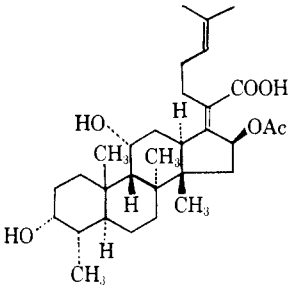
On the Mode of Action of Fusidic Acid*

C. L. Harvey,† S. G. Knight, and Charles J. Sih

ABSTRACT: Fucidin, a steroidal antibiotic, inhibited protein synthesis in whole cells of *Staphylococcus aureus* and inhibited polyphenylalanine synthesis in cell-free systems from *Escherichia coli*, *Bacillus stearothermophilis*, and a diploid yeast. The incorporation of tyrosine, alanine, and lysine into protein in cell-free systems from *E. coli* was also inhibited by this antibiotic. It was necessary that the antibiotic be present at the time of peptide synthesis for inhibition to occur and this inhibition was not reversed by increasing amounts of polyuridylic acid (poly-U), magnesium ions, phenylalanine soluble ribonucleic acid (s-RNA), guanosine triphosphate, ribosomes, or supernatant fraction. Binding of fucidin to ribosomes or polysomes from *E. coli* could not be found with our experimental conditions. In attempting to determine the exact step in protein synthesis that was inhibited by fucidin, it was found that fucidin did not inhibit the activation or transfer of phenylalanine to s-RNA, or the attachment of poly-U and phenylalanyl s-RNA to the ribosome.

Fusidic acid was isolated from *Fusidium coccineum* by Godtfredsen *et al.* (1962a) and its structure was determined by Godtfredsen and Vangedal (1962), and Arigoni *et al.* (1963, 1964). The structure of this antibiotic is shown below.



Fusidic acid is chemically related to cephalosporin P_1 and helvolic acid (Umezawa, 1964). The similarity in structure of these three steroid antibiotics is reflected in their activity against gram-positive bacteria but not against gram-negative bacteria, yeasts, or molds (Burton and Abraham, 1951; Godtfredsen *et al.*, 1962b). These facts indicate that the steroidal antibiotics possess a similar mode of action, and fusidic acid, because of its greater potency and clinical value, was selected for study. During the course of this study, Yamaki (1965) independently reported that fusidic acid is an inhibitor of protein synthesis. This paper further defines the site within the over-all process of protein synthesis at which fusidic acid inhibits. A preliminary report on this work already appeared (Harvey *et al.*, 1965).

Experimental Procedure

Cultures. Two bacterial cultures were used in these studies. Nonpigmented, coagulase negative *Staphylococcus aureus* (K. R., 1962) and *Escherichia coli* C were obtained from Professors W. B. Sarles and D. Pratt, respectively (Department of Bacteriology, University of Wisconsin, Madison, Wis.). These cultures were transferred monthly to slants of tryptose phosphate agar (Difco) and incubated overnight at 37°. They were stored at 3°.

Fucidin Sensitivity Assays. The sensitivity of the cultures to fucidin was determined by serial dilution in tryptose phosphate broth. The broth (1 ml) containing the antibiotic was inoculated with 1 ml of a 1:100 dilution of a 20-hr cultures of the organism in the same broth. The tubes were incubated at 37° for 20 hr, and the minimum inhibitory concentration[1] of the antibiotic was determined.

Conditions for Determining Macromolecule Synthesis. Tryptose phosphate broth (100 ml) was inoculated from a slant of *S. aureus* and incubated overnight at 25°. This culture was transferred to 600 ml of the same medium in a 2-l. erlenmeyer flask, which then was incubated for 6 hr on a rotary shaker at 25°. When the logarithmic growth phase was reached [optical density of 0.4 at 600 mμ, Spectronic 20 (B and L)] 0.019 mM fucidin was added and 100-ml samples were taken every 15 min. The samples were cooled rapidly to 5° in a salt–ice mixture and sedimented at 10,000*g* for approximately 10 min at 3° in a Servall centrifuge.

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† Present address: Chemistry Department, Hoffmann-La Roche, Nutley, N. J.

[1] Abbreviations used: MIC, minimum inhibitory concentration; TCA, trichloroacetic acid; ATP and GTP, adenosine and guanosine triphosphate.

The supernatant was decanted and the cells were washed twice by centrifugation in 10-ml volumes of cold, distilled water, followed by washing with 5 ml of cold 5% TCA. After centrifugation, 2 ml of 5% TCA was added to the residue; the suspension was heated for 20 min at 100°, cooled, and sedimented by centrifugation. The supernatant fluid was saved for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) determinations. The residue was mixed with 5 ml of 1 N NaOH, left overnight at 37°, and saved for protein assay. The same procedure was used for the control culture except fucidin was omitted. All determinations were corrected to the starting optical density.

Conditions for the Uptake of L-[^{14}C]*Lysine and Fractionation of the Cells. S. aureus* was grown overnight on a rotary shaker at 25°. The medium contained 5 g of glucose, 5 g of peptone (Difco), 2 g of yeast extract (Difco), and 0.3 g of K_2HPO_4/l. This culture (5 ml) was added to 45 ml of fresh medium, diluted tivefold in each of two 250-ml erlenmeyer flasks, and incubation was continued for 2 hr. At this time (zero time), 5 μc of L-[^{14}C]lysine was added to each flask and after 10 min, 0.019 mM fucidin was added to one of the flasks. Samples (5 ml) were withdrawn from each flask and the optical density at 600 mμ was read; the samples were then added to 5 ml of 10% TCA. The cells that had been precipitated with TCA were fractionated by the following modification of the procedure of Park and Hancock (1960). The TCA precipitate was heated for 30 min at 100° and sedimented by centrifugation. The residue was washed by centrifugation in two 5-ml volumes of 5% TCA, followed by 5 ml of ethanol–ether (3:5). The washed residue was dried and digested with 1 ml of a buffered solution of trypsin. After 3 hr of digestion at room temperature, the residue was separated by centrifugation and the supernatant fluid containing the protein fraction was saved for determination of radioactivity. The sides of the tube containing the trypsin-digested residue were wiped dry, and 1 ml of 1 N NaOH was added. After dissolving the residue overnight at 37°, the solution, which contained the mucopeptide fraction, was used for ^{14}C determination.

Preparation of Extracts. The procedure of Nirenberg (1963) was used to grow cells of *E. coli* C and to prepare the extracts and fractions. The extracts were not preincubated unless otherwise stated. They were, however, separated into a S100 fraction, which is the supernatant fluid from 107,000*g* centrifugation, and a W-RIB fraction, the washed ribosomes. These fractions were frozen rapidly in a Dry Ice–acetone mixture and stored at −20°. *Saccharomyces fragilis* x *Saccharomyces dobzanskii*, a diploid yeast, was obtained from Professor H. O. Halvorson (Department of Bacteriology, University of Wisconsin). The conditions used for the preparation of the extracts and for the assay of the [^{14}C]phenylalanine incorporation directed by polyuridylic acid (poly-U) were the same as those used by Bretthauer *et al.* (1963). *Bacillus stearothermophilus* NCA 2184 extracts were prepared by the method of Friedman and Weinstein (1964).

Composition of Reaction Mixture for in Vitro Protein Synthesis. The basic reaction mixture for *in vitro* incorporation of [^{14}C]amino acids was a modification of Nirenberg's (1963). The mixture contained in 0.5 ml: 50 μmoles of Tris–HCl buffer (pH 7.8), 7 μmoles of Mg acetate, 80 μmoles of NH_4Cl, 0.5 μmole of ATP, 0.015 μmole of GTP, 1.9 μmoles of phosphoenolpyruvate, 4 μg of pyruvate kinase, 20 μg of poly-U, 0.2 μl of 2-mercaptoethanol, 0.05 μmole of each of the L-amino acids (omitting the appropriate [^{14}C]amino acid), 1 mg of supernatant (S100) protein, and 1 mg of ribosomal protein.

Procedure for Cell-Free Protein Synthesis. The reaction mixture was incubated at 37° for 15 min. The reaction was terminated by the addition of 3 ml of 10% TCA and the mixture was then heated at 100° for 20 min. After cooling for 30 min in ice water, the precipitate was removed by centrifugation and washed with 3–5-ml volumes of 5% TCA followed by 5 ml of ethanol–ether (3:5). The precipitate was drained until dry and dispersed in 1 ml of 10× hyamine hydroxide (Packard Instrument Co.). When the precipitate had dissolved (heating at 65° for 10 min was sometimes necessary), the solution was transferred quantitatively to 10 ml of toluene scintillant solution and the radioactivity counted in a Packard scintillation counter.

Preparation of [^{14}C]*Phenylalanyl Soluble Ribonucleic Acid (s-RNA). E. coli* B s-RNA (Calbiochem) was freed of residual amino acids by the method of Nathans and Lipmann (1961). The s-RNA was charged in 5 ml of the following mixture: 500 μmoles of Tris–acetate buffer (pH 7.4), 50 μmoles of Mg acetate, 20 μmoles of ATP (pH 7.0), 7 mg of *E. coli* C supernatant (S100) protein, 10 mg of s-RNA, and 0.5 μc of L-[^{14}C]phenylalanine (302 mc/mM). After incubation at 35° for 30 min, the s-RNA was purified by phenol extraction and ethanol precipitation using the method of Conway (1964). The precipitate was dissolved in 3 ml of buffer (0.01 M NH_4 maleate, pH 7.4, and 0.01 M KCl) and dialyzed overnight at 3° against two changes of the same buffer. The s-RNA, charged with L-[^{14}C]phenylalanine, was stored at −20° in 1-ml quantities.

Sucrose Density Gradients. Exponential density gradients were made using the procedure of Spyrides and Lipmann (1962). The mixing flask contained 5.5 ml of 5% sucrose and the reservoir contained 11.5 ml of 22% sucrose; both sucrose solutions contained 0.05 M Tris–HCl buffer (pH 7.4), 0.16 M NH_4Cl, and 0.012 M Mg acetate. This quantity was used to fill three 5-ml centrifuge tubes simultaneously by using a three-way stopcock. The sucrose density gradients were made at 3° and equilibrated overnight at this temperature before using. After equilibration, the sample to be separated was carefully layered onto the gradient. The tubes containing the gradients were placed in a Spinco SW 39 rotor that had been cooled to 3°. After centrifugation for 60 min at 125,000*g*, fractions from the gradient were collected by lowering a thin polyethylene tube (PE 90 Intramedic, Clay-Adams, Inc.) to the bottom of the tube and siphoning off 5-drop samples. Alternate samples were used to measure

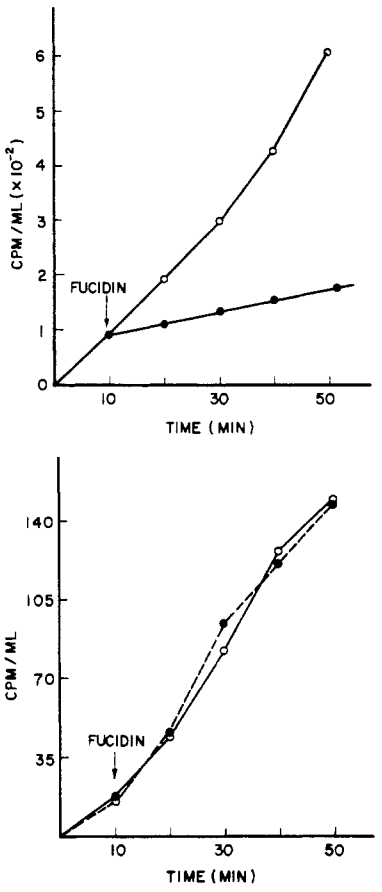

FIGURE 1: Effect of fucidin on incorporation of L-[^{14}C]-lysine into the protein (upper) and mucopeptide (lower) fractions of *S. aureus*. Cells of *S. aureus* were grown for 2 hr as described in Materials and Methods. At zero time, 0.1 μc/ml of L-[^{14}C]lysine was added to each of two cultures and 10 min later, 0.019 mM fucidin was added to one. Samples of 5 ml were withdrawn at 10-min intervals and added to 5 ml of 10% TCA. Samples were fractionated into protein and mucopeptide as described in Materials and Methods. Radioactivity was corrected to optical density at zero time. O—O, control, ●—●, fucidin added at 10 min.

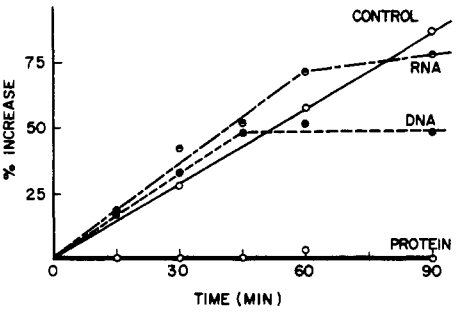

FIGURE 2. Effect of fucidin on the synthesis of DNA, RNA, and protein in *S. aureus*. Cells were grown as described in Materials and Methods. At midlog phase (zero time), 0.019 mM fucidin was added and samples were taken every 15 min. The samples were assayed for DNA, RNA, and protein as described in Materials and Methods. O—O, DNA, RNA, and protein of control; ●—●, DNA after addition of fucidin; ⊖—⊖, RNA after addition of fucidin; O—O, protein after addition of fucidin.

absorbancy at 260 mμ. The contents of the other tubes were precipitated with 3 ml of cold 5% TCA, filtered thru Millipore filters (0.45 μ, 25 mm, and washed with 10 ml of cold 5% TCA. After the filters were dried under an infrared lamp for 30 min, they were counted in the scintillation counter in 10 ml of toluene scintillant solution (counting efficiency, 80% ^{14}C and 30% ^{3}H).

Analytical Methods. Protein was determined by the method of Lowry *et al.* (1951) with purified albumin (Fisher Scientific Co.) as a standard. The orcinol method (Albaum and Umbreit, 1947) was used for determination of RNA with purified yeast RNA (Calbiochem) as a standard. DNA was assayed by the diphenylamine procedure of Burton (1956) with a salmon sperm DNA as a standard. Growth was estimated by measuring the optical density at 600 mμ.

Determination of Radioactivity. Radioactivity was determined with a Geiger-Muller gas-flow counter for the experiment in Figure 1. The gas-flow counter had a counting efficiency of 33% for ^{14}C. Samples were pipetted onto 1-in. copper planchets and dried overnight at room temperature before counting. A scintillation counter (Packard, Model 524) was used for other experiments.

Reagents. Reagents for the cell-free protein synthesis were obtained from the same sources as recommended by Nirenberg(1963). Fucidin (sodium salt of fusidic acid) was supplied through the courtesy of Dr. Godtfredsen at Leo Pharmaceutical Products, Copenhagen, Denmark. Radioactive amino acids were obtained from Calbiochem. Tritiated fucidin was prepared by the New England Nuclear Corp., using the tritium gas exposure method of Wilzbach (1957) and was subsequently purified by column chromatography using silica gel and acetone for elution (Harvey, 1966).

Results

Effect of Fucidin on the Synthesis of RNA, DNA, and Protein in S. aureus Cells. Cells of *S. aureus* were grown at a temperature of 25° to slow down the physiological processes and facilitate the study of their kinetics. The generation time of *S. aureus* under these conditions was 1.6 hr. Figure 2 shows that fucidin stopped RNA synthesis after a 75% increase in the RNA present at the time the antibiotic was added. The synthesis of DNA was blocked after an increase of 40–50%, while no protein synthesis was detected

after the addition of fucidin. When growth, as determined by optical density at 600 mμ, was followed, no inhibition by fucidin was apparent until the optical density had increased 100–150%. No evidence of cell lysis at this concentration of antibiotic was noted even after 24 hr; these results indicated that the antibiotic blocked protein synthesis first. The cessation of RNA and DNA synthesis appeared to be secondary effects resulting from blocking protein synthesis; however, a direct effect of fucidin on the synthesis of RNA and DNA cannot be excluded except by *in vitro* assays.

Effect of Fucidin on the Incorporation of L-[^{14}C]*Lysine into the Protein and Mucopeptide Fractions of S. aureus.* Since L-lysine is found in the cell wall mucopeptide of *S. aureus* (Cummins and Harris, 1956), the incorporation of this amino acid into the mucopeptide fraction was used as a criterion for measuring cell wall synthesis. Figure 1 (upper) shows that 0.019 mM fucidin inhibited the incorporation of L-[^{14}C]lysine into the protein fraction of *S. aureus*, while the uptake of this amino acid into the mucopeptide fraction was not affected (Figure 2 lower). Hancock and Park (1958) obtained similar results when they added chloramphenicol to growing cells of *S. aureus* and followed the incorporation of labeled glycine and lysine into the protein and mucopeptide fractions.

Effect of Fucidin on in Vitro Protein-Synthesizing Systems. Table I shows that fucidin inhibited the incorporation of [^{14}C]phenylalanine into [^{14}C]polyphenylalanine as directed by poly-U, in cell extracts of a gram-positive, a gram-negative bacterium, and a hybrid yeast. The effectiveness of chloramphenicol and fucidin as an inhibitor of protein synthesis in the *E. coli* system was compared. Table II shows that fucidin was a more powerful inhibitor in the range of 2×10^{-5} to 4×10^{-3} M. Table III shows that fucidin also inhibited protein synthesis in a system in which polyadenylic acid (poly-A) was substituted for poly-U and L-[^{14}C]-lysine was substituted for L-[^{14}C]phenylalanine. The effect of fucidin on the incorporation of other amino acids into peptides by cell-free extracts of *E. coli* was studied by using ribosomes with endogenous messenger. The data, presented in Table IV, show that fucidin inhibited the incorporation of phenylalanine, alanine, tyrosine, and lysine into peptides approximately to the same extent. Fucidin also inhibited peptide synthesis in the poly-U-directed system at a level comparable to that in the system with only endogenous messenger. Yamaki (1965), however, found that inhibition by fucidin was decreased in a system without added external messenger.

Effect of Fucidin on the Kinetics of [^{14}C]*Phenylalanine Incorporation.* The effect of adding fucidin either be-

TABLE I: Comparison of the Effect of Fucidin on Various *in Vitro* Protein-Synthesizing Systems.

System	Cpm		% Inhibn
	Control	Fucidin	
Yeast[a]	1,040	167	84
B. stearothermophilus[b]	1,828	428	77
E. coli[c]	11,410	1,556	86

[a] The yeast system (*S. fragilis* x *S. dobzanskii* diploid) was described by Bretthauer *et al.* (1963). [b] *B. stearothermophilus* was assayed in the same manner as *E. coli* except 1 mg of S-30 supernatant protein was used in place of S100 supernatant and ribosomes. Each tube contained 0.2 μc of [^{14}C]phenylalanine (4 mc/mM) and treated samples had 2 mM fucidin. [c] The *E. coli* reaction mixture and procedure were described in Materials and Methods.

TABLE II: Comparison of Fucidin and Chloramphenicol Concentrations on Protein Synthesis by *E. coli* Extracts.[a]

Concn (M)	% Inhibition	
	Fucidin	Chloramphenicol
2×10^{-5}	0	—
1×10^{-4}	34	14
4×10^{-4}	65	28
2×10^{-3}	86	46
4×10^{-3}	97	68

[a] The reaction mixture and procedure is given in Materials and Methods. Each tube contained 0.1 μc of L-[^{14}C]phenylalanine (302 mc/mM).

TABLE III: Effect of Fucidin on *in Vitro* Protein Synthesis by *E. coli* Extracts with Poly-U or Poly-A as Messenger.[a]

Messenger	Cpm		% Inhibn
	Control	Fucidin	
Poly-U	15,757	2,113	86
Poly-A	1,053	331	69

[a] The procedure and reaction mixture were the same as in Materials and Methods except the [^{12}C]amino acids were omitted. In the first sample, 0.2 μc of L-[^{14}C]-phenylalanine (4 mc/mM) and 20 μg of poly-U were used. For the second sample, poly-A was substituted for poly-U and 0.5 μc of L-[^{14}C]lysine (10.9 mc/mM) was substituted for [^{14}C]phenylalanine. The treated tubes contained 1 mM fucidin.

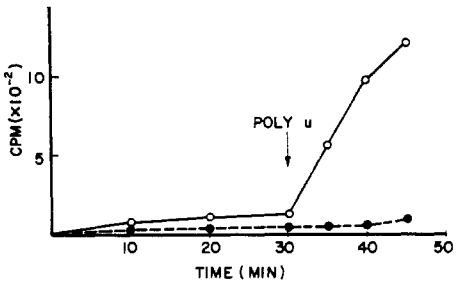

FIGURE 3: Effect of adding fucidin prior to poly-U on the rate of [^{14}C]polyphenylalanine formation. The reaction mixture and procedure are given in Materials and Methods except poly-U was omitted from the mixture and each sample contained 0.2 μc of L[^{14}C]-phenylalanine (4 mc/mM). To one set of tubes 2 mM fucidin was added at zero time. Tubes were incubated and at indicated times, a tube was removed, and the reaction was stopped with 3 ml of 10% TCA. Poly-U (10 μg) was added at 30 min to the remaining tubes. O—O, control; ●—●, plus fucidin.

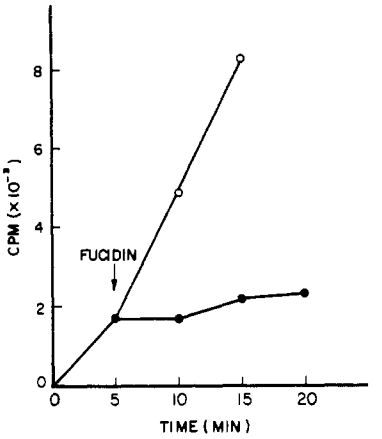

FIGURE 4: Effect of adding fucidin after poly-U on the rate of [^{14}C]polyphenylalanine formation. Reaction mixture and procedure were the same as Figure 1 (upper) except 10 μg of poly-U was added at zero time and 0.4 mM fucidin was added 5 min later to one set of tubes. O—O, control; ●—●, plus fucidin.

TABLE IV: Effect of Fucidin on Incorporation of Various Amino Acids with Endogenous Messenger in *E. coli* Extracts.[a]

[^{14}C]Amino Acid	Cpm		%
	Control	Fucidin	Inhibn
Alanine	2,406	981	59
Tyrosine	11,846	4,812	59
Lysine	1,727	781	55
Phenylalanine	324	150	54

[a] The reaction mixture and procedure are given in Materials and Methods except poly-U was omitted and 0.1 μmole of L-[^{12}C]phenylalanine was added to the samples without labeled phenylalanine. Each tube contained 0.5 μc of appropriate [^{14}C]amino acid (L-[^{14}C]-alanine (75 mc/mM), L-[^{14}C]tyrosine (363 mc/mM), L-[^{14}C]lysine (166 mc/mM), and L-[^{14}C]phenylalanine (4 mc/mM)). The treated samples contained 0.4 mM fucidin.

fore or after the addition of poly-U is shown in Figures 3 and 4. Figure 3 shows the incorporation of [^{14}C]-phenylalanine slowly increased and reached a plateau. When poly-U was added at 30 min, a rapid increase of incorporation was noted. If fucidin was included in the reaction mixture before incubation, it inhibited the incorporation of [^{14}C]phenylalanine, directed by endogenous messenger and when poly-U was added at 30 min, no further increase of incorporation was observed. If fucidin was added 5 min after the addition of poly-U, as shown in Figure 4, the incorporation of [^{14}C]phenylalanine was arrested at the time fucidin was added. These results suggest that fucidin inhibited [^{14}C]polyphenylalanine synthesis regardless whether it was added before or after the poly-U messenger.

Effect of Fucidin on Transfer of [^{14}C]*phenylalanine to s-RNA.* The effect of fucidin on the activation and transfer of [^{14}C]phenylalanine to s-RNA is shown in Table V. These results show that 1 mM fucidin had no effect on the transfer of [^{14}C]phenylalanine to [^{14}C]-phenylalanyl s-RNA, whereas this concentration of

TABLE V: Effect of Fucidin on the Formation of L-[^{14}C]-Phenylalanyl s-RNA by *E. coli* Extracts.[a]

Incubn Time (min)	Cpm/mg of RNA	
	Control	Fucidin
10	8,000	7,280
40	9,660	10,400

[a] The incubation mixture contained in a final volume of 1 ml: 50 μmoles of Tris–HCl (pH 7.5), 10 μmoles of ATP (pH 7.0), 1.2 mg of *E. coli* s-RNA, 0.4 μc of L-[^{14}C]phenylalanine (4 mc/mM), and 0.34 mg of *E. coli* supernatant S-100 protein. The treated samples contained 2 mM fucidin. The tubes were incubated at 37° for 10 min, the reaction was terminated with 1 ml of cold 10% TCA, and the tubes were centrifuged. The cold TCA precipitate was washed with 3-ml volumes of cold 5% TCA, heated for 20 min at 95°, cooled, and centrifuged. The supernatant fluid was extracted with 3-ml volumes of ether, and 1 ml of the aqueous phase was added to 10 ml of dioxane scintillant fluid for counting. Activity in the control minus s-RNA was 90 cpm.

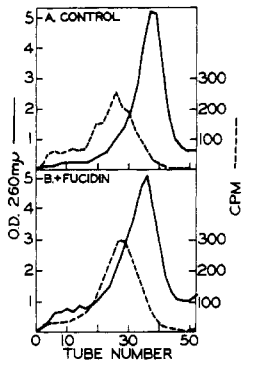

FIGURE 5: Effect of fucidin on the attachment of [^{14}C]-poly-U to *E. coli* ribosomes. The samples contained: 12.5 μmoles of Tris–HCl (pH 7.4), 40 μmoles of NH_4Cl, 3 μmoles of Mg acetate, 0.8 mg of preincubated ribosomal protein (*E. coli*), and 25 μg of [^{14}C]poly-U (18,550 cpm) in 0.25 ml. The samples were incubated for 30 min at 3° and added to 5-ml sucrose gradients as described in Materials and Methods. The treated sample contained 2 mM fucidin which was added to the ribosomes 5 min before the [^{14}C]poly-U. The sucrose gradient containing the treated sample had 4 mM fucidin.

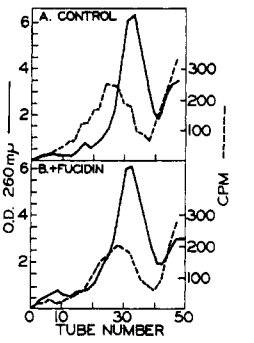

FIGURE 6: Effect of fucidin on the formation of the ternary complex. The 0.25-ml samples contained: 12.5 μmoles of Tris–HCl (pH 7.4), 40 μmoles of NH_4Cl, 3 μmoles of Mg acetate, 10 μg of poly-U, 0.8 mg of preincubated ribosomal protein (*E. coli*), and 124 μg of L-[^{14}C]phenylalanyl s-RNA (16,980 cpm). The tubes were incubated for 10 min at 25° with and without 2 mM fucidin which was added before the poly-U. The mixtures were separated in sucrose gradients as described in Materials and Methods. The sucrose gradient containing the treated sample had 4 mM fucidin.

antibiotic caused a 70–80% inhibition of [^{14}C]polyphenylalanine synthesis (Table II).

Effect of Fucidin on the Attachment of Messenger Ribonucleic Acid (m-RNA) to E. coli Ribosomes. The attachment of m-RNA to ribosomes is required for the formation of polyribosomes, which are believed to be the functional units for protein synthesis (Barondes and Nirenberg, 1962; Gilbert, 1963). The attachment of [^{14}C]poly-U to ribosomes was studied by means of sucrose density gradients. As shown in Figure 5, a radioactivity peak in the polyribosome region, which is slightly to the left of the 70S peak as shown by absorbancy at 260 mμ, was formed. When 4 mM fucidin was added to the ribosomes 5 min before the addition of [^{14}C]poly-U, there was no decrease in the size of this radioactive peak (lower graph). This experiment indicates that fucidin did not prevent the attachment of [^{14}C]poly-U to ribosomes and the formation of polyribosomes.

Effect of Fucidin on the Formation of the Ternary Complex. Spyrides (1964) found that there was a specific binding of aminoacyl s-RNA to ribosomes in the presence of complementary template. He showed that K^+ or NH_4^+ ions were necessary to stabilize the binding and that Mg^{2+} ions were necessary to maintain the integrity of the ribosomes. The binding took place before the polymerization of the amino acids; thus, it was considered to be the final step before the initiation of the peptide chain. In this work, two different methods were used to determine if fucidin affected the formation of this ternary complex.

Figure 6 shows the density gradient pattern obtained with the control (upper graph) and when fucidin (2 mM) was added to the reaction mixture prior to the addition of poly-U, ribosomes and [^{14}C]phenylalanyl s-RNA (lower graph). Fucidin did not appreciably affect the size of the radioactive peak, found in the polyribosome region.

The second procedure used to assay for the binding of [^{14}C]phenylalanyl s-RNA was that of Nirenberg and Leder (1964). Using *E. coli* ribosomes, poly-U, and [^{14}C]phenylalanyl s-RNA, it was found that a concentration of 0.2 and 2 mM fucidin had no effect on the binding of [^{14}C]phenylalanyl s-RNA to the poly-U–ribosome complex.

Effect of Adding Fucidin to the Supernatant S100 and Ribosome Fraction Prior to Protein Synthesis. To determine whether fucidin was irreversibly bound to, or inactivated by some component of the supernatant S100 or ribosome fraction, fucidin (10 mM) was added to both fractions prior to dialysis for 8 hr in either ribosome buffer (Nirenberg, 1963).

Table VI shows that, when either untreated ribosomes combined with treated supernatant fraction, treated ribosomes combined with untreated supernatant fraction, or treated ribosomes combined with treated supernatant fraction were used for *in vitro* protein synthesis, all gave the same amount of [^{14}C]polyphenylalanine formation as the control. These data indicate that fucidin did not appear to inactivate polyphenylalanine synthesis by binding irreversibly to any of the components needed for protein synthesis.

When tritiated fucidin was incubated with *E. coli* ribosomes, ribosomes and poly-U, or the complete system for protein synthesis, no radioactive peaks were detectable in either the 70S or polysome region in any

TABLE VI: Treatment of *E. coli* Ribosomes and Supernatant Fraction with Fucidin and Dialysis Prior to Protein Synthesis.[a]

Supernatant	Ribosomes	Cpm
Untreated	Untreated	2822
Treated	Treated	2600
Untreated	Treated	2500
Treated	Untreated	2450

[a] In this experiment 10 μmoles of fucidin was added to 1 ml of supernatant fraction (12.8 mg of protein) and also to 1 ml of ribosome suspension (20.4 mg of protein). These two samples were dialyzed at 3° for 8 hr against 1 l. of ribosome buffer (Nirenberg, 1963). The procedure for the untreated sample was the same except no fucidin was added. After dialysis, the samples were assayed for formation of [^{14}C]polyphenylalanine by the procedure given in the Materials and Methods. Each tube contained 0.2 μc of L-[^{14}C]phenylalanine (4 mc/mM).

of these three mixtures upon analysis on sucrose density gradients. The method of Wolfe and Hahn (1965) was also used to detect binding of [^{3}H]fucidin to *E. coli* ribosomes. The same three mixtures as in the previous experiment were sedimented at 107,000*g* and the residue was examined for radioactivity. At the same time, tubes were incubated with nontritiated fucidin in addition to the tritiated fucidin on the premise that if [^{3}H]fucidin were bound to the ribosomes, the counts should be lower when the specific activity was lowered. However, it was found that the level of radioactivity was the same in all samples. This indicated that the counts found were due to [^{3}H]fucidin entrapped in the ribosomes and not bound. These experiments indicate that if there is any binding of [^{3}H]fucidin to ribosomes, it must be extremely reversible.

Discussion

Experiments with intact cells show that fucidin inhibits protein synthesis immediately after the addition of the antibiotic and prior to the cessation of RNA and DNA synthesis. Cell wall mucopeptide synthesis continued after inhibition of protein synthesis, and "growth" as measured by absorbance at 600 mμ increased for a period of time corresponding to one or two generations. Although the absorbance at 600 mμ continued to increase after the addition of fucidin, there was no multiplication of the bacterial cells. Experiments here, as well as by Godtfredsen *et al.* (1962b), showed that 0.019 mM fucidin stopped cell division at the time of addition, hence the continued increase in optical density may have been caused by the failure of fucidin to immediately block cell wall mucopeptide and nucleic acid synthesis. This explanation was advanced by Hash *et al.* (1964) who found that tetracycline, an inhibitor of protein synthesis, caused *S. aureus* cells to stop dividing immediately although the absorbance continued to increase.

Experiments with cell-free extracts of *E. coli*, *B. stearothermophilus*, and a diploid yeast showed that 2 mM fucidin inhibited the incorporation of [^{14}C]-phenylalanine into [^{14}C]polyphenylalanine. However, intact cells of *E. coli* and the diploid yeast were insensitive to a concentration of fucidin 300 times that required to inhibit the growth of *B. stearothermophilus*. These results suggest that *E. coli* and the diploid yeast are impermeable to fucidin.

Fucidin was found to inhibit [^{14}C]polyphenylalanine synthesis by cell-free extracts of *E. coli* when added either before or after the poly-U messenger. The per cent of inhibition by fucidin was approximately the same with either a system containing poly-U or one containing only endogenous messenger. The inhibition of polyphenylalanine synthesis was not reversed by the addition of increasing amounts of magnesium ions, poly-U, phenylalanyl s-RNA, GTP, ribosomes, or supernatant fraction.

Treatment of the supernatant fraction and ribosomes with fucidin followed by dialysis, revealed that the antibiotic does not inactivate or irreversibly bind to any component of these fractions. Thus, fucidin must be present at the time peptide bond formation is taking place in order for the antibiotic to inhibit protein synthesis. This is in agreement with the known bacteriostatic action of fucidin (Barber and Waterworth, 1962).

These facts seem to indicate that fucidin does not irreversibly bind to any known constituent, needed for *in vitro* protein synthesis. Wolfe and Hahn (1965) showed that the binding of [^{14}C]chloramphenicol to ribosomes of *E. coli* could not be demonstrated with sucrose density gradients even if the gradient contained the labeled antibiotic. However, they were able to observe binding by adding [^{14}C]chloramphenicol to ribosomes and determining radioactivity in the sediments after centrifugation. Using [^{3}H]fucidin both of these procedures were unsuccessful. The high specific activity of the tritiated fucidin (193 mc/mM) used should detect any binding that occurred. Thus, it appears that any binding of fucidin to the ribosome or polysome must be extremely reversible.

Although the binding of fucidin to any of the components needed for protein synthesis could not be established, it was possible to narrow the possible mechanisms of action. Fucidin did not affect the specific activation or transfer of phenylalanine to s-RNA, and studies with sucrose density gradients showed that the formation of the polyribosome and the binding of phenylalanyl s-RNA to this complex was also not inhibited. At this stage, the ternary complex has been formed and the actual synthesis of the peptide chain will start if no inhibitor is present. Fucidin, therefore, appears to affect the final polymerization of amino acids after the formation of the ternary complex. This stage may be arbitrarily divided into three steps; the initiation of the peptide chain by polymerization of the amino acids; the movement of the ribosome to the successive codons, and finally, the termination or release

of the completed peptide chain from the ribosome. The exact mechanisms by which these three processes take place are not yet understood. Further investigations on the mode of action of antibiotics such as fucidin may help to elucidate these final steps of protein synthesis.

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References

Albaum, H. G., and Umbreit, W. W. (1947), *J. Biol. Chem. 167*, 369.

Arigoni, D., von Daehne, W., Godtfredsen, W. O., Marquet, A., and Melera, A. (1963), *Experientia 19*, 521.

Arigoni, D., von Daehne, W., Godtfredsen, W. O., Melera, A., and Vangedal, S. (1964), *Experientia 20*, 344.

Barber, M., and Waterworth, P. W. (1962), *Lancet 1*, 931.

Barondes, S. H., and Nirenberg, M. W. (1962), *Science 138*, 813.

Bretthauer, R. K., Marcus, L., Chaloupka, J., Halvorson, H. O., and Bock, R. M. (1963), *Biochemistry 2*, 1079.

Burton, K. (1956), *Biochem. J. 62*, 315.

Burton, H. S., and Abraham, E. P. (1951), *Biochem. J. 50*, 168.

Conway, T. W. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1216.

Cummins, C. S., and Harris, H. (1956), *J. Gen. Microbiol. 14*, 583.

Friedman, S. M., and Weinstein, I. B. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 988.

Gilbert, W. (1963), *J. Mol. Biol. 6*, 374.

Godtfredsen, W. O., Jahnsen, S., Lorck, H., Roholt, K., and Tybring, L. (1962a), *Nature 193*, 987.

Godtfredsen, W. O., Roholt, K., and Tybring, L. (1962b), *Lancet 1*, 928.

Godtfredsen, W. O., and Vangedal, S. (1962), *Tetrahedron 18*, 1029.

Hancock, R., and Park, J. T. (1958), *Nature 181*, 1050.

Harvey, C. L. (1966), Ph.D. Thesis, University of Wisconsin, Madison, Wis.

Harvey, C. L., Sih, C. J., and Knight, S. G. (1965), *Bacteriol. Proc.*, P3.

Hash, J. H., Wishnick, M., and Miller, P. A. (1964), *J. Biol. Chem. 239*, 2070.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Nathans, D., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 497.

Nirenberg, M. W. (1963), *Methods Enzymol. 6*, 17.

Nirenberg, M. W., and Leder, P. (1964), *Science 145*, 1399.

Park, J. T., and Hancock, R. (1960), *J. Gen. Microbiol. 22*, 249.

Spyrides, G. J. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1220.

Spyrides, G. J., and Lipmann, F. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1977.

Umezawa, H. (1964), *Recent Advan. Chem. Biochem. Antibiotics*, 26.

Wilzbach, K. E. (1957), *J. Am. Chem. Soc. 79*, 1013.

Wolfe, D. A., and Hahn, F. E. (1965), *Biochem. Biophys. Acta 95*, 146.

Yamaki, H. (1965), *J. Antibiotics (Tokyo) 18A*, 228.