CERTAIN ASPECTS OF STREPTOMYCIN INHIBITION OF PROTEIN SYNTHESIS IN *SERRATIA MARCESCENS**

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The action of streptomycin on protein synthesis was examined *in vivo* and *in vitro* with *Serratia marcescens*. At lethal concentrations of streptomycin (10 μg/ml and above) inhibition of protein synthesis and cell death appear to be causally related. Lower concentrations of the antibiotic (4~5 μg/ml) do not reduce protein synthesis, growth or the viability of the sensitive cells even after prolonged incubation in the presence of the drug. However, ribosomes isolated from cells so treated show partial reduction of amino acid incorporation *in vitro* in the absence of added streptomycin. Evidence suggests that subtle structural defects induced during ribosomal synthesis in the presence of the antibiotic and binding of streptomycin to the ribosomes together account for the defective ribosomal functioning during protein synthesis *in vitro*.

Numerous studies have shown that streptomycin (SM) appears to have several effects upon bacterial cells: inhibition of protein synthesis, permeability changes, breakdown of RNA, *etc*[1]. Recent evidences suggest that SM interferes with the initiation step of proten synthesis and causes irreversible blockage of the ribosomal cycle[2,3]. The comparative extent of these effects depends upon the relative concentration of the antibiotic and upon the susceptibility of the bacterial species or strain.

The site of action on protein synthesizing system is localized to a single protein in 30S ribosomal subunits[4], which presumably accounts for both the sensitivity of ribosomes to SM and its ability to bind the drug. The close correlation between bacterial sensitivity to SM *in vivo* and the results of ribosomal interactions with the antibiotic *in vitro* makes it likely that this interaction is a significant step in the antibiotics' lethal action on living bacterial cells. The mechanism is not yet fully understood. Data presented in this communication confirm the previous findings that as the concentration of SM is increased to kill sensitive cells, protein synthesis is inhibited simultaneously with and proportional to the killing of cells. However, cells grown in sub-lethal concentrations of SM produce ribosomes with reduced ability to

* A major portion of this work was done at the University of Houston, Houston, Texas.

synthesize proteins *in vitro*. These ribosomes seem to possess an altered structure as they exhibit an enhanced capacity to bind the drug *in vitro*.

Materials and Methods

S. marcescens strain Nima, streptomycin-sensitive, was grown on BUNTING's complete medium (CM) as modified by WILLIAMS *et al*[5]. Strain Nima was kindly provided by Dr. R. P. WILLIAMS, Baylor University School of Medicine, Houston, Texas. Inoculum was produced by growing the cells in 50 ml of CM on a rotary shaker at 27°C. The overnight culture was harvested by centrifugation and suspended in half-volume of 0.85 % saline. One ml of this suspension was used as inoculum in 50 ml of CM. After incubation on a rotary shaker at 27°C for the desired period to time, total cell counts were made in a Petroff-Hauser counting chamber, and, for viable cell counts, 0.1 ml of various dilutions in 0.85 % saline were plated on complete agar plates. Colonies were counted after overnight incubation at 27°C. The pigment, prodigiosin, was measured by the procedure described by HUBBARD and REMINGTON[6].

Incorporation of [^{14}C] proline into protein was determined by adding 2.5 μC of the amino acid (Sp. activity, 165 mC/mM) into 50 ml of mineral salt medium. This medium consisted of all the mineral salts of CM with 1 % glycerol and 0.5 % amino acid mixture. One ml of exponentially growing cells, harvested and suspended in saline, was added as inoculum, and SM was added in desired concentrations. At intervals, 5 ml samples were harvested to determine protein content by LOWRY's method[7], and the radioactivity of the precipitated protein samples was counted.

The procedure for isolation of ribosome and amino acid incorporation was essentially similar to that described by NIRENBERG and MATTHAEI[8]. The complete reaction mixture, 0.5 ml, contained ribosomes, 6 A_{260} units, supernatant protein 200 μg, poly-U 50 μg, tRNA 10 mμmole, 18 [^{12}C] amino acids 5×10^{-4} M, [^{14}C] phenylalanine (Sp. act. 170.5 mC/mmole) 5×10^{-4} M, ATP 10^{-3} M, GTP 10^{-4} M, phosphoenolpyruvate 5×10^{-3} M, and PEP-kinase 18.8 μg in Tris-HCl 10^{-1} M, pH 7.8, containing magnesium acetate 10^{-2} M, KCl 6×10^{-2} M and mercaptoethanol 6×10^{-3} M. The incubation was for 40 minutes at 37°C and the reaction was terminated by adding 2 ml of 10 % TCA. The activity of labeled aimino acid incorporated into TCA-insoluble material was counted in a Packard Scintillation Spectrometer with 50 % efficiency.

For experiments on ribosomes from cells grown in the presence of SM, strain Nima was grown overnight in 100 ml of CM containing 4~5 μg/ml of the antibiotic to produce the inoculum which was then added to 2 liters of CM supplemented with the same concentration of SM. Cells were harvested at mid-log phase and ribosomes isolated as before. Control cells were treated identically except for omission of SM. One sample of ribosomes from SM-grown cells was dialyzed overnight against 1,000 volume of SM-free Tris-Mg buffer. Amino acid incorporation with ribosomes from SM-grown cells, from cells grown with SM and then dialyzed, and from control cells were carried out as above. The supernatant fraction in the reaction mixture was common for all ribosomal samples and came from SM-free control cells.

[^{14}C] Phenylalanine was purchased from the New England Nuclear Corporation. Poly-U was purchased from Miles Chemical Laboratory. [^{14}C] Streptomycin was a gift from Dr. ROSENBLUM, Merck and Co., New Jersey. PEP-kinase was purchased from Sigma Chemical Co.: ATP, GTP, PEP, DNase were purchased from Mann Research Laboratory.

Results

The lethal effect of SM, already observed by serveral workers on *Escherichia coli* was studied for *S. marcescens* strain Nima. The growth of strain Nima was not affected up to 6~8 μg/ml of SM, but concentrations above 10 μg/ml caused a sharp

decline in cell count (Fig. 1). The lethal effect at this concentration does not appear to be due to permeability changes in membrane, as no significant increase in the 260 mμ-absorbing material was found in the medium below a SM concentration of 20 μg/ml[9]. The loss of viability in a SM-resistant strain D 1 of *S. marcescens* seems, however, to accompany the concomitant lysis of the cells due to very high concentrations of the antibiotic[9]. WHITE and FLAKS[10] have reported the cessation of protein synthesis in *E. coli* before the onset of killing by SM, while HURWITZ *et al*[11]. have shown that the killing effect of SM coincides with the inhibition of protein synthesis. A comparison of the inhibition of protein synthesis with loss of viable cell count in *S. marcescens* suggests that the effect on protein synthesis is closely related to cell death (Fig. 2). There is a sharp reduction in the labeled amino acid incorporation coinciding with the loss of viable cells within 20~30 minutes after exposure of sensitive cells to 10 μg/ml of SM. The effect with lower concentration, 5 μg/ml, was negligible. Therefore, the killing of cells by SM at this concentration appears to be the result of an abrupt reduction in protein synthesis and is not due to extensive damage of cell membrane.

Sub-lethal concentrations of SM may have other effects. One of them observed in our system is inhibition of prodigiosin, the red pigment, at significantly low concentrations (see Fig. 1 and ref. 12). Whether this unique effect could be related to any of the known effects of SM is not known. The second effect is shown in Table 1 where ribosomes isolated from cells grown in 4~5 μg/ml of SM showed partial inhibition of amino acid incorporation in the absence of added SM. Addition of SM inhibitied poly-U directed phenylalanine incorporation significantly in *S. marcescens* as in the *E. coli* system. To test the possibility that the reduced activity of ribosomes could be due to SM that was bound to ribosomes during growth and remained bound after isolation, ribosomes from SM-grown cells were dialyzed for 15 hours against 1,000 volume SM-free Tris-Mg buffer. Using supernatant fraction from SM-free control cells, the incorporation activities of ribosomal samples from (1) SM-grown cells, (2) SM-

Fig. 1. Effect of varying concentrations of SM on growth and pigment production in *S. marcescens*. Cells were grown, as described in Materials and Methods, in the presence of varying concentration of SM. After 8~10 hours growth duplicate samples were analyzed for total and viable cell counts and prodigiosion content.

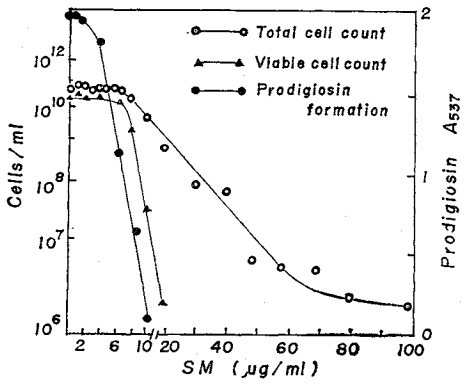


Fig. 2. Kinetics of SM inhibition of [^{14}C] proline incorporation into protein in *S. marcescens*. The growth conditions and estimation of protein content are described in Materials and Methods.

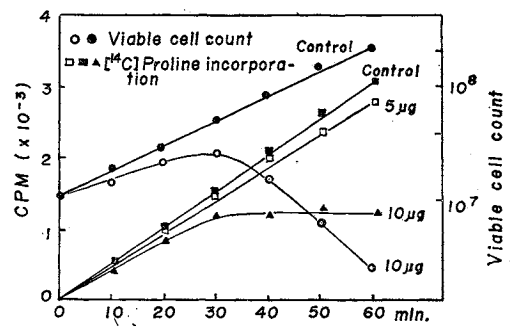


Table 1. Poly-U-directed phenylalanine incorporation by streptomycin-grown and -free cell-free components of *S. marcescens*

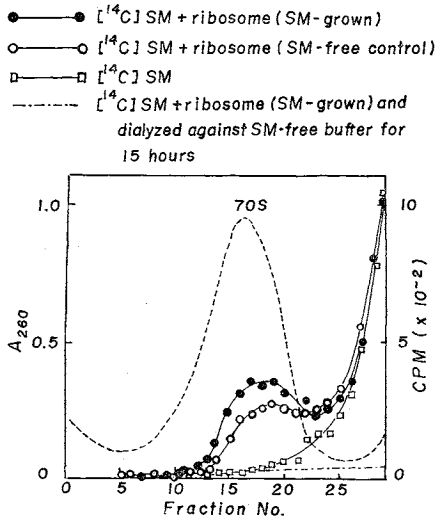
Ribosomes	S-100 Supernatant	SM (2.7×10^{-5} M) added to reaction mixture	pmoles [^{14}C] Phe incorporated*
C	C	—	60.2 (100 %)
C	C	+	20.6 (34 %)
C(d)	C	—	60.7 (101 %)
Sm	C	—	43.0 (71 %)
Sm(d)	C	—	50.0 (83 %)
C	SM	—	57.3 (95 %)
C	SM	+	19.0 (32 %)

Experimental conditions were same as described in Materials and Methods. SM=streptomycin, C=control (streptomycin-free), Sm=streptomycin-grown, -(d)=grown under the conditions described but dialyzed against streptomycin-free buffer.

Note: Regular procedure for ribosome isolation and incorporation experiments includes one dialysis of the components for 15 hours against 1,000 volume of Tris-Mg buffer; -(d) refers to a second dialysis.

* These figures are average of three determinations. All counts were corrected for zero time incubation.

Fig. 3. Binding of [^{14}C] SM to ribosomes from SM-grown and control cells of *S. marcescens*. 200 A_{260} units of 70S ribosomes were incubated with 2 mg of [^{14}C] SM (Sp. act. 0.054 μC/mg) in Tris-Mg buffer. Samples were layered on 28 ml of 5~20 % sucrose gradient in Spinco SW 25 rotor and centrifuged for 5 hours at 23,000 rpm to isolate 70 S particles. The activity of the measured aliquots from each fraction was counted in a Packard scintillation spectrometer.



grown cells followed by extensive dialysis against SM-free buffer, and (3) the control SM-free cells, were examined. Ribosomes from SM-grown cells showed consistently (three independent determinations) less incorporation of [^{14}C] phenylalanine (Table 1). The second dialysis restored the activity partially, but never completely as subsequent dialysis had no effect. Similar dialysis of the control ribosomes did not affect the incorporation activity of the ribosomes. The fact that reduction of incorporation activity was associated with ribosomes and was not due to contamination of supernatant fraction was demonstrated by interchanging the components, *i.e.*, ribosomes and supernatant. The possibility that SM caused gross conformational changes in ribosomes was tested by applying measured amounts of ribosomes to density gradient analysis. No measurable differences were observed in the density profiles of 50S and 30S particles[9]. Analyses of RNA and protein content of the ribosomes did not reveal any significant difference between ribosomes produced in the presence and the absence of SM nor was there any difference in the acrylamide gel-electrophoretic pattern of ribosomal proteins.

Ribosomes from cells grown in the presence of SM bound more SM than did control ribosomes. Two mg of [^{14}C] SM were added to about 200 A_{260} units of 70 S ribosomes and the mixture was left for 30 minutes at 4°C. A sample of ribosomes from untreated cells served as control. Both of these samples were layered on a linear sucrose gradient to isolate the 70S peak fractions. After pooling the peak fractions, activity was counted. At least 1.8~2 times more cpm/A_{260} units of ribosomes were associated with SM-grown samples than control ribosomes. Sucrose gradient profile (Fig. 3) also showed significantly high counts with the ribosomes from SM-grown cells.

Most of the labeled material remained at the top of the gradient tubes.

Discussion

The effects of sub-lethal concentrations of SM on intact cells, on protein synthesis, and especially on ribosomal function were examined in the present investigation. Protein synthesis was demonstrated in cell-free extracts of this organism and the data (unpublished) showed that protein synthesis in *S. marcescens* was similar to that observed in *E. coli*.

The kinetics of the inhibition of protein synthesis caused by the presence of SM in the growth medium suggest that this effect is related to cell death. Similar observations were made by HURWITZ *et al*[11]. The killing effect of SM is complicated by its action on the permeability of the cell membrane. Results (unpublished) indicate that damage to the cell membrane, measured in terms of nucleotide excretion into the medium starts at a concentration of about 20 μg/ml of SM, a cancentration higher than that which causes the onset of protein inhibition and loss of viability.

Evidence presented here indicates that cells grown in sub-lethal concentrations of SM are deficient in prodigiosin formation (Fig. 1) and contain ribosomes with reduced ability to incorporate [^{14}C] phenylalanine into protein *in vitro* in the absence of added SM (Table 1). While the selective inhibition of prodigiosin formation by SM cannot be explained in terms of the known effects of SM there are two possible explanations for the defective functioning of ribosomes:

(1) the presence of SM in the growth medium in sub-lethal concentration, induces the synthesis of ribosomes defective for cell-free protein synthesis.

(2) a small but significant amount of SM remains bound to ribosomes with forces sufficiently strong to resist washing and dialysis.

However, no significant changes in RNA and protein content were noted. The acrylamide gel-electrophoretic pattern of ribosomal protein was identical to that of normal ribosomes. The sedimentation pattern on sucrose density gradients did not indicate any grossly abnormal ribosomal particle. Nevertheless, a slight structural change, non-detectable by conventional methods of density gradient analysis, is not excluded. The ability of ribosomes from cells grown in SM to bind greater amounts of SM (1.8×) after isolation and dialysis, than control ribosomes, suggests structural changes and/or changes in state or distortion.

When the ribosomes were centrifuged on a sucrose density gradient after prior incubation with [^{14}C] SM, a significant amount of activity remained associated with the ribosomes; however, after dialysis the activity was very close to the background (Fig. 3). Since the specific activity of [^{14}C] SM was very low, the cpm above background remaining bound to ribosomes after dialysis could not be considered significant. Calculations based on the particle weight of the 70 S ribosomes as 2.6×10^6 indicated that, before dialysis, the ratio of SM molecules bound to ribosomes was 20 : 1. A report of LEON and BROCK[13] states that even after washing the ribosomes, at least one molecule remains firmly bound to each ribosome. WOLFGANG and LAWRENCE[14] have also found a small but significant amount of [^{14}C] SM bound to ribosome that resists dialysis. If this is the case, the reduced capacity of ribosomes to incorporate [^{14}C] phenylalanine can be explained on the basis of one molecule bound per ribosome. PESTKA[15] has estimated that a concentration of approximately one molecule per ribosome is sufficient to produce amino acid substitution errors *in vitro*. This reasoning, however, does not totally explain our data, particularly since dialysis partially restored the incorporation activity.

The defective ribosomes in this system are not identical to those formed by treatment of cells with chloramphenicol as reported by YOUNG and NAKADA[16]. The concentration of chloramphenicol in their system caused cessation of protein synthesis, and the resulting ribosomes were significantly deficient in proteins. What seems to be the common feature, however, is that incubation of the sensitive cells in the presence of the antibiotic causes

alterations in ribosomes making them functionally defective. The defect seems to be reversible in the case of chloramphenicol treatment and irreversible in the case of SM.

The conclusion most consistent with the data as interpreted here is that the presence of low (sub-lethal) concentrations of SM induces *in vivo*, the synthesis of ribosomes with slightly abnormal structure and results in SM binding to ribosomes, a binding reversible only in part. These dual effects reduce the ability of the altered ribosomes to function in polyphenylalanine synthesis *in vitro*, in the absence of added SM. Whether incorporation of amino acid directed by natural messengers and some other aspects of ribosomal functions are affected similarly is not presently known.

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