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Actinomycin D Inhibition of Deoxyribonucleic Acid-Dependent Synthesis of Ribonucleic Acid

Abstract. Minute amounts of actinomycin D inhibit the synthesis of ribonucleic acid by nuclear extracts of HeLa cells in a ribonucleic acid–synthesizing system that is dependent on deoxyribonucleic acid and requires the presence of all four ribonucleoside triphosphates. The inhibition can be reversed by adding deoxyribonucleic acid to the enzymatic reaction. These findings support the work of others on the mode of action of actinomycin D in vivo.

Actinomycin D, one of the most potent anticancer agents discovered so far, exerts a profound influence on cellular nucleic acid (*1*). Actinomycin C forms complexes with DNA, and to a much lesser extent with RNA, similar to those formed by the acridine dyes with nucleic acids (*2*). It is by such complex formation that the antibiotic is thought to act. Although actinomycin is an inhibitor of bacterial DNA polymerase (*3*), in the bacterial or mammalian cell DNA synthesis appears to be unaffected by the antibiotic (*4*, *5*). However, Reich *et al.* have found a specific irreversible suppression of RNA formation in L cells exposed to this compound (*5*). Furthermore, their studies on mammalian cells infected with either RNA or DNA virus indicate that actinomycin D interferes with that RNA synthesis which is dependent on either cellular or viral DNA. The experiments reported here on the effect of antinomycin D on the cell-free syntheisis of RNA support such a mechanism.

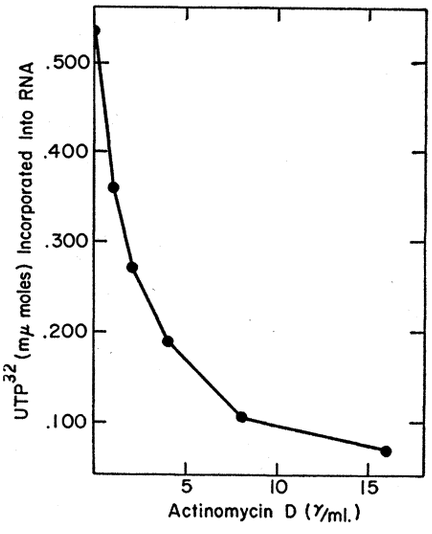

Fig. 1. Inhibition of incorporation of P^{32}-labeled uridine triphosphate (UTP^{32}) into RNA by varying concentrations of actinomycin D. Concentration of actinomycin D is given in micrograms (γ) per milliliter.

An RNA-synthesizing system that is dependent on DNA and requires the presence of all four ribonucleoside triphosphates has been found in nuclear extracts of HeLa cells (*6*). The enzymatically active component is composed of protein tightly bound with DNA and smaller amounts of RNA. This "aggregate" is capable of incorporating P^{32} from ribonucleoside triphosphate (labeled in its innermost phosphate) into RNA. In the experiments reported here proximally labeled uridine triphosphate was used to incorporate radioactive uridylic acid into RNA. The HeLa "aggregate" preparation, as well as the method of assay for RNA synthesis, have been described in a previous publication (*6*).

Small amounts of antinomycin D strikingly inhibited the incorporation of P^{32}-labeled uridine triphosphate into RNA (Fig. 1). The HeLa "aggregate" used in this experiment contained 1.44 mg of protein, 390 μg of DNA, and 144 μg of RNA. The reaction mixture contained 3 μmole of $MnCl_2$; 100 μmole of Tris buffer (*p*H 8.1); 20 μmole of NaF; 0.8 μmole each of adenosine triphosphate, guanosine triphosphate, and cytidine triphosphate; 0.1 μmole of uridine triphosphate-P^{32} (2.8×10^6 count/min μmole); and 0.1 ml of saturated $(NH_4)_2SO_4$ (4°C), at *p*H 8.0. The final volume was 1.0 ml, and incubation was for 20 minutes at 37°C. A similar curve of inhibition was obtained with P^{32}-labeled 5-ribosyluracil triphosphate (*7*). Actinomycin D was an effective inhibitor in this system at considerably lower concentrations than reported for the DNA polymerase (*3*). Mitomycin (100 μg/ml) and vancomycin (200 μg/ml) had no effect on the RNA-synthesizing system. With the same amount of "aggregate" with which 2 μg/ml of actinomycin D produced a 51-percent inhibition of incorporation of label into RNA, proflavine (200 μg/ml) caused a 35-percent and acriflavine (200 μg/ml) a 69-percent decrease in incorporation. In these experiments the RNA was isolated by repeated acid washes, since in the presence of high concentrations of acriflavine, ethanol precipitation of RNA was found to be incomplete.

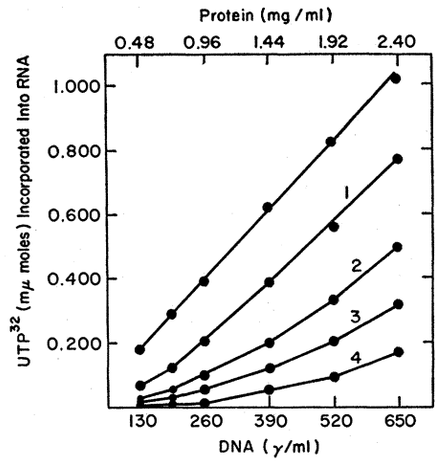

Fig. 2. Effect of varying the HeLa "aggregate" concentration on the degree of inhibition of RNA synthesis produced by different concentrations of actinomycin D. Actinomycin D concentrations: curve No. 1, 2 μg/ml; No. 2, 4 μg/ml; No. 3, 8 μg/ml; and No. 4, 16 μg/ml. Concentration of DNA is given in micrograms (γ) per milliliter.

The study of the kinetics of inhibition by actinomycin D is made difficult by the presence of considerable amounts of DNA bound to the HeLa RNA polymerase. It has been possible, however, to examine the effect of different concentrations of the antibiotic with

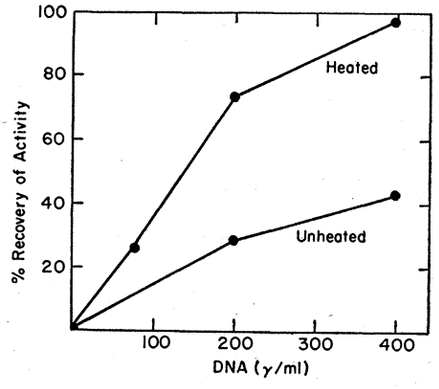

Fig. 3. Reversal of actinomycin D inhibition by addition of calf thymus DNA. Concentration of DNA is given in micrograms (γ) per milliliter.

varying "aggregate" concentrations (Fig. 2). The degree of inhibition bears an inverse relationship to the concentration of "aggregate" or probably more properly DNA.

The actinomycin D effect can be reversed by the addition of calf thymus or HeLa DNA to the enzymatic reaction (Fig. 3). In this experiment 2.4 mg protein of HeLa "aggregate" and 4 μg/ml of actinomycin D were used. Heat-denaturation was accomplished by heating a solution of calf thymus DNA (2 mg/ml) in 0.01*M* Tris buffer, *p*H 7.4 and 0.01*M* NaCl at 100°C for 3 minutes and then rapidly cooling it in ice. Heat-denatured DNA appeared to be more effective than native DNA. Single-stranded DNA, which complexes less readily with the antibiotic (*3*), may act as a substitute for the inactivated natural DNA template. Yeast RNA at about 100 times the concentration of DNA used in the experiment described above, relieved the inhibition only in part.

The inhibition of the RNA polymerase system in HeLa cells by minute amounts of actinomycin D and its reversal by addition of DNA support the mode of action proposed by Reich *et al.* (*5*) for this antibiotic in the intact cell (*8*).

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8. The RNA and DNA determinations were performed by Edwin Rosenbloom. This study was aided by grants from the National Science Foundation (G14383) and from the United States Public Health Service (H-4442 and HTS-5447).
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2 November 1961

Magnesium Binding as an Explanation of the Mode of Action of Novobiocin

Abstract. Novobiocin affects a wide variety of biochemical and biological processes in bacteria, whereas most other antibiotics seem to affect one specific process. All of the processes known to be affected by novobiocin require magnesium ions, and in a number of cases magnesium deficiency mimics the action of the antibiotic. Evidence is presented that novobiocin complexes with magnesium ions.

Earlier work has shown that the action of novobiocin is reversed by magnesium ions (*1*) and that the antibiotic affects the permeability of *Escherichia coli* ML 35 (*2*). Recently a number of other effects of novobiocin have been noticed. A search for a common element in these effects revealed that all of the processes inhibited require magnesium ions. In addition, novobiocin induces some effects that are also brought about by magnesium deficiency.

Since novobiocin is an inhibitor of growth, it is essential that any effects noted should be attainable at concentrations of the antibiotic which inhibit growth. However, when the antibiotic is added to growing cells of a sensitive organism (*Streptococcus faecium*), growth continues for a period of time. The degree of residual growth is less at higher concentrations of novobiocin. However, it is only at a concentration of $10^{-3}M$ that growth stops immediately after the antibiotic is added, although a concentration of $10^{-6}M$ will eventually bring about complete inhibition. In short-term biochemical experiments with nongrowing cells, inhibitions achieved at $10^{-3}M$ are therefore considered significant in explaining growth inhibition.

A summary of the magnesium-related processes affected by novobiocin is given in Tables 1 and 2.

Novobiocin is not a general cytotoxic substance, since cells can be incubated for several hours in the presence of $10^{-3}M$ concentrations with no significant killing. Further, the accumulation of C^{14}-uracil in the cell pool is not inhibited but is in fact three times higher than in the control. The incorporation of C^{14}-lysine into the cell wall is only slightly affected by concentrations that completely prevent lysine incorporation into soluble RNA.

Webb (*3*) has shown that zinc ions potentiate magnesium deficiency. Zinc ions also potentiate novobiocin action against *Escherichia coli*. Sodium azide inhibits most of the same processes inhibited by novobiocin and, when used at subinhibitory concentrations, also potentiates novobiocin action against *E. coli*.

Novobiocin is able to form insoluble salts with a variety of metal ions (magnesium, calcium, manganese, and zinc). In addition, novobiocin is able to form weak complexes of another type with magnesium ions, as shown by the following observations:

1) An aqueous mixture of 0.01*M* novobiocin monosodium salt and 0.01*M* $MgCl_2$ at *p*H 7.5 or 10.0 fluoresces under ultraviolet light when frozen or when dried on filter paper but does not fluoresce in the liquid state. A fluorescent complex is not formed with sodium or calcium ions.

2) Mixtures of 0.01*M* novobiocin and 0.01*M* $MgCl_2$ are more yellow than solutions of the antibiotic alone. Difference spectra reveal a peak for the mixture at 360 mμ at *p*H 7 or 10,

Table 1. Processes requiring magnesium that are inhibited by novobiocin.

Magnesium-requiring process	Novobiocin inhibits completely at
Growth	$10^{-3}M$
Nitrate reductase in *Escherichia coli* (*2*)	$10^{-3}M$
Succinic dehydrogenase in *Micrococcus lysodeikticus**	$10^{-3}M$
Ethanol dehydrogenase in *M. lysodeikticus**	$10^{-3}M$
Adenosine triphosphatase in *E. coli*† and *Streptococcus faecium**	$10^{-2}M$
Oxidative phosphorylation in rat liver (*5*)	$7.8 \times 10^{-4}M$
Amino acid incorporation into soluble RNA in *S. faecium**	$10^{-4}M$
RNA synthesis in *S. faecium**	$10^{-4}M$
Amino acid transport in *S. faecium**	$10^{-3}M$

* My observations. † D. Kessler and H. V. Rickenberg, unpublished observations.

Table 2. Effects induced by both magnesium deficiency and novobiocin.

Effect induced by magnesium deficiency	Same effect induced by novobiocin at
Increased permeability to ortho-nitrophenyl-β-D-galactoside in *Escherichia coli* ML 35*	$10^{-3}M$
Loss of 260 mμ absorbing material in *E. coli* ML 35*	$10^{-3}M$
Filamentation in *E. coli* (*1*)	$10^{-3}M$–$10^{-5}M$
Chaining in *Streptococcus faecium**	$10^{-6}M$
Slow death of cells in *E. coli* (*2*) and *S. faecium**	$10^{-3}M$
Slow autolysis of cells in *E. coli* ML 35*	$10^{-3}M$

* My observations.