effects of actinobolin on labeling of ribosomes by [^{14}C]phenylalanine (purified ribosomes)*

The conditions were those of the standard assay, except for the presence of poly U (400 μg/ml) or actinobolin (3.9 mM). Actinobolin, when present, was added before the addition of the enzyme fraction. After a 40-min incubation, the ribosomes were separated by centrifugation and resuspended in buffer; the suspension was then layered on 30 ml of 10–30% sucrose, and separations and assays were performed as describde in the text. Left, control; Right, actinobolin present. ●- - -●, Counts per minute per fraction; ●——●, optical density at 260 mμ.

the Mg^{++} concentration would be reduced by only about 30%.

Moncrief and Heller (25) recently pointed out that a number of biologically active compounds contain lactone rings and postulated that they may act by acylation of cellular constituents. Since actinobolin contains a lactone ring, which apparently is readily opened at pH values of 7 or higher (1), such a mechanism is also conceivable for actinobolin. That acylation or other covalent bond formation is not responsible for the inhibition of protein synthesis produced by actinobolin is indicated by the failure of cells to concentrate actinobolin and by the failure of ribosomes, tRNA, and other constituents of the cell-free system to change the pattern of elution of actinobolin from Sephadex columns.

The chief interest in the observation that actinobolin inhibits protein synthesis lies in the fact that actinobolin bears no obvious close structural relationship to other known classes of inhibitors of protein synthesis, such as the glutarimide antibiotics, streptomycin and related compounds, and chloramphenicol. Actinobolin is similar to puromycin in that it contains an aminoacyl moiety, although its mechanism of action is clearly different, as shown in the present work. The only known class of inhibitors of protein synthesis to which actinobolin bears significant structural similarity is that represented by lincomycin (26) and its close relatives celesticetin and desalicetin (27); lincomycin has been shown to act at the ribosomal level and to inhibit the binding of aminoacyl-tRNA to polysomes (28–31). The structural similarities, which are reasonably extensive, are shown in Fig. 10. Both antibiotics contain a threonine-related dipeptide unit. Munk *et al.* (7) isolated L-alanyl-L-threonine as a permanganate oxidation product of actinobolin. Hoeksema *et al.* (26) identified a derivative of D-allothreonine during structural studies on lincomycin. A configurational difference therefore appears to exist at the α-carbon atoms of the two threonine-like units of the two antibiotics. In addition, both molecules con-

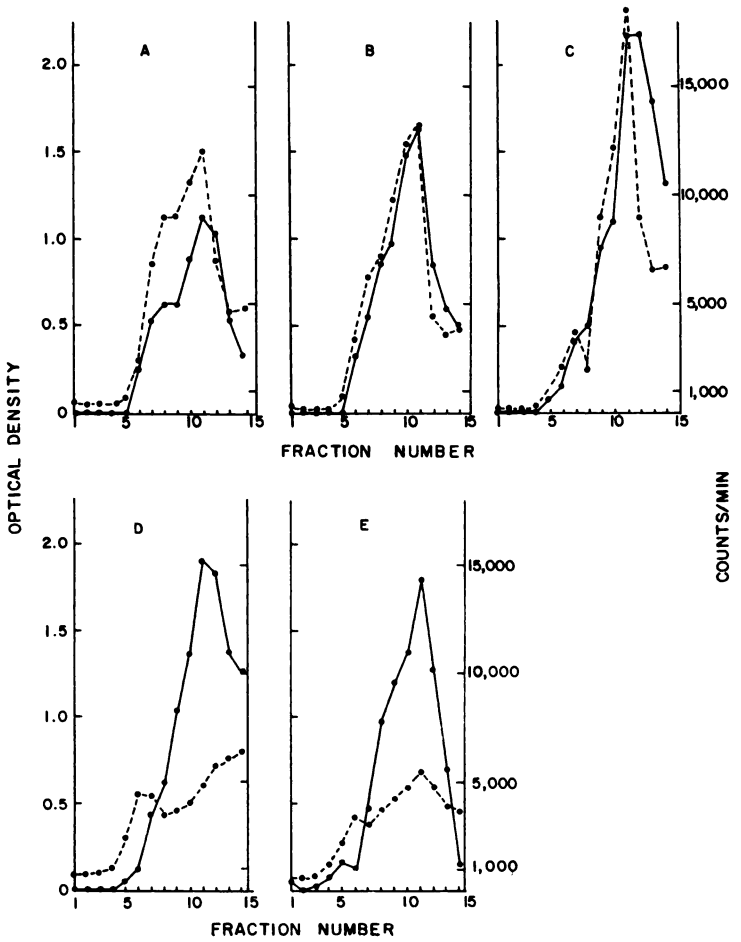

FIG. 8. *Effects of actinobolin and puromycin on release of peptides from previously labeled ribosomes*

Ribosomes were labeled by incubation for 20 min in the complete standard mixture containing [^{14}C]phenylalanine and poly U (600 μg/mg of ribosomal RNA), after which the ribosomes were separated by centrifugation. The labeled ribosomes were then divided into several portions. One portion was analyzed directly on a 10–40% sucrose gradient (A). The other portions were incubated with one of the following: fresh batches of the standard incubation mixture without an amino acid or poly U (B); fresh batches of standard mixture containing actinobolin sulfate (2.6 mM) (C); fresh batches of standard incubation mixture containing puromycin (1.0 mM) (D); or actinobolin sulfate (2.6 mM) plus puromycin (0.4 mM) (E). After 30 min, portions of the incubation mixture were assayed on 10–40% sucrose gradients as described in the text. ●– – –●, Counts per minute per fraction; ●——●, optical density at 260 mμ.

tain vicinal secondary hydroxyl groups and yielded isopropylidene derivatives at identical sites in the same portion (7, 26). Another interesting feature is that both possess one basic function with an identical pK'_a value (1, 26). Since results of this work suggest that actinobolin inhibits protein synthesis by competition with aminoacyl-tRNA for polysomes without covalent bond formation, a logical reactive site is the basic moiety of the antibiotic. The identical pK'_a value for lincomycin would suggest the possibility of a similar weak association between the same sites in the antibiotic and on the polysome.

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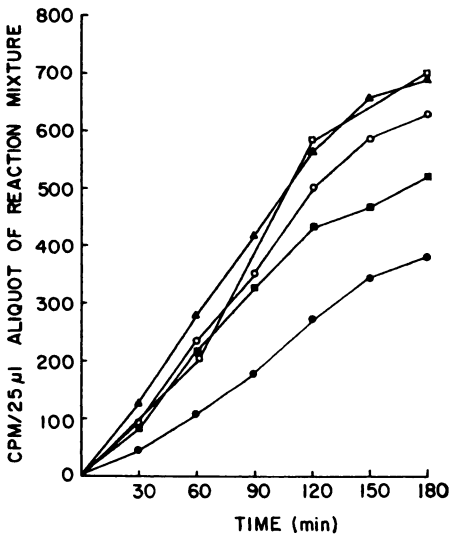

FIG. 9. *Effects of actinobolin and urethane on incorporation of [2-^{14}C]dTMP into acid-insoluble material by a DNA synthetase system from H. Ep. No. 2 cells*

See the text for references to methods used. ○——○, Control, no actinobolin; ■——■, actinobolin, 3.4 mM; ●——●, actinobolin, 10 mM; ▲——▲, urethane, 17 mM; □——□, Na_2SO_4, 7.3 mM.

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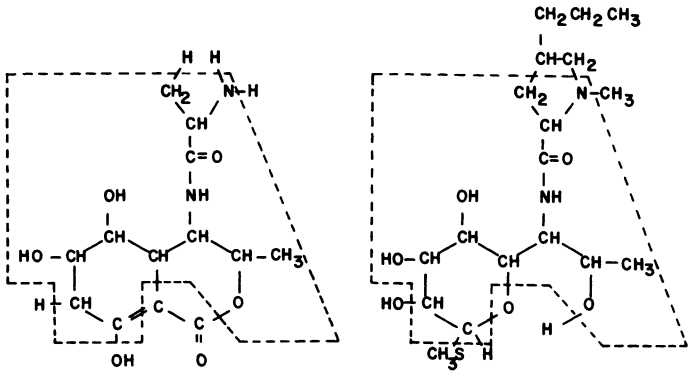

FIG 10. *Structural similarity of actinobolin and lincomycin*

The dashed lines indicate areas of the same structure in the two molecules.

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