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Mode of Action of Antibiotic U-20,661

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ABSTRACT

Antibiotic U-20,661 was shown to inhibit predominantly deoxyribonucleic acid (DNA)-directed ribonucleic acid (RNA) synthesis by binding to the double-stranded DNA template. Specific binding to DNA was verified by difference spectroscopy, reversal of the RNA polymerase inhibitory effect by increasing concentrations of DNA template, and by moderately increasing the melting temperature of double-stranded DNA in the presence of the antibiotic. The RNA polymerase reaction primed with synthetic poly dAT was inhibited considerably, but not completely even with high concentrations of antibiotic. Thus, the agent might bind to adenine or thymidine or both bases in the double-stranded DNA helix.

Antibiotic U-20, 661, a new antibacterial agent, was isolated from the culture broth of *Streptomyces steffisburgensis* sp. n. (Dietz, *in preparation*). The neutral compound crystallizes as orange-red needles which have very limited solubility in aqueous solutions. Preparation, isolation, characterization, and biological properties will be described elsewhere (Bergy and Reusser, *in preparation*). The compound is highly inhibitory against gram-positive bacteria *in vitro*, but was ineffective in the treatment of experimental infections caused by gram-positive organisms in mice. The antibiotic is remarkably nontoxic in mice and extremely cytotoxic in mammalian cell cultures.

This paper describes the effects of antibiotic U-20,661 on protein and nucleic acid synthesis in *Bacillus subtilis* cells as well as in cell-free macromolecular biosynthetic systems of bacterial origin.

MATERIALS AND METHODS

B. subtilis strain 23 cells were grown in 2% peptone broth (Difco). Antibiotic was added to the culture during the early log phase of growth. Cell growth was followed by measuring the optical density of the bacterial suspension at 570 mμ.

The cellular protein fraction was isolated essentially as described by Park and Hancock (10), except that the trypsinization step of the protein fraction was replaced by treatment with 1 N NaOH at 37 C overnight. Protein in this fraction was determined by the method of Lowry et al. (6).

Nucleic acids from the *B. subtilis* cells were isolated and separated into deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) fractions according to the Schmidt-Thannhauser method (13). DNA was determined with the diphenylamine reagent and RNA by the orcinol method (13).

Uniformly labeled valine-C^{14} (208.5 mc/mmole) incorporation was studied in a cell-free *B. subtilis* system (strain 23), which was prepared essentially as described by Matthaei and Nirenberg (8) for their *Escherichia coli* system. The 30*S* fraction was used as such without further purification, and no extraneous soluble RNA (sRNA) or templates were added. The reaction was run at room temperature in a total of 2 ml and contained 2 mg of 30*S* protein per ml of reaction mixture. The cell-free polyuridylic acid (poly U)-directed phenylalanine-C^{14} incorporation system was prepared as described by Nirenberg (9). The 30*S* fraction was used as an enzyme source.

DNA-dependent RNA polymerase was purified partially as described by Chamberlin and Berg (2). The purification included precipitations with streptomycin, protamine, and ammonium sulfate, followed by diethylaminoethyl (DEAE) chromatography. The purified enzyme incorporated approximately 325 mμmoles of adenosine triphosphate (ATP) per mg of protein per hour with native salmon sperm DNA as a primer. The assay mixtures(0.25 ml) were prepared as described by Chamberlin and Berg (2) with the following exceptions: DNA (salmon sperm), 6.6 μg; ATP-*8*-C^{14} (31 mc/mmole), 0.045 μc; polymerase, 35 μg of protein (6). The system was strictly DNA-dependent. The reaction was stopped by the addition of 3 ml of cold 3.5% perchloric acid containing 60 mg of Celite per 100 ml. The acid-insoluble product was collected on 0.45-μ filter paper discs, type HA (Millipore Filter Corp., Bedford, Mass.), and the discs were washed extensively with cold 0.1 N HCl. The papers were dissolved in 15 ml of diotol and counted in a scintillation counter. Diotol contained 30 ml of toluene, 35 ml of dioxane, 21 ml of methanol, 7.3 g of naphthalene, and 5 ml of liquifluor (Pilot Chemicals, Inc., Watertown, Mass.).

DNA polymerase was purified partially as de-

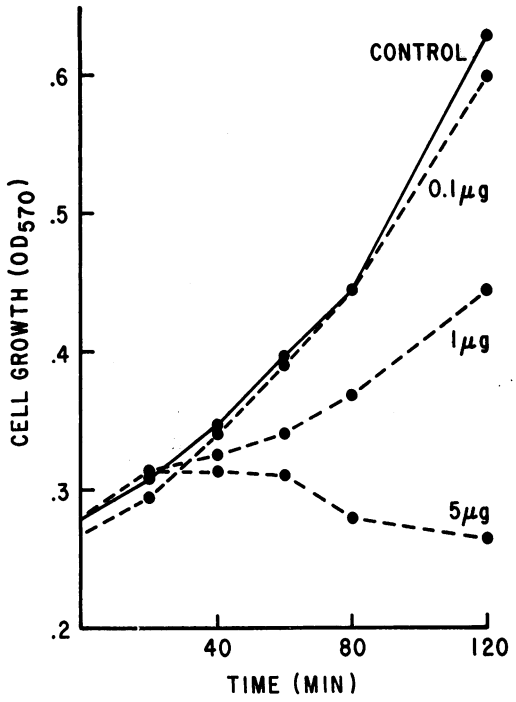

FIG. 1. *Effect of antibiotic U-20,661 on Bacillus subtilis cell growth. The antibiotic was added to the culture during the early log phase of growth.*

scribed by Richardson et al. (12). Fraction 4 was used throughout these experiments as an enzyme source. The purified polymerase incorporated approximately 9 mμmoles of thymidine triphosphate (TTP) per mg of protein per hour with denatured salmon sperm DNA as a primer. The compositions of the assay mixtures (0.5 ml) were as described by Richardson et al. (12) with the following exceptions: salmon sperm DNA, 6.6 μg; TTP-H^3 (1.2 c/mmole), 0.22 μc; polymerase, 291 μg of protein (6). The reaction was strictly DNA-dependent. The acid-insoluble fraction was recovered as described by Magee (7).

Salmon sperm DNA was denatured by heating a DNA solution at 100 C for 10 min followed by rapid cooling in ice.

Difference spectra were measured in a Cary spectrophotometer by the method of Goldberg et al. (4) in the cuvettes described by Trowne and Rabin (15).

Measurements of the melting temperature of calf thymus DNA (T_m) were made with a Gilford recording thermospectrophotometer. Detailed experimental conditions are give in the legend of Table 2.

RESULTS

Experiments with B. subtilis cells. The effects of different concentrations of antibiotic U-20,661 on cell growth of *B. subtilis* cells are shown in Fig. 1. At a concentration of 5 μg/ml, growth of exponentially dividing cells ceased after addition of the drug. Lower concentrations caused proportional inhibitions. Cells exposed to 5

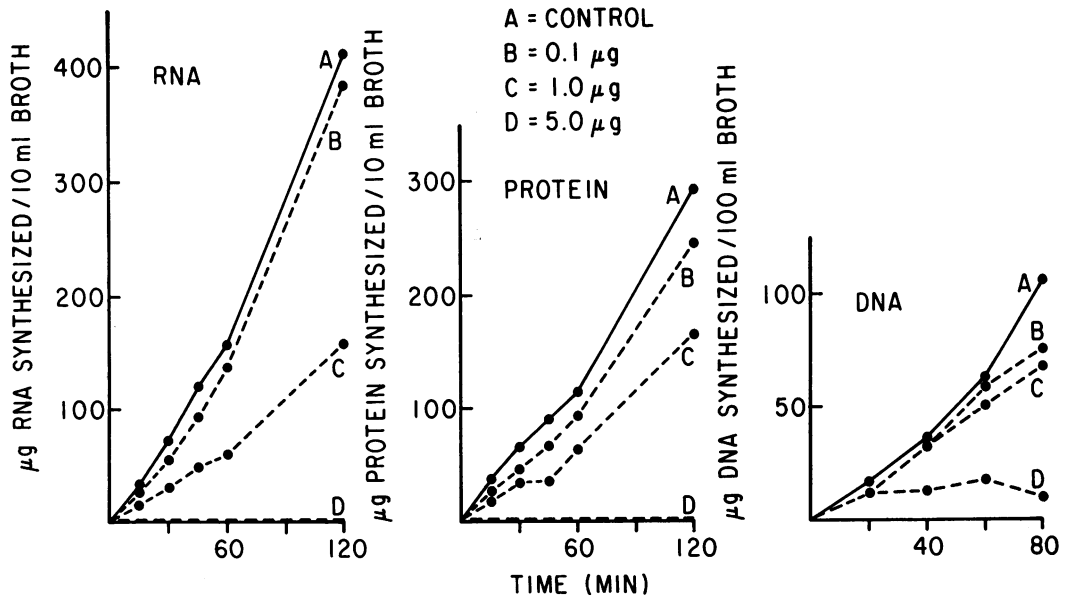

FIG. 2. *Effect of antibiotic U-20,661 on RNA, DNA, and protein synthesis in whole Bacillus subtilis cells. The cellular protein fraction was isolated according to Park and Hancock (10), and the protein content was determined by the Lowry method. The nucleic acid fraction was recovered and separated into DNA and RNA by the Schmidt-Thannhauser method. RNA was estimated with the orcinol; DNA, with the diphenylamine reagent.*

μg/ml remained viable for at least 2 hr. The primary effect of the antibiotic on *B. subtilis* is bacteriostasis.

Syntheses of cellular protein and RNA (Fig. 2) were inhibited completely and immediately after addition of 5 μg of antibiotic per ml. At this same concentration, traces of DNA (Fig. 2) appear to have been synthesized during the first 15 min after addition of the drug. Lower concentrations caused proportional inhibitions of the three macromolecular biosynthetic processes.

Effect of antibiotic U-20,661 on macromolecular biosynthetic processes in cell-free systems. Concentrations of 40 or 20 μg of antibiotic per ml of reaction mixture caused approximately 40% inhibition of valine-C^{14} incorporation in a cell-free *B. subtilis* amino acid incorporation system after 30 min of incubation (Fig. 3). Poly U-directed phenylalanine-C^{14} incorporation in a cell-free *E. coli* system was affected similarly (Table 1). Amounts of 50 μg of antibiotic per ml caused 30% inhibition; 25 μg/ml caused approximately 16% inhibition. Thus, both systems were inhibited somewhat at higher antibiotic concentrations. The DNA polymerase reaction (Fig. 4) was inhibited only marginally at relatively high antibiotic concentrations. At a dose of 200 μg/ml of reaction mixture, only 15% inhibition of DNA polymerase was obtained with denatured salmon DNA primer and 28.3% inhibition occurred with native salmon DNA primer. Low concentrations (e.g., 20 to 50 μg/ml) were even slightly stimulatory with both primers. By contrast, the DNA-directed RNA polymerase system was profoundly affected by the antibiotic (Fig. 5). With the native DNA primer, 50% inhibition of the reaction was achieved at a dose of 2.5 μg/ml. When the reaction was primed with denatured DNA, significantly less inhibition occurred, and a dose of 30 μg/ml gave 50% inhibition of the reaction. The inhibitory effect of antibiotic U-20,661 on RNA polymerase was reversed by increasing amounts of DNA template in the reaction mixture. Thus, DNA concentrations of 6.6, 13.3, and 20 μg per sample gave inhibitions of 46.2, 45.4, and 26.9% in the presence of 5 μg of antibiotic per ml of sample. With synthetic poly dAT primer, 75% inhibition was obtained at an antibiotic concentration of 20 μg/ml with 0.60 optical density units (at 260 mμ) of primer per ml and 90% inhibition at a concentration of 40 μg/ml with 0.24 units of primer per ml (Table 2).

Effect of antibiotic U-20,661 on thermal transition of DNA (T_m). The antibiotic had little effect on the melting temperature of calf thymus DNA under the conditions used except when

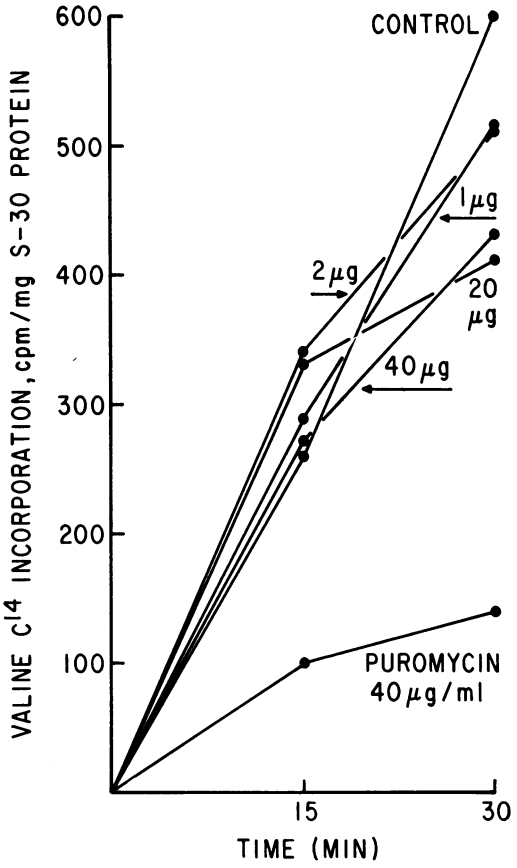

FIG. 3. *Effect of antibiotic U-20,661 on in vitro amino acid incorporation system. The reaction mixture (2.0 ml) contained, per ml: 100 μmoles of tris(hydroxymethyl) aminomethane (pH 7.8); 10 μmoles of magnesium acetate; 50 μmoles of KCl; 6 μmoles of mercaptoethanol; 1 μmole of ATP; 5 μmoles of phosphoenolpyruvate; 20 μg of pyruvate kinase (crystalline); 0.05 μmole of 20 L-amino acids minus valine; 0.03 μmole each of guanosine triphosphate, cytidine triphosphate, and uridine triphosphate; ~100,000 counts/min of L-valine-C^{14}; and 2 mg of 30S protein. Incubation was at room temperature. Samples (0.5 ml) were withdrawn at appropriate times, and the reaction was stopped by the addition of equal volumes of cold 10% trichloroacetic acid.*

present in high amounts (see Table 3). It was observed that the antibiotic alone yielded a linear increase in optical density upon slow heating, amounting to approximately twice the original value in a 57 μg/ml solution when heated from room temperature to 60 C.

Difference spectroscopy with antibiotic-DNA mixtures. Difference spectra obtained with calf thymus DNA, salmon sperm DNA, and *E. coli* DNA mixed with antibiotic U-20,661 were similar, and showed an absorption shift from 440 to

TABLE 1. *Effect of antibiotic U-20,661 on in vitro poly U-directed phenylalanine incorporation system*[a]

Addition (per ml of reaction mixture)	Counts per min per mg of 30S protein	Per cent of control
U-20,661, 50 μg	3,640	69.5
U-20,661, 25 μg	4,372	83.5
U-20,661, 10 μg	5,314	101.5
U-20,661, 5 μg	5,537	105.7
None, control	5,234	100.0
Control, 0 min	18	.3
Less poly U	57	1.1

[a] Assay mixtures (0.25 ml) were those described by Nirenberg (9) with the following exceptions: poly U, 15 μg; 30S protein, 1 mg. The reactions were conducted at 37 C for 15 min.

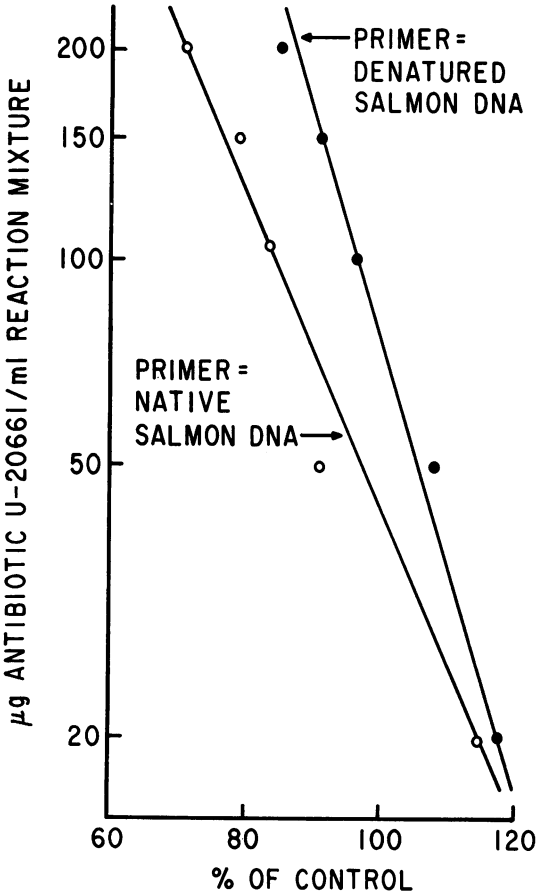

FIG. 4. *Effect of antibiotic U-20,661 on cell-free DNA polymerase system. Assay mixtures (0.5 ml) were those described by Richardson et al. (12) with the following exceptions: DNA, 6.6 μg; TTP-H³, 0.22 μc; polymerase, 291 μg.*

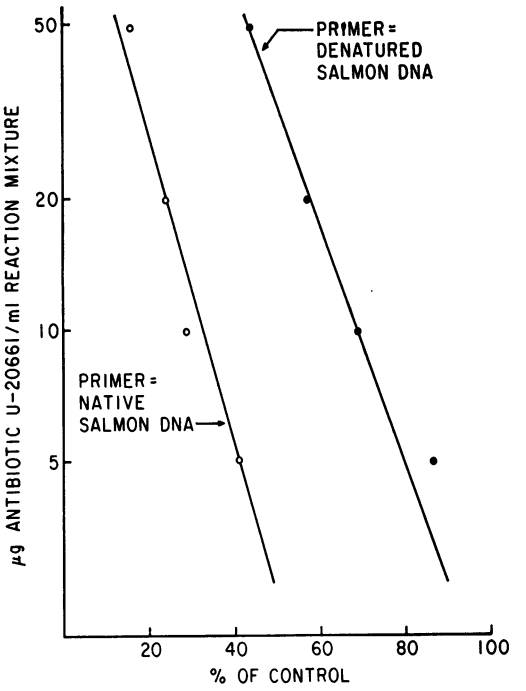

FIG. 5. *Effect of antibiotic U-20,661 on cell-free DNA-dependent RNA polymerase system. Assay mixtures (0.25 ml) were those described by Chamberlin and Berg (2) with the following exceptions: DNA, 6.6 μg; ATP-8-C¹⁴, 0.045 μc; polymerase, 35 μg.*

TABLE 2. *Effect of antibiotic U-20,661 on RNA polymerase primed with synthetic poly dAT*

Amt of primer (OD units/sample)[a]	Antibiotic U-20, 661	Inhibition
	μg/ml	%
0.15	20	74.6
0.06	40	89.6

[a] Optical density determined at 260 mμ.

TABLE 3. *Effect of antibiotic U-20,661 on thermal transition of calf thymus DNA (T_m)*[a]

Antibiotic	T_m
μg/ml	C
None	62.5
1.15	63.5
11.5	68.0
114.8	72.0

[a] Reaction mixtures contained 25 μg/ml of calf thymus DNA in 0.002 M tris(hydroxymethyl) aminomethane-HCl buffer (*p*H 7.6) containing 0.002 M NaCl.

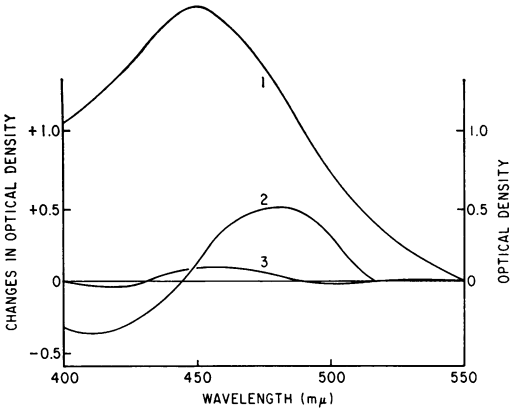

FIG. 6. *Difference spectra of antibiotic U-20,661 and DNA. All substances were dissolved in 0.01 M Tris-HCl (pH 7.6) containing 0.01 M NaCl. Regular spectrum of antibiotic U-20,661 (100 μg/ml) read against buffer blank = curve 1. A 1-ml amount of antibiotic solution containing 200 μg/ml was mixed with 1 ml of a 1 mg/ml solution of salmon sperm DNA (curve 2) or 1 ml of 2 mM cytosine solution (curve 3).*

480 mμ (Fig. 6). Apurinic DNA prepared from calf thymus DNA (13), *E. coli* sRNA, yeast RNA, the usual nucleic acid bases, ribose- and deoxyribonucleosides or nucleotides, ribose, deoxyribose, and inorganic PO_4^{-} additions caused small, nonspecific changes in the spectrum identical to that shown in Fig. 6 for cytosine.

DISCUSSION

Antibiotic U-20,661 was shown to inhibit protein and nucleic acid synthesis in *B. subtilis* cells. Experiments in cell-free systems indicated that the antibiotic predominantly affects DNA-directed RNA synthesis by binding to the DNA template. The fact that RNA synthesis is more profoundly affected with native template than with denatured DNA suggests that the degree of helicicity of the template is important for the antibiotic to bind to DNA. Specific binding to DNA was verified by difference spectroscopy, reversibility of the RNA polymerase inhibition by increasing concentrations of DNA template, and a moderate increase of the melting temperature of double-stranded DNA-antibiotic complexes. Difference spectroscopy with several bases did not indicate to which bases, if any, the antibiotic might be bound specifically. However, such an absence of change in spectrum does not preclude base-specific binding. The RNA polymerase reaction was inhibited significantly by the antibiotic when primed with synthetic poly dAT, but synthesis was not abolished completely despite high doses of the drug. Thus, antibiotic U-20,661 might react with either or both of these bases, but the absence of complete inhibition indicates some degree of specificity of the antibiotic toward the secondary structure of the primer DNA. These experiments do not exclude the possibility of additional interactions of the antibiotic with guanine or cytosine residues in the DNA helix.

The experiments in whole *B. subtilis* cells have shown that both RNA and protein synthesis ceased immediately after addition of the antibiotic. The results derived from the cell-free systems would allow one to expect that some protein synthesis would continue after RNA synthesis ceased until full depletion of preformed messenger RNA (mRNA) was attained. This immediate cessation of protein synthesis in intact cells cannot be explained at this time. One might postulate a very short half-life for mRNA in this *B. subtilis* strain, or consider that the cellular fractionation methods used are gross; hence, small amounts of newly formed protein in the presence of the antibiotic might have escaped detection. However, the possibility of secondary effects of the antibiotic upon the cellular protein synthetic mechanism cannot be excluded, since moderate inhibition was also observed in the cell-free amino acid incorporation systems at higher antibiotic concentrations.

The predominant suppression of DNA-directed RNA synthesis by binding to the DNA primer suggests that antibiotic U-20,661 is related in its mode of action to other antibiotics known to inhibit the same reaction, such as actinomycin D, daunomycin, nogalamycin, chromomycin, etc. (1, 3, 5, 11). Although the described experiments in cell-free systems have established the primary site of antibiotic U-20,661 inhibition, they do not provide an explanation for the remarkable nontoxic behavior of the agent in mice.

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