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Antibiotic Glycosides. VI. Definition of the 50S Ribosomal Subunit of *Bacillus subtilis* 168 as a Major Determinant of Sensitivity to Erythromycin A*

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ABSTRACT: Two strains of *Bacillus subtilis* have been used for the preparation of ribosomes or subribosomal particles and a partially purified enzyme fraction, which together with synthetic polynucleotides, amino acids, and an energy source will synthesize polypeptides. One bacterial strain is sensitive to the medium-ring macrolide antibiotic erythromycin A, and the other strain is a mutant which is less sensitive. The effect of erythromycin A on polypeptide synthesis has been measured as a function of the source of the ribosomes, subribosomal particles, and the enzyme fraction. The concentration of erythromycin A that is required for maximal inhibition of polypeptide formation depends markedly on both the source of the 50S subribosomal particle and the nature of the polymer being synthesized. In particular, the 50S ribosomal subunits differ. Reconstituted systems with 50S subunits from erythromycin-sensitive *B. subtilis* show a 15-fold greater sensitivity to the antibiotic than those which employ 50S subunits from the resistant strain of bacteria. The bacterial mutation which has led to a reduced sensitivity to erythromycin A probably has produced some change in the structure of the 50S subribosomal particle, and this affects the interaction of the antibiotic with the reactions of protein synthesis.

Brock and Brock (1959) were the first to suggest that erythromycin A (EaDC)[1] acts as a selective inhibitor of protein synthesis. This conclusion was based on the observation that both protein synthesis and growth of an *Escherichia coli* strain ceased in the presence of 1000 μg/ml of erythromycin A, while nucleic acid synthesis continued. Taubman *et al.* (1963) continued the investigation of the mechanism of bacterial sensitivity to this macrolide antibiotic with two strains of *Bacillus subtilis* 168, one which is sensitive to less than 1 μg/ml of erythromycin A and the other which is relatively resistant (about 100-fold less sensitive) as the result of a mutation. It was shown that erythromycin A, at concentrations which are of physiological significance, blocked protein biosynthesis in the bacteria which are sensitive, and that these same concentrations had little effect on the strain which had acquired resistance. The studies were extended (Taubman *et al.*, 1963, 1964), employing a cell-free system derived from the antibiotic-sensitive strain of *B. subtilis*, and erythromycin A was found to inhibit the incorporation of amino acids into polypeptides. Wolfe and Hahn (1964), who studied an *E. coli* strain similar in sensitivity to that used by Brock and Brock (1959), have reported a similar finding.

Taubman *et al.* (1964) have defined the action of erythromycin A as an inhibition of the ribosome-dependent transfer of activated amino acids (aminoacyl-

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[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; EaDC, erythromycin A; TCA, trichloroacetic acid.

tRNAs) into polypeptide linkage. Moreover, Taubman and Corcoran (1965) and Taubman *et al.* (1966) recently have described a specific binding of erythromycin A to the 50S subribosomal particle of *B. subtilis*, under conditions where protein synthesis is occurring and erythromycin A is effective. Under the same conditions, there is a much reduced binding of erythromycin A to ribosomes from the "resistant" bacterial strain. Tanaka *et al.* (1966) also have demonstrated binding of the antibiotic to ribosomes of *E. coli* and *B. subtilis*, but no studies of resistant organisms were reported. All of these findings suggest that the ribosome is the site of action of the drug, and the results of Taubman *et al.* (1966) provide indirect evidence that an alteration in the ribosome of the bacterial mutant is responsible for its resistance to the antibiotic. Circumstantial evidence for this is also provided by genetic mapping experiments of Dubnau *et al.* (1965a,b), who have shown that the chromosomal region of *B. subtilis* which is responsible for the development of the RNA in 50S subribosomal particles is also the site of mutations leading to resistance to erythromycin A.

In the present communication we wish to present the results of some further studies of polypeptide synthesis by the strains of *B. subtilis* described by Taubman *et al.* (1963). The cell-free systems have been fractionated, and the constituents purified and reconstituted in a manner which permits a comparison of the ribosome function. According to this criterion the ribosome, specifically the 50S subunit, does indeed play a major role in determining the degree of inhibition of protein synthesis produced by erythromycin A.

Experimental Section

Materials. The two strains of bacteria used, designated "EaDC-sensitive" or "sensitive" and "EaDC-resistant" or "resistant," have been described previously (Taubman *et al.*, 1963). Amino acids uniformly labeled with carbon-14 were purchased from the New England Nuclear Corp. and Schwarz BioResearch, Inc., and used without further purification. ATP, GTP, poly U, poly A, poly C, enolpyruvate phosphate, and pyruvate kinase were products of Sigma Chemical. Antibiotic medium 3 was purchased from Difco Laboratories, and foaming during the growth of bacteria was controlled by Hodag PPG-2000 (Hodag Chemical Corp., Skokie, Ill.).

Analytical Methods. In all the procedures described below, bacterial growth was determined by measurement of turbidity using a Klett-Summerson colorimeter with a 540-mμ filter. Protein was determined by the method of Lowry *et al.* (1951), RNA by the orcinol reaction (Mejbaum, 1939), and DNA by the method of Burton (1956). Centrifugal force values (g) are average values.

Growth of Bacteria and Preparation of Cell-Free Extracts (S-30 Supernatant Suspension). The bacteria were routinely grown at 37° in a Microferm Laboratory fermentor (New Brunswick Instruments) with an impeller speed of 400 rpm and aeration through a ring sparger at 0.5 l./min per l. of medium. The bacteria were harvested (Sharples centrifuge) in mid-log phase at an approximate density of 3×10^7 cells/ml and quickly chilled. All subsequent operations for the preparation of ribosomes and enzyme fraction were carried out at 4° unless noted otherwise. The cells were suspended in three times their wet weight of buffer A (Tris-HCl, 0.01 M, pH 7.6; magnesium acetate, 0.012 M; ammonium chloride, 0.04 M; and mercaptoethanol, 0.002 M) and washed three times by centrifugation (30,000g, 10 min) and resuspension in the same buffer. Before the final wash, bentonite (prepared according to Fraenkel-Conrat *et al.*, 1961) was added to the cell suspension to a concentration of 10 mg/g of wet cells. The cells were broken by grinding one part (wet weight) of cells with two parts of Alumina A-305 (Aluminum Co. of America) for 5 min in a prechilled mortar. The resultant paste was extracted with buffer A (2 ml/g of wet cells). The extract was centrifuged at 30,000g for 60 min to remove cell debris, leaving the S-30 supernatant suspension.

Preparation of Washed Ribosomes. The ribosomes were collected from the S-30 supernatant suspension by centrifugation for 90 min at 100,000g. The remaining supernatant solution (S-100 solution) was retained for the preparation of the enzyme fraction. The ribosomal pellet was resuspended in buffer B (composition similar to buffer A, except that the concentration of magnesium acetate is 1.2×10^{-4} M) at approximately 200 A_{260} units/ml and allowed to stand for 6 hr at 0°. The suspension was then dialyzed for 12 hr against 100 volumes of buffer A, and the ribosomes were collected by centrifugation at 100,000g for 90 min. The ribosomes were stored at −80° in buffer A at 100 A_{260} units/ml. The preparation retains 100% of its activity for at least 1 month. The above-described procedure, especially prolonged incubation of the ribosomes in the presence of a reduced concentration of magnesium ions, is necessary if minimal contamination with nonribosomal RNAs and enzyme fraction activities is to be achieved. The times indicated were determined empirically as a compromise between further purification of the ribosomes and loss of their function in promoting polypeptide formation. For many purposes more active ribosomes are useful, and these are obtained by omitting the incubation and dialysis steps. This alternate preparation is described in the following section.

Isolation of 50S and 30S Subribosomal Units. Ribosomes were collected directly from the S-30 supernatant suspension by centrifugation at 100,000g for 90 min. The ribosomes were resuspended in buffer A at 400 A_{260} units/ml and stored at −80°. For separation and recovery of the ribosomal subunits, the ribosome suspension (1 ml) was thawed and layered on the top of a centrifuge tube (3.1 × 8.7 cm) containing a sucrose solution (50 ml) varying in concentration linearly from 10 to 25%. The sucrose solution also contained Tris-HCl buffer (0.01 M, pH 7.6), magnesium acetate (1.2×10^{-4} M), and ammonium chloride (0.04 M). After centrifugation at 3° for 10 hr at 25,000 rpm (75,500g) in a Spinco SW 25.2 rotor, successive 2.0-ml fractions were siphoned from the bottom of the centrifuge tube, and

their absorbancy was measured at 260 mμ. Fractions comprising each of the two peaks were pooled separately and centrifuged for 2 hr at 50,000 rpm (150,000*g*) in a Spinco no. 50 rotor. The upper two-thirds of the supernatant solution was removed and discarded. The sedimented ribosomal particles were resuspended in the remaining solution and used immediately without further treatment. The residual sucrose has no effect on the assay procedure.

Preparation of the Enzyme Fraction. The S-100 supernatant solution from 15 g of wet *B. subtilis* cells was taken to pH 5.3, by the addition of acetic acid (0.1 M) over a period of 30 min with constant stirring. After standing for 5 hr, a precipitate was collected by centrifugation at 30,000*g* for 30 min. The precipitate was resuspended in approximately 10 ml of buffer A. The fraction prepared in this manner contains most of the RNA and DNA and approximately one-fifth of the protein present in the original S-100 supernatant solution. For further purification, the pH 5.3 fraction was applied to the top of a column (25 × 1.5 cm) of DEAE-cellulose which had been previously equilibrated with buffer A. This buffer (40 ml) was passed through the column and elution was carried out at pH 7.6 with a logarithmic gradient of ammonium chloride (0.04–0.67 M). Initially, the mixing chamber contained 200 ml of buffer A and the reservoir contained 200 ml of a buffer containing Tris-HCl, pH 7.6, 0.01 M; magnesium acetate, 0.012 M; and ammonium chloride, 1.0 M. Fractions (5 ml) were collected at a flow rate of 1 ml/min. The main protein peak appeared between ammonium chloride concentrations of 0.35 and 0.45 M. The fractions constituting this peak were pooled and brought to 45% of saturation with ammonium sulfate by slow addition of the solid salt to a stirred solution. After standing for 30 min, the mixture was centrifuged at 30,000*g* for 30 min and a precipitate was discarded. The supernatant solution was brought to 65% of saturation with ammonium sulfate, as before, and allowed to stand for 1 hr. A precipitate was collected, as above, and dissolved in buffer A at a protein concentration of 2 mg/ml. This preparation was dialyzed against three changes of 100 volumes of buffer A and designated as the enzyme fraction. It contains no appreciable DNA or RNA and is stable for at least 1 month at −80°.

Preparation of B. subtilis tRNA. The bulk tRNA was derived from the S-100 supernatant solution prepared from the sensitive *B. subtilis*. It was isolated by phenol extraction and differential precipitation with isopropanol, as described by Zubay (1962). In some cases, a commercial preparation of *B. subtilis* tRNA from General Biochemicals, Inc., was substituted. The two preparations gave virtually identical results.

Assay of Incorporation of [^{14}C]Amino Acids into Polypeptides. Reaction mixtures (0.5 ml) contained Tris-HCl (pH 7.6, 5 μmoles), magnesium acetate (6 μmoles), ammonium chloride (20 μmoles), mercaptoethanol (3 μmoles), ATP (0.5 μmole), enolpyruvate phosphate (2.5 μmoles), pyruvate kinase (25 μg), GTP (0.04 μmole), a mixture of 20 [^{12}C]amino acids minus the particular amino acid under investigation (2 mμmoles each), tRNA (200 μg), washed ribosomes (100 μg of ribosomal protein), and enzyme fraction (40 μg). In addition to this standard assay mixture, synthetic polynucleotides, [^{14}C]amino acids, and erythromycin A were added, as specified in the appropriate figure (below). Reactions were intiated by the addition of the enzyme fraction and incubated at 37°, generally for 30 min.

The incorporation of [^{14}C]phenylalanine into polypeptides was determined as follows. After incubation, an aliquot of the mixture was pipetted onto a 2.0-cm disk of Whatman No. 3MM filter paper. The disks were treated as described by Mans and Novelli (1960), to precipitate hot TCA-insoluble polypeptides. The washed disks were placed in scintillation vials and soaked with 0.6 ml of 1 M ammonia for 30 min. Radioactivity was determined by adding 10 ml of a dioxane-based scintillation fluid (Hayes, 1960) and counting in a Nuclear-Chicago Mark I liquid scintillation system. Counting efficiency was determined for each sample and was routinely 74–78%.

The incorporation of [^{14}C]lysine into polypeptides was determined as above, except that the TCA washing solution was adjusted to pH 2 and contained sodium tungstate (0.5 mg/ml). The assay of [^{14}C]proline incorporation into polypeptides proceeded differently since prolyl polypeptides are soluble in the TCA washing solutions. The reaction was terminated by the addition of 1 M sodium hydroxide (0.02 ml), and incubated for an additional 30 min to hydrolyze prolyl-tRNA. The mixtures were neutralized by the addition of 1 M hydrochloric acid (0.02 ml) and the sample was applied along the origin of a strip (4 × 40 cm) of Whatman No. 3MM filter paper. The strip was chromatographed in descending fashion for 16 hr in a solvent system consisting of 1-butanol–methanol–water (2:2:1, v/v) (Hardy *et al.*, 1955). The polypeptides containing [^{14}C]proline remained at the origin of the chromatogram. This area was cut into strips (1 × 4 cm) for radioactivity measurement as described above.

Results and Discussion

In order to study the details of the mechanism of action of erythromycin A, we have fractionated and partially purified the components of the cell-free protein-synthesizing systems (Taubman *et al.*, 1964) from an antibiotic-sensitive and -resistant strain of *B. subtilis* 168. We have prepared ribosomes, subribosomal particles, and an enzyme fraction which contains both aminoacyl-tRNA synthetase activity for a variety of amino acids and the enzymatic acitivity for transfer of these activated amino acids into peptide linkage. The components which we have obtained can be used in several different ways to construct "reconstituted" systems that are capable of synthesizing polypeptides. For these experiments, we have made reciprocal pairings of the ribosomes and the enzyme fractions, as well as similar pairings of the subribosomal units. In the latter case the assay for activity employed the enzyme fraction from the sensitive bacteria.

We have studied the utilization of L-lysine, L-proline, and L-phenylalanine for polypeptide synthesis, as

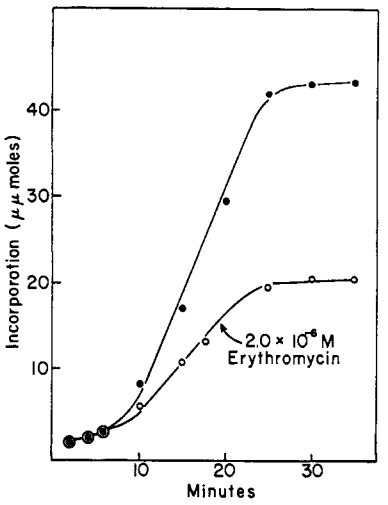

FIGURE 1: Kinetics of the incorporation of [^{14}C]lysine into polypeptides with ribosomes from EaDC-sensitive *B. subtilis:* (●—●) Standard reaction mixture (see Experimental Section) plus 50 μg of poly A and 1.6 mμmoles of [^{14}C]lysine (0.4 μc); aliquots were taken at times indicated after addition of the enzyme fraction and assayed as described in the Experimental Section. (○—○) As above but with 2.0 × 10^{-6} M erythromycin.

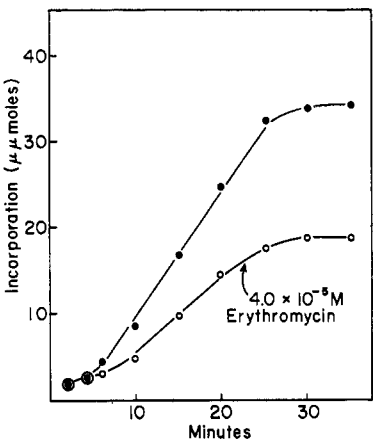

FIGURE 2: Kinetics of the incorporation of [^{14}C]lysine into polypeptide with ribosomes from EaDC-resistant *B. subtilis.* (●—●) Standard reaction mixture (see Experimental Section) plus 50 μg of poly A and 1.6 mμmoles of [^{14}C]lysine (0.4 μc); aliquots were taken at times indicated after addition of the enzyme fraction and assayed as described in the Experimental Section. (○—○) As above with 4.0 × 10^{-5} M erythromycin.

mediated by the appropriate mRNAs, poly A, poly C, and poly U. The general characteristics of the system which was constructed from the sensitive ribosomes and enzyme fraction are shown in Table I. The incorporation of the amino acids into polypeptide linkage is seen to be dependent on a source of energy, the synthetic polynucleotide, bulk tRNA, and the enzyme fraction. All of the reconstituted systems exhibit similar requirements. The extent of the utilization of the amino acids is limited by the quantity of ribosomes which are present. The incorporation increases linearly as the ribosomal protein concentration is increased to 0.6 mg/ml. An increase in the amount of tRNA, [^{14}C]amino acid, or of the enzyme fraction does not stimulate the formation of polypeptides. Other factors probably affect the extent of polypeptide synthesis by the systems which have been studied (*cf.* Taubman *et al.*, 1964). In particular, the magnesium ion concentration used represents the center of a rather narrow range of values (1.0–1.5 × 10^{-2} M) which are compatible with a maximal rate of protein synthesis.

TABLE I: Requirements for Incorporation of [^{14}C]-Amino Acids into Polypeptides.

Conditions	μμmoles of [^{14}C]Amino Acid Incorporated into Polypeptides		
	Phenylalanine	Lysine	Proline
Complete system[a]	41.8[b]	31.0[c]	5.0[d]
—ATP, pyruvate kinase, phosphoenolpyruvate	0.8	0.5	0.3
—Synthetic polynucleotides	3.0	2.0	0.4
—tRNA	2.1	1.1	0.4
—Enzyme fraction I	1.0	0.5	0.3

[a] Complete system contains standard reaction mixture (see Experimental Section) plus: [b] poly U (100 μg) and [^{14}C]phenylalanine (1.26 mμmoles, 0.4 μc), [c] poly A (50 μg) and [^{14}C]lysine (1.6 mμmoles, 0.4 μc), and [d] poly C (40 μg) and [^{14}C]proline (2.7 mμmoles, 0.4 μc).

The duration of polypeptide synthesis by each of the systems which have been described was determined to be about 30 min after the addition of the enzyme fraction. In each case, a lag of 5–6 min was observed before synthesis began. No attempt has yet been made to determine the reason for either the lag or the cessation of synthesis. The presence of the inhibitor, erythromycin A, affected neither the length of the lag nor the duration of active polypeptide synthesis. The situation, as measured for the utilization of L-lysine in the presence of poly A, is shown in Figures 1 and 2. The first of these shows the system employing both ribosomes and the enzyme fraction from sensitive *B. subtilis.* The second is that system which is formed from the same enzyme fraction, but with ribosomes from resistant bacteria. In the light of the observations mentioned, either the rate of protein synthesis (slope of the linear portion of the time *vs.* incorporation plot) or the extent of incorporation of an amino acid into polypeptides by the end of active synthesis can be cited as evidence for the behavior of a

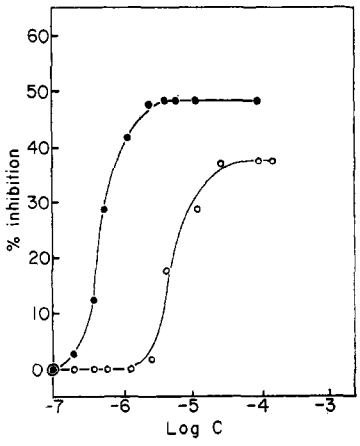

FIGURE 3: Comparison of EaDC-sensitive and EaDC-resistant ribosomes and the inhibitory effect of EaDC on incorporation of [^{14}C]lysine into polypeptides. (●—●) Standard reaction mixture incubated and assayed as described in the Experimental Section employing ribosomes from the EaDC-sensitive strain of *B. subtilis* plus 50 μg of poly A and 1.6 mμmoles (0.4 μc) of [^{14}C]lysine in 0.5 ml. (O—O) As above but with ribosomes from the EaDC-resistant strain.

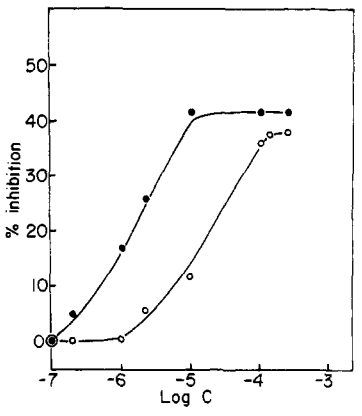

FIGURE 4: Comparison of EaDC-sensitive and EaDC-resistant ribosomes and the inhibitory effect of EaDC on incorporation of [^{14}C]proline into polypeptides. (●—●) Standard reaction mixtures incubated and assayed as described in the Experimental Section, employing ribosomes from the EaDC-sensitive strain of *B. subtilis* with 40 μg of poly C and 2.7 mμmoles (0.4 μc) of [^{14}C]proline in 0.5 ml. (O—O) As above but with ribosomes from the EaDC-resistant strain.

given cell-free system. We have chosen to report total incorporation of the amino acid into polypeptides as the measure of activity.

The data presented in Table I show that the polypeptide synthesis which depends on washed *B. subtilis* ribosomes requires a protein fraction from the S-100 supernatant solution. This enzyme fraction contains aminoacyl-tRNA synthetase activity for all L-amino acids thus far studied: arginine, aspartic acid, glutamic acid, lysine, phenylalanine, proline, and valine. Evidence exists that the enzyme fraction also contains the enzymes which catalyze the transfer of these amino acids into polypeptide linkage. For example, we found that the supernatant solution remaining after the initial acid precipitation of the crude enzyme fraction possesses appreciable phenylalanyl-tRNA synthetase activity, but almost no ability to convert the activated amino acid into polypeptide linkage. When the protein (40 μg) soluble at pH 5.3 was substituted for the enzyme fraction in an experiment similar to that described as the complete system of Table I, but without ribosomes, 95 $\mu\mu$moles of [^{14}C]phenylalanyl-tRNA (as assayed by precipitation with cold 5% trichloroacetic acid) was formed. Of this, however, only 0.9 $\mu\mu$mole of the amino acid was converted into polypeptide linkage in the presence of ribosomes. On the other hand, the same amount of protein as the purified enzyme fraction will not only catalyze the formation of 70 $\mu\mu$moles of [^{14}C]-phenylalanyl-tRNA in 30 min but it also can promote the transfer of 41.8 $\mu\mu$moles into polypeptide linkage. Comparable demonstrations with [^{14}C]proline and [^{14}C]lysine were not feasible, since the protein which is soluble at pH 5.3 possesses essentially no aminoacyl-tRNA synthetase activity for these amino acids.

The difference in the binding of erythromycin A to ribosomes derived from the two strains of *B. subtilis* (Taubman *et al.*, 1966) had suggested to us that the ribosome of this microorganism may be the major factor which determines the degree of inhibition of protein synthesis which is produced by the antibiotic. The validity of this presumption was shown in experiments which used the enzyme fraction from the bacteria which are sensitive to erythromycin A, and ribosomes from either the antibiotic-sensitive or -resistant strains. The synthesis of polylysine, as mediated by poly A, is shown in Figure 3. In the presence of ribosomes from the antibiotic-sensitive *B. subtilis* a maximal inhibition of polypeptide synthesis of 48% is found at about 2.0×10^{-6} M erythromycin A (1.4 μg of EaDC/ml). With the ribosomes from antibiotic-resistant *B. subtilis* about 14 times more erythromycin A (2.8×10^{-5} M) is required for maximal inhibition of polylysine synthesis. The results of the study of poly C dependent polyproline synthesis by the two cell-free systems are shown in Figure 4. This process appears qualitatively to be similar to that described for the synthesis of the polylysines (Figure 3). The main difference is that higher concentrations of erythromycin A are required to produce equivalent inhibitions of polypeptide synthesis. With sensitive ribosomes maximal inhibition (42%) is observed at 1.0×10^{-5} M erythromycin A. As before, when resistant ribosomes are used more erythromycin A (1.3×10^{-4} M) is needed to produce equivalent inhibition of polypeptide synthesis. The reaction which is least sensitive to the antibiotic is

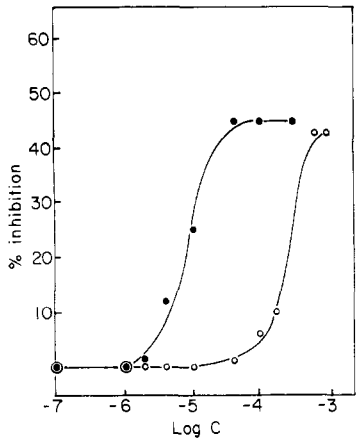

FIGURE 5: Comparison of EaDC-sensitive and EaDC-resistant ribosomes and the inhibitory effect of EaDC on the incorporation of [^{14}C]phenylalanine into polypeptides. (●—●) Standard reaction mixture incubated and assayed as described in the Experimental Section, employing ribosomes from the EaDC-sensitive strain of *B. subtilis* plus 100 μg of poly U and 1.26 mμmoles (0.4 μc) of [^{14}C]phenylalanine in 0.5 ml. (O—O) As above but with ribosomes from resistant strain.

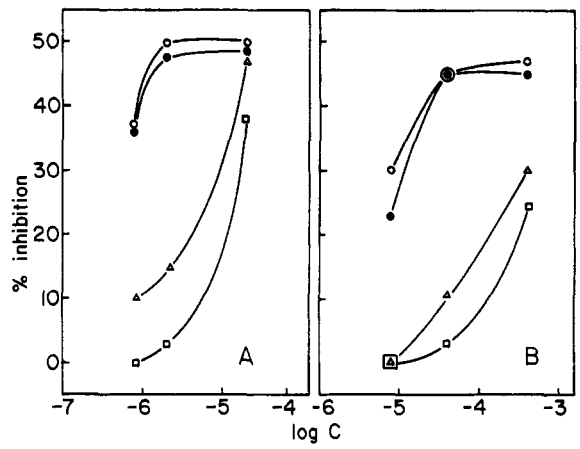

FIGURE 6: Inhibition by erythromycin A of polypeptide synthesis with various combinations of resistant and sensitive *B. subtilis* ribosomes and enzyme fractions. (A) Inhibition of polylysine synthesis; incubations contained standard reaction mixture (see Experimental Section) ribosomes, enzyme fraction, and erythromycin as specified plus poly A (50 μg) and [^{14}C]lysine (1.6 mμmoles, 0.4 μc). (●) Sensitive ribosomes and sensitive enzyme fraction. (O) Sensitive ribosomes and resistant enzyme fraction. (△) Resistant ribosomes and resistant enzyme fraction. (□) Resistant ribosomes and sensitive enzyme fraction. (B) Inhibition of polyphenylalanine synthesis, incubations as in A but with poly U (100 μg) and [^{14}C]phenylalanine (1.26 mμmoles, 0.4 μc); symbols have the same meaning as in A.

the formation of polyphenylalanines, promoted by poly U (Figure 5). The system using the sensitive ribosomes is maximally inhibited (45%) at 4.0×10^{-5} M erythromycin A. A 15-fold increase in the concentration of the antibiotic (to 6.0×10^{-4} M EaDC) was needed to give a similar maximal (40%) inhibition of polyphenylalanine synthesis when resistant ribosomes promoted the synthesis.

Since the properties of the enzyme fraction used in these experiments might also vary with its source, we repeated some of the experiments described above with the enzyme fraction from resistant *B. subtilis*. Polylysine and polyphenylalanine formation without erythromycin was comparable to the values reported in Table I. The results of the reciprocal pairings of ribosomes and enzyme fractions and the effect of erythromycin A on polypeptide formation are presented in Figure 6. It is apparent that the difference in sensitivity to the antibiotic is primarily a function of the source of the ribosome.

Previous studies of erythromycin A binding to *B. subtilis* ribosomes had suggested that the 50S subunit may be a site of difference between the sensitive and resistant bacterial strains. To test whether these units are different functionally, we recovered the 30S and 50S ribosomal subunits from 70S ribosomes of each strain of bacteria and made reciprocal pairings of the subunits. Experiments were carried out to measure both polyphenylalanine and polylysine formation. The results were similar in both sets of experiments, but the activity of the reconstituted systems is much greater when assayed with phenylalanine and poly U. These results, shown as Table II, demonstrate that it is the 50S ribosomal subunit which determines the sensitivity of the cell-free systems to erythromycin A.

Although the ribosomes of *B. subtilis*, particularly the 50S subunit, do seem to differ in their function when erythromycin A is present, the action of the antibiotic on both varieties may be similar. This is not certain, but it is suggested by the fact that the limiting inhibition of protein synthesis produced by erythromycin A is 40–50% in the case of each amino acid studied. This is irrespective of the source of the ribosome, and is reproducible for the reconstituted systems when assayed as described above. It is in contrast to the variable degrees of inhibition observed during studies of crude preparations (Taubman *et al.*, 1964).

All of the lines of evidence which are available at this moment suggest that erythromycin A interacts with the ribosomes of sensitive bacteria (*cf.* Taubman *et al.*, 1966). Direct measurements in the case of *B. subtilis* ribosomes show that the degree of interaction is related to the sensitivity of the parent bacteria to erythromycin A (Oleinick and Corcoran, 1967). It is tempting to speculate that this difference in interaction (binding) is related both to the difference in sensitivity to the antibiotic shown by these strains and to the normal mech-

TABLE II: The Effect of Erythromycin A on the Synthesis of Polyphenylalanine with Recombined 30S and 50S Particles from Sensitive and Resistant *B. subtilis*.[a]

Subribosomal Particles Employed[b]				Inhibn (%) of Polyphenylalanine Synthesis[c] by Erythromycin A as a Function of the Concn (M) of Antibiotic				
50 S_{sen}	50 S_{res}	30 S_{sen}	30 S_{res}	10^{-6}	10^{-5}	4×10^{-5}	10^{-4}	4×10^{-4}
+		+		0	14	15	25	40
+			+	0	15	20	34	
	+		+	0	0	0	10	25
	+	+		0	0	0	15	20

[a] Incubations contained standard reaction mixture (see Experimental Section), specified 30S particle (110 μg), specified 50S particle (165 μg), poly U (100 μg), and L-[^{14}C]phenylalanine (1.1 mμmoles, 0.4 μc). [b] Sedimentation coefficients are nominal values given to identify particle types. [c] In the absence of erythromycin A approximately 25 $\mu\mu$moles of L-[^{14}C]phenylalanine was incorporated into polypeptides with the complete systems. With either sensitive or resistant 30S particles alone, 1.0 $\mu\mu$mole was incorporated, and with either type of 50S particle alone, 1.5 $\mu\mu$moles was incorporated.

anism whereby erythromycin inhibits protein formation. The results discussed in the present communication support the hypothesis, in that they show the 50S subribosomal unit of *B. subtilis* to be a major determinant of the inhibitory effect produced by erythromycin A on cell-free protein synthesis. However, it is not clear from these data whether there is any simple relationship between the binding of erythromycin to 50S subunits and the observed degree of inhibition of polypeptide formation. Indeed, the data shown above (Figures 3–5) indicate that some factor concerned with the nature of the amino acid being incorporated into polypeptide linkage plays a considerable role in determining the amount of antibiotic required for a given degree of inhibition. This is so even though the amount of antibiotic bound to 50S subunits is not much affected by changes in the polynucleotide message.[2]

Another major problem which remains unanswered is that of why erythromycin A is effective in inhibiting the multiplication of both *B. subtilis* strains, whereas in the various cell-free systems we have studied, the inhibition of protein synthesis is always less than complete. First of all, it may well be that protein synthesis does not need to be inhibited completely for cell multiplication to be affected. Secondly, the process measured in cell-free systems with either "natural" mRNA or "synthetic" mRNAs is hardly physiological. On the assumption that cell-free studies do at least mirror the normal case, one can visualize several factors which may affect the extent of erythromycin A inhibition of polypeptide (protein) synthesis. If binding of erythromycin A to 50S subribosomal particles is prerequisite to an inhibition of protein synthesis, it may be that the resultant protein-synthesizing complex is not devoid of ability to form protein. Such a complex may function at a low rate or in some differential manner where some amino acids (aminoacyl-tRNAs) are affected more than others. A further complication seems to be that reversible binding of the antibiotic to the 50S ribosomal subunit may occur best when this unit is not associated with 30S subunits and/or mRNA.[2] If so, a given ribosomal population may represent an equilibrium mixture of erythromycin A bound and unbound 50S subunits. Thus, experimental design may play a considerable role in the maximal inhibition of polypeptide synthesis produced by erythromycin A in cell-free systems. Obviously, the physiological action of the antibiotic may be much more complex than suggested in this analysis. It must, however, be compatible with two major facts. One is that the antibiotic binds specifically to 50S ribosomal subunits from *B. subtilis* and the extent of binding reflects the sensitivity of the parent bacteria. Secondly, the same sensitivity to erythromycin A is shown by the 50S ribosomal subunits when they are functioning in protein synthesis.

[2] N. L. Oleinick and J. W. Corcoran, unpublished observations.

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Characterization of Some of the Proteins of the Large Subunit of Rat Liver Ribosomes*

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ABSTRACT: The four chief electrophoretic components of the proteins of the large subunit of rat liver ribosomes have been separated and their molecular weights measured. Two of these components were obtained as electrophoretically homogeneous fractions with molecular weights of 14,800 and 28,600. For the other two, molecular weights were obtained by extrapolation of measurements on mixtures but the values, 16,000 and 19,000, probably are fair estimates of the true molecular weights. The similar sizes of electrophoretically distinguishable components suggest that the ribosomal proteins differ in composition as well as size.

The electrophoretic heterogeneity of ribosomal proteins in starch and polyacrylamide gels is well known (Waller and Harris, 1961; Leboy *et al.*, 1964; Hamilton and Ruth, 1966; MacQuillan and Bayley, 1966; Low and Wool, 1966). Because of the sieving properties of the gels, migration is affected by molecular size and shape as well as by net charge and it is difficult to interpret the patterns. Thus: (1) the components might be all the same size, but differ in composition; (2) they might be alike in composition, but form complexes of various sizes; (3) they might vary in both size and composition; and (4) as an added complication, they may exhibit conformational isomerism. To determine which of these descriptions applies to the population of ribosomal proteins, the mixture has been fractionated electrophoretically on a polyacrylamide column and the molecular weights of the fractions have been measured by equilibrium centrifugation. Because the electrophoretic distributions of the proteins from the two ribosomal subunits differ, the subunits were first separated and the more stable large subunit of rat liver ribosomes has been used as a source of protein. Two of the fractions were monodisperse both in the ultracentrifuge and on reexamination by electrophoresis. These had molecular weights of 14,800 and 28,600. The other fractions were not monodisperse by electrophoresis although some appeared to be monodisperse in the ultracentrifuge.

Experimental Procedures

Isolation of Ribosomes and Separation of Subunits.[1] Ribosomes were isolated from the livers of fasted, adult male rats and purified by the procedures of Petermann and Pavlovec (1963) and Schwartz and Petermann (1966) which involve the use of sodium deoxycholate, bentonite, a pH 8 wash, and $MgCl_2$ precipitation. This method yields electrophoretically pure ribosomes. Ribosomes were dissociated to subunits by passage through a Sephadex G-100 column in 0.1 M KCl–0.001 M potassium phosphate (pH 7.0) (Petermann and Pavlovec, 1966), dialyzed overnight against

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[1] All procedures were carried out in a cold room at temperatures from 5 to 10°. Stock solutions of urea 10 (M) were treated with charcoal before use.