STREPTOMYCIN ACTION AND THE RIBOSOME*

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There is a growing body of evidence which links streptomycin (SM) action to ribosomal function in bacteria. SM and dihydrostreptomycin (DSM) will inhibit both protein synthesis in intact bacteria and amino acid incorporation into polypeptides in cell-free extracts.[1–9] With the latter system, extremely low concentrations of the antibiotic are effective.[8] It has also been shown that the difference between sensitivity to SM and either resistance to it, or dependence upon it, is due to a difference in the ribosomes obtained from the different bacterial strains.[7, 9] No other component of the protein synthetic machinery has thus far been implicated, at least in so far as high level resistance to the antibiotic is concerned. This present knowledge implicates the ribosome, but most of the mechanistic details are unknown. Evidence is presented in this report which bears on two important questions concerning mechanism.

The first of these deals with the question of whether or not a SM-sensitive ribosome, in the presence of SM, can still attach to a strand of messenger RNA (mRNA). Obviously, prevention of such an attachment would explain the inhibition of protein synthesis, and in fact just such a proposal has been made by Spotts and Stanier.[10] This question has been approached by examining the sedimentation distribution of the major cytoplasmic constituents after a brief pulse-labeling with P^{32}-phosphate of bacteria growing in the presence and absence of DSM. It has been found that ribosomes will attach to mRNA in the presence of DSM, and that such a complex can be extracted from cells treated with the antibiotic. An *in vitro* demonstration of this same point is presented elsewhere in these Proceedings by J. Davies.[10a]

Second, a question was asked as to whether it is possible to localize a SM-binding site on the ribosome. It has been known since the work of Tissières and Watson[11] that the bacterial ribosome may be reversibly dissociated into 30S and 50S subunits, and since our previous work[8] had shown that one to two molecules of DSM per ribosome inhibited amino acid incorporation, the question is whether one or both of the subunits from a SM-sensitive ribosome is required for a response to DSM. This has been examined by first separating the 30S and 50S ribosomal subunits from bacteria of the various SM-genotypes, reforming 70S ribosomes with one or the other of the subunits derived from a different bacterial strain, and then testing such ribosomal hybrids for their response to DSM with the *in vitro* amino acid incorporating system.[12] Ribosomal sensitivity to SM has been found to be associated exclusively with the 30S subunit, and further, the 30S subunits derived from high level SM-resistant and from SM-dependent strains are both resistant to the inhibition of amino acid incorporation by SM.

The two observations reported allow us to make some definitive statements with regard to the mechanism of SM action.

Materials and Methods.—Bacterial strains: *E. coli* B was used throughout the experiments reported here. A SM-resistant mutant (*Str*^{R}-11, previously designated *Sr*/11) and a SM-dependent

mutant (*Str*^{D}-9, previously designated *Sd*/9) were isolated in this laboratory and have been used in previous studies.[8, 9] They are single-step spontaneous mutants derived from the parent, strain B.

Preparation of ribosomes and supernatant fraction: Log-phase cells grown on a broth medium were chilled, harvested, and washed once with 0.85% NaCl. The cells were frozen and stored at $-20°$ until needed. Ribosomes and supernatant fraction were prepared according to the procedure of Matthaei and Nirenberg,[12] with the following modifications. The ribosomes were washed once with 0.01 *M* Tris-HCl, pH 7.8, containing 0.006 *M* mercaptoethanol, 0.08 *M* KCl, 0.01 *M* Mg^{++} and 0.1% sodium deoxycholate. Insoluble debris present in the ribosomal suspension (about 20% of the total protein) was removed by centrifugation at 20,000 $\times$ *g* for 10 min. Ribosomal concentrations were estimated by absorbancy measurements at 260 mμ.[13] The 100,000 $\times$ *g* supernatant fraction was further centrifuged for 6 hr at 100,000 $\times$ *g*, the rotor decelerated without braking, and the tubes (containing 12 ml) carefully removed. The upper 2 ml was discarded, the next 6 ml was saved, and the remainder of the tube's contents was discarded. Suitable aliquots of the supernatant fraction were frozen and stored at $-20°$. Prior to an experiment a supernatant fraction was thawed and centrifuged in a 2-ml tube at 100,000 $\times$ *g* for 2 hr. The upper $^2/_3$ of the sample was removed and used; the remainder (containing 30S ribosomes) was discarded. This special treatment of the supernatant fraction was found necessary in order to reduce the incorporation of phenylalanine by 50S subunits in the presence of the supernatant to an acceptable level. Protein concentrations were determined by the Lowry procedure using bovine serum albumin as the standard.[14]

Isolation of 30S and 50S subunits: 70S ribosomes were dissociated to 30S and 50S subunits by overnight dialysis against a minimum of 1,000 volumes of 0.05 *M* Tris-HCl, pH 7.4, 10^{-4} *M* Mg^{++}, 0.06 *M* KCl. One ml of the resulting ribosomal solution (containing 30 mg of ribosomes) was layered as an inverted linear gradient (5–0% sucrose) on 28 ml of a 5–20% linear sucrose gradient, and the 30S and 50S particles isolated by zone centrifugation for 10 hr at 23,000 rpm in the Spinco SW 25 rotor.[15] One-ml fractions from the peak concentrations of 30S and 50S subunits were isolated with the aid of a UV flow analyzer and used without further purification. These fractions were of greater than 95% purity as estimated from ultraviolet absorption patterns obtained with the analytical ultracentrifuge.

Recombination of subunits and synthesis of polyphenylalanine: The polyuridylic acid (poly U) directed synthesis of polyphenylalanine was carried out in a total volume of 0.3 ml essentially as described by Nirenberg and Matthaei,[16] using a poly U preparation with an $S_{20,w}$ of 6.8. The following modifications were adopted: the total KCl concentration was raised to 0.08 *M*, and the Mg^{++} concentration was adjusted to 0.0175 *M*. The Mg^{++} concentration is important, as the degree of inhibition by DSM of polypeptide synthesis directed by different ribopolynucleotides varies with the Mg^{++} concentration, inhibition in the poly U system being greatest at the optimum Mg^{++} concentration for polypeptide synthesis.[17] Recombined 30S and 50S subunits from the strains listed were preincubated at 0° for 30 min in the absence of poly U to allow 70S hybrid ribosome formation. The reaction vessels were then incubated 10 min at 37° with all components but poly U, chilled, poly U added, and the vessels then incubated a further 30 min at 37°. The reactions were terminated by chilling the vessels, 5 ml of 5% trichloroacetic acid was added, followed by 1 mg of bovine serum albumin as carrier protein and the hot-acid insoluble material processed by a modification of the Siekevitz procedure.[18] The insoluble protein residue was collected on a glass fiber filter disk and assayed for radioactivity in a Packard scintillation spectrometer.

Pulse-labeling of bacteria and density gradient centrifugations: For the experiment shown in Figure 1, *E. coli* B in 1.4 liters of the Tris-buffered medium of Hershey[19] (modified to contain 10 μg P/ml) with 0.2% glucose was grown exponentially to a cell density of 5 $\times$ 10^8 cells/ml. A 350-ml aliquot was removed and mixed vigorously for 20 sec with 1 mc of P^{32}-orthophosphate. The pulse was terminated with rapid chilling by adding crushed ice and Na azide (0.01 *M* final concentration). DSM (80 μg/ml) was added to the remainder of the culture, and three similar P^{32}-pulses were carried out at 10, 20, and 40 min after the addition of DSM. The experiment cited in Figure 2 was carried out in similar fashion using half the initial total volume of bacterial cells. The pulses with P^{32}-orthophosphate were carried out just prior to, and 60 min after, the addition of DSM.

All further operations were carried out at 2–4°. The cells in each aliquot were harvested and washed twice with 200 ml of the Tris-buffered media containing the appropriate Mg^{++} concentration (see legends to Figs. 1 and 2), resuspended in 3 ml of the same media containing 5 μg/ml of DNase, and disrupted in a French pressure cell. The wall-membrane fraction was removed by centrifuging the extract for 15 min at 20,000 × *g*, and 1 ml of the resulting supernatant solution was layered as an inverse gradient on 28 ml of a sucrose density gradient as described above. The Mg^{++} in the sucrose gradients is indicated in the legends to Figures 1 and 2. The sucrose gradients were centrifuged for 6 hr at 25,000 rpm in the Spinco SW 25 rotor, and 15 drop fractions were collected from the bottom of each tube and assayed for cold acid-insoluble radioactivity and absorbancy at 260 mμ.

Results.—The synthesis of rapidly labeled RNA in DSM-inhibited bacteria: The results of two experiments which are presented in Figures 1 and 2 demonstrate that with short pulses of P^{32}-phosphate a rapidly labeled RNA fraction, presumably with

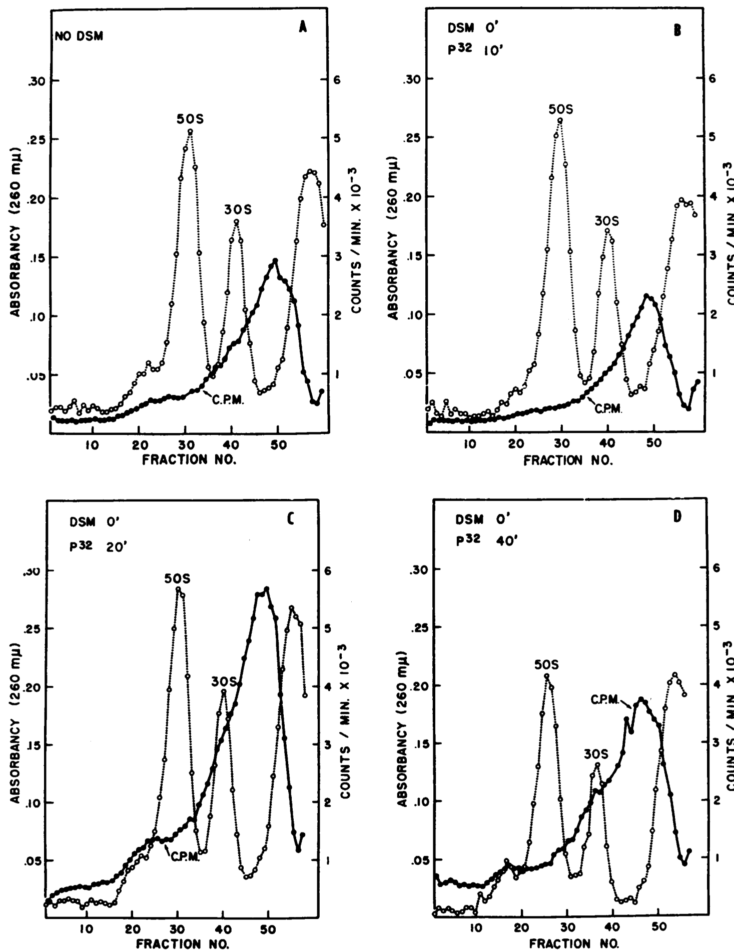

FIG. 1.—Rapidly labeled RNA in DSM-inhibited cells of *E. coli* B. Aliquots from a log-phase culture treated with 80 μg/ml DSM were removed at 10, 20, and 40-min intervals and pulse-labeled with P^{32}-orthophosphate for 20 sec (*B*, *C*, *D*). A control sample from the same culture was removed immediately prior to the addition of DSM and pulse-labeled in an identical manner (*A*). The cells were harvested, and cell-free extracts prepared as described in *Methods* with 1×10^{-4} *M* $MgCl_2$ in the buffers and in the sucrose gradients.

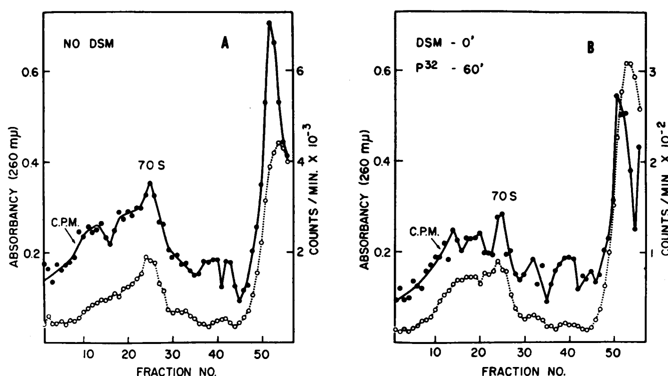

FIG. 2.—Rapidly labeled RNA in DSM-inhibited cells of *E. coli* B. A log-phase sample of *E. coli* B was pulse-labeled for 20 sec with P^{32}-orthophosphate 60 min after the addition of 80 μg/ml DSM (*B*). A control culture was pulse-labeled for 20 sec immediately before the addition of DSM (*A*). The cells were harvested, and extracts prepared with 1×10^{-2} *M* $MgCl_2$ in all of the buffers as described under *Methods*.

mRNA in predominance, is synthesized for periods of up to 60 min after the addition of DSM. With the concentration of DSM used here (80 μg/ml), 99.9 per cent of the viable cells are killed by the fifth min after addition of the antibiotic. The rapidly labeled RNA fraction is identified as mRNA on the basis of its transient and reversible Mg^{++}-dependent association with the ribosome. Other experiments, not reported here, were tried in which a larger number of pulses were carried out, and the cold acid-precipitable P^{32} incorporation into whole cells was measured. They revealed that not only is a mRNA fraction synthesized after DSM but that the rate of its synthesis is about the same for periods up to about 40 min after DSM addition. Thereafter, the rate of synthesis falls, but as late as 60 min after DSM a synthetic rate approximately 5 per cent that of the control is found (Fig. 2). The RNA polymerase therefore appears not to be inhibited by DSM, at least not early in DSM action. However ribosomal- and transfer-RNA synthesis are stopped some several min after killing of the cells is complete.[3]

When the extracts are centrifuged in the presence of 1×10^{-2} *M* Mg^{++}, it is clear from the results of Figure 2 that a large fraction of the mRNA sediments at 70S or greater. This indicates that not only does the mRNA attach to the 70S ribosome in the presence of DSM, but that a polyribosome can also be found.

Another interesting point is revealed in a comparison of *C* and *D* with *A* and *B* of Figure 1. After a longer exposure to DSM, the fraction of mRNA sedimenting at 70S or greater does not dissociate as readily from the ribosomes on lowering of the Mg^{++} level to 1×10^{-4} *M*. After DSM, there also is an apparent accumulation of material sedimenting above 100S as revealed by the absorbancy curves in Figure 2.

Recombination of ribosomal subunits derived from Str^R and Str^S strains of E. coli B: Polyphenylalanine synthesis utilizing hybrid ribosomes reconstituted with subunits derived from resistant and sensitive strains is shown in Table 1. The hybrid 70S ribosome is sensitive to DSM inihibition only when it contains a 30S subunit derived from a SM-sensitive strain. The SM-sensitivity of the strain from which the 50S subunit originates is of no consequence. The error be-

TABLE 1

Synthesis of Polyphenylalanine by Hybrid Ribosomes Reconstituted from Streptomycin-Resistant and Sensitive Subunits

No.	Ribosome source* 30S	50S	DSM†	Cpm‡	% Inhibition
1	Sens.	Sens.	−	5082	—
2	Sens.	Sens.	+	1493	70
3	Res.	Res.	−	3873	—
4	Res.	Res.	+	4089	0
5	Res.	Sens.	−	4113	—
6	Res.	Sens.	+	3603	10
7	Sens.	Res.	−	5084	—
8	Sens.	Res.	+	885	88
9	Res.	—	−	102	—
10	—	Res.	—	324	—
11	Sens.	—	−	244	—
12	—	Sens.	−	201	—
13	Sens.	Sens.	−	116	—
14	Res.	Res.	−	144	—

* The following ribosomal concentrations were used: 32 μg 50S and 19 μg 30S of the sensitive strain (*E. coli* B); 36 μg 50S and 18 μg 30S of the resistant strain (*E. coli* Str^R-11).
† Dihydrostreptomycin sulfate: 2.2×10^{-5} *M* when present.
‡ The counts per minute have been corrected for 741 cpm incorporated by the supernatant alone in the presence of poly U and the buffer-energy components. Cpm are reported as the average of duplicate determinations. Poly U was omitted in the incubations for vessels 13 and 14.
Each reaction vessel contained, in a final volume of 0.3 ml: Tris buffer, pH 7.8, 0.10 *M;* KCl, 0.08 *M;* mercaptoethanol, 0.006 *M;* ATP, 0.0017 *M;* GTP, 0.043 mM; phosphoenolpyruvate, 0.003 *M;* magnesium acetate, 0.0175 *M;* transfer RNA, 0.2 mg; C^{14}-L-phenylalanine, 0.2 μc, specific activity of 1 μc/μg; pyruvate kinase, 7.5 μg; 100,000 × *g* supernatant, 0.46 mg protein; ribosomes as specified above and 6.57 μg poly U.

tween duplicates in these experiments sometimes attains 10 per cent, and hence the 6–10 per cent inhibition observed with hybrid ribosomes containing Str^R or Str^D subunits is not considered to be significant.

C^{14}-phenylalanine incorporation requires both subunits (vessels 9–12) and is dependent on poly U as messenger (vessels 1–8 compared to vessels 13 and 14 where poly U is omitted).

Recombination of ribosomal subunits derived from Str^D and Str^S strains of E. coli B: Polyphenylalanine synthesis utilizing hybrid ribosomes reconstituted with subunits derived from dependent and from sensitive bacteria is shown in Table 2. Only

TABLE 2

Synthesis of Polyphenylalanine by Hybrid Ribosomes Reconstituted from Streptomycin-Dependent and Sensitive Subunits

No.	Ribosome source* 30S	50S	DSM†	Cpm‡	% Inhibition
1	Sens.	Sens.	−	4096	—
2	Sens.	Sens.	+	1026	74
3	Depen.	Depen.	−	5612	—
4	Depen.	Depen.	+	5308	6
5	Sens.	Depen.	−	3035	—
6	Sens.	Depen.	+	1363	55
7	Depen.	Sens.	−	6119	—
8	Depen.	Sens.	+	5678	8
9	—	Depen.	−	434	—
10	Depen.	—	−	158	—
11	—	Sens.	−	732	—
12	Sens.	—	−	93	—
13	Sens.	Sens.	—	67	—
14	Depen.	Depen.	−	302	—

* The following ribosomal concentrations were used: 36.5 μg 50S and 20.6 μg 30S from the dependent strain (*E. coli* Str^D-9); 37.0 μg 50S and 19.7 μg 30S from the sensitive strain (*E. coli* B).
† Dihydrostreptomycin sulfate: 2.2×10^{-5} *M* when present.
‡ Cpm have been corrected for 680 cpm incorporated by supernatant plus poly U. The volumes and concentrations are the same as described in Table 1. Cpm are reported as the average of duplicate determinations.

those vessels containing 30S particles isolated from SM-sensitive ribosomes are inhibited. 70S hybrids composed of dependent 50S and dependent 30S subunits or of dependent 30S and sensitive 50S subunits are not inhibited by DSM to a significant extent. 30S subunits isolated from dependent mutants behave in this system in a manner identical to the behavior of 30S subunits isolated from a resistant mutant.

Discussion.—We believe that the two major observations presented here are in fact closely related to each other. Current theories of the mechanism of protein synthesis picture the active synthetic unit as a polyribosome complex, with the group of ribosomes attached to a single strand of messenger RNA. The ribosomes migrate along the message strand in the act of translating the information contained therein to specify a particular polypeptide sequence.[20-24] The growing polypeptide chain is carried on the 50S subunit of the ribosome,[25] which also appears to be the site of attachment of transfer RNA.[26] The 30S ribosomal subunit is involved in the attachment of the ribosome to the mRNA strand.[27] It is this latter observation which is of interest in regard to the phenomena concerned with SM. The findings reported here, as well as those of J. Davies,[10a] implicate the 30S ribosomal subunit in the phenomena associated with SM. This suggests that the events concerned with ribosomal attachment and possibly its sequential movement on the message strand are crucial, and that the transfer of amino acids to the ribosome and their polymerization are unaffected by SM.

There are several other facts concerned with SM action which have been established. In order for SM to inhibit polypeptide synthesis *in vitro,* it is necessary for the sensitive ribosome to see SM prior to the addition of mRNA.[8] If the ribosome and mRNA are premixed and SM subsequently added, the resultant inhibition is considerably lessened in degree. The implication from this is that the binding site for SM on the ribosome is relatively inaccessible to the antibiotic when such a ribosome is already on the message strand. It becomes accessible only when the ribosome completes its current round of polypeptide synthesis and leaves the messenger. SM may well be competing for a site on the 30S subunit normally occupied by either Mg^{++} or a polyamine.[17] If such is the case, then the association between SM and the ribosome is a much tighter one than that between the ribosome and the normal cation which occupies this site, since it is impossible to reverse an established SM-inhibition with these cations.[8] It has also been found that the rate at which SM kills a sensitive population of bacteria is directly related to the momentary capacity for protein synthesis exhibited by the bacterial population.[28]

The requirement for protein synthesis could serve one or possibly both of two likely functions. Those ribosomes attached to a mRNA strand at the time SM is added to a growing population of sensitive bacteria must complete their round of protein synthesis before the SM-sensitive site is made available. Secondly, it may be that not only must the site be made available, but that the SM-containing ribosome must actually attach to a new message strand. In effect this would inactivate not only the ribosome containing SM which actually attaches, but also the message strand itself and all subsequent ribosomes which attach. For such ribosomes, assuming they do not contain SM, would be able to migrate along the message strand only up to that point to which a SM-containing ribosome is fixed. Some evidence suggesting that this latter point is, in fact, the case has been obtained and will be presented shortly.[17,28]

The findings presented here that polyribosome-like material is found in cells after DSM, implies attachment of ribosomes to mRNA in the presence of SM as Davies[10a] has directly shown. This will necessitate a modification in the proposal of SM action made recently by Spotts and Stanier.[10] We modify their proposal regarding ribosomes from SM-sensitive bacteria to state that *a SM-sensitive ribosome in the presence of SM does attach to mRNA*. Preliminary evidence suggests that in fact such an attachment is required for a lethal effect of SM.

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