

## Streptomycin Dependence in *Escherichia coli*: Effects of Antibiotic Deprivation on Ribosomes

DACE I. VICEPS<sup>1</sup> AND BARBARA L. BROWNSTEIN\*

Temple University, Biology Department, Philadelphia, Pennsylvania 19122

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The inhibition of cell division and the ultimate loss of viability after removal of streptomycin from growing cultures of streptomycin-dependent bacteria are not the result of "unbalanced growth" or of the breakdown of ribosomes. The streptomycin-dependent strain of *Escherichia coli* K-12 continued to synthesize ribonucleic acid (RNA) and protein during streptomycin starvation. There was no evidence of a gross imbalance in the ratio of RNA to protein synthesized or of selective degradation of either protein or RNA. Using the sedimentation of subunits in sucrose as the criterion, normal ribosomes were synthesized even after 18 h of streptomycin deprivation, although the rates of appearance of mature 30S and 50S subunits decreased with time of deprivation. Once formed, these ribosomes appeared stable, as did those synthesized before the onset of starvation. Ribosomes isolated from starved dependent cells were as "functional" as ribosomes from cells grown with streptomycin in their capacity to bind aminoacyl-transfer RNA in response to polyuridylic acid or natural messenger RNA, to interconvert between active and inactive transfer RNA binding states, and to synthesize proteins in cell-free systems. The effects are consistent with an impaired rate of synthesis of ribosomal components or assembly of ribosomes resulting in a continually diminishing rate of protein synthesis. The effect on cell division may be the result of a decreased rate of protein synthesis in general and the requirement for a specific protein(s) in particular.

The mutation from streptomycin sensitivity to dependence has been observed in a number of bacterial strains. In *Escherichia coli*, the mutation is in the *strA* locus and results in an alteration in ribosomal protein S12; high-level streptomycin resistance and streptomycin dependence are allelic. Physiological and in vitro studies have been used to determine the mechanism whereby this single-step mutation causes a number of phenotypic changes resulting in a failure of streptomycin-dependent *E. coli* to survive in the absence of the antibiotic (1, 9, 29, 30). Spotts (29, 30) has reported an imbalance in the synthesis of cellular macromolecules in streptomycin-dependent bacteria and proposed a streptomycin requirement for the normal functioning of the ribosomes. Subsequent in vitro protein synthesis studies with isolated and reconstituted ribosomes have indicated that the ribosomes from streptomycin-dependent cells do differ in functional activity from those of the isogenic, sensitive strains (3, 6, 15, 16, 18). Although studies have

not been performed on dependent strains, Misikin et al. (20, 21, 32, 33) showed that streptomycin interferes with certain conformational interconversions of the sensitive ribosomes while not affecting the ribosomes from the resistant strains. Although the conclusions of Misikin et al. can be interpreted as a generalized mode of action of streptomycin, our work indicates that the role of streptomycin in dependent cells is not to correct an altered capacity of the mutant ribosomes to undergo these cyclic conformational changes.

In this study on the effects of streptomycin withdrawal on ribosome synthesis and function in dependent cells, we found that the imbalance in ribonucleic acid (RNA) and protein synthesis reported by Spotts (29, 30) does not occur in all dependent strains. Such an imbalance in cellular regulatory systems cannot, therefore, be the basis of cell death upon antibiotic withdrawal, although streptomycin does appear to affect some conformational change in the ribosome required for a translational step(s) and/or proper assembly. Under conditions of streptomycin deprivation, the rates of RNA and

<sup>1</sup> Present address: Wistar Institute of Anatomy and Biology, Philadelphia, Pa. 19104.

protein synthesis were depressed to an equal extent and ribosome assembly appeared normal, although occurring, as does the rest of protein and RNA synthesis, at a slower rate than under optimal growth conditions. The loss of viability on removal of streptomycin seemed to be a result of a decreased rate of protein synthesis in general and the requirement for some protein(s) needed for cell division in particular. Attempts to detect a difference in functionality between ribosomes from dependent and starved dependent cells to account for this decreased growth rate suggested that streptomycin acts directly on the translational machinery.

### MATERIALS AND METHODS

**Bacterial strains and media.** AB-468d is a spontaneous streptomycin-dependent mutant of the wild-type *E. coli* K-12 streptomycin-sensitive parental strain, AB-468. This strain is stringent in its RNA control, auxotrophic for adenine, histidine, proline, and thiamine, and requires streptomycin for growth. Of the classes of dependent strains described by Bjare and Gorini (4, 19), 468d belongs to class Str<sup>P</sup>, growing on streptomycin but not on ethanol or paromomycin. AB-468r, a high-level streptomycin-resistant mutant, was isolated from the same parental strain.

Tryptose phosphate broth (Difco) was used for cell growth rate and viability determinations. For ribosome isolation, mass cultures were grown in complex medium (5). For measurement of RNA and protein synthesis and sucrose gradient analysis, the minimal medium of Davis (2) was used. Glucose was added to a final concentration of 0.1%. Amino acids and thiamine supplements were added to satisfy the auxotrophic requirements at final concentrations of 20 and 5  $\mu\text{g/ml}$ , respectively. When specified, streptomycin was present at a concentration of 100  $\mu\text{g/ml}$ .

**Growth conditions.** All cultures were grown at 37 C. The mass doubling time was determined by optical density measurements of logarithmically growing cultures. The doubling time for AB-468d is 45 min in tryptose phosphate broth and 60 min in minimal medium. Viability was determined by the number of colonies on tryptose phosphate agar plates supplemented with 100  $\mu\text{g}$  of streptomycin per ml after 2 days of incubation. For growth under conditions of streptomycin deprivation, logarithmically growing dependent cells were collected on sterile membrane filters (Millipore Corp.) and washed four times with 10 ml of phosphate buffer (20 mM; pH 7.4). The washed cells were used to inoculate a medium containing all of the growth requirements except streptomycin.

**RNA and protein synthesis.** Dependent cells were grown in Davis minimal medium supplemented with the nutritional requirements, [<sup>3</sup>H]uracil (0.88 mM, 5.6 Ci/mol), [<sup>14</sup>C]arginine (0.056 mM, 0.89 Ci/mol), and with or without streptomycin. At 30-min intervals, duplicate 0.5-ml samples were removed into 5 ml of ice-cold 5% trichloroacetic acid. One drop of bovine

serum albumin (1 mg/ml) was added to each sample to facilitate precipitation. Precipitates were collected on glass-fiber filters (Schleicher & Schuell, Inc.) and washed, and the filters were dried and counted.

**Sucrose gradient analysis of ribosomes.** Bacteria were grown for 1 h in the presence of streptomycin and 5.7  $\mu\text{M}$  [<sup>14</sup>C]uracil (52 mCi/mol) in Davis minimal medium supplemented with the growth requirements. The culture was washed free of unincorporated label and streptomycin and suspended in fresh Davis medium (plus amino acid supplements), and incubation was then continued. Ten-minute [<sup>3</sup>H]uracil (0.11  $\mu\text{M}$ ; 16.8 Ci/mmol) pulses were given to portions of [<sup>14</sup>C]labeled culture after 0 and 6 h of streptomycin deprivation. One portion labeled with [<sup>3</sup>H]uracil at 6 h of starvation was exposed to 0.89 mM cold uracil for 1 h after the pulse. The double-labeled cells were disrupted in a French pressure cell in a solution of 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (Tris) (pH 7.8) and 0.1 mM MgCl<sub>2</sub> containing 25  $\mu\text{g}$  of deoxyribonuclease (ribonuclease free, Worthington Biochemicals Corp.) per ml. Whole cells and debris were removed by centrifugation, and a 0.2-ml sample of the supernatant was layered on a 5 to 20% (wt/vol) sucrose gradient (in 10 mM Tris-hydrochloride [pH 7.8]-0.1 mM MgCl<sub>2</sub>) and centrifuged at 48,000 rpm for 115 min in a Beckman SW50.1 rotor. Drops from the gradient were collected into scintillation vials and counted.

**Ribosome isolation.** Ribosomes were prepared by a modified method of Kurland (17); the ribosomes underwent only one cycle of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation. They were further purified by two 1 M NH<sub>4</sub>Cl washes (10 mM Tris-hydrochloride, pH 7.4; 6 mM 2-mercaptoethanol; 1 M NH<sub>4</sub>Cl; 3 and 10 mM magnesium acetate, respectively).

**Measurement of phenylalanyl-tRNA binding.** Inactivation and reactivation procedures were modified by the method of Miskin and Zamir (20).

(i) **Inactivation of ribosomes.** NH<sub>4</sub>Cl-washed ribosomes (500 absorbancy units at 260 nm [ $A_{260}$ ] per ml) were reactivated for 30 min at 40 C in a mixture containing 20 mM magnesium acetate, 0.2 M NH<sub>4</sub>Cl, 10 mM Tris-hydrochloride (pH 7.5), and 2 mM dithiothreitol. They were immediately diluted into an inactivation medium at 25 C so that the final concentrations were: 1 mM magnesium acetate; 20 mM NH<sub>4</sub>Cl; 10 mM Tris-hydrochloride (pH 7.5); 2 mM dithiothreitol; and 0, 2, or 20  $\mu\text{g}$  of streptomycin per ml. At the stated times, samples were removed and placed into the binding assay mixture where incubation proceeded for 60 min at 0 C. The final concentrations in the 50- $\mu\text{l}$  binding mixture were the following: 20 mM magnesium acetate; 0.15 M NH<sub>4</sub>Cl; 50 mM Tris-hydrochloride (pH 7.5); 2 mM dithiothreitol; 12  $\mu\text{g}$  of polyuridylic acid; 5.8 pmol of [<sup>14</sup>C]phenylalanyl-transfer RNA (tRNA) (466 mCi/mmol; 862 counts/min per pmol); and 0, 2, or 20  $\mu\text{g}$  of streptomycin per ml. The amount of [<sup>14</sup>C]phenylalanyl-tRNA bound was determined by filtering the binding solution through membrane filters (Millipore Corp.) pre-soaked in wash buffer (10 mM Tris-hydrochloride, pH 7.8; 10 mM magnesium acetate; 0.1 M NH<sub>4</sub>Cl; 6 mM 2-mercaptoethanol). Filters were dried and

counted. Background, as determined by a binding reaction with all components except polyuridylic acid, was subtracted from all samples. The conversion factor used was: 25 pmol of 70S = 1  $A_{260}$  unit (10).

(ii) **Reactivation of ribosomes.**  $\text{NH}_4\text{Cl}$ -washed ribosomes were inactivated by a 20-h dialysis at 4 C against a buffer containing 0.1 mM magnesium acetate, 0.1 M  $\text{NH}_4\text{Cl}$ , 10 mM Tris-hydrochloride (pH 7.5), and 6 mM 2-mercaptoethanol. Inactive ribosomes (500  $A_{260}$  units per ml) were reactivated by incubation at 40 C in a medium containing 20 mM magnesium acetate; 0.1 M  $\text{NH}_4\text{Cl}$ ; 10 mM Tris-hydrochloride (pH 7.5); 2 mM dithiothreitol; and 0, 2, or 20  $\mu\text{g}$  of streptomycin per ml. Samples were removed from the reactivation medium at the given times and diluted into the binding mixture as described above.

**Cell-free systems of protein synthesis.** Cell-free polypeptide synthesis system was modified by the method of Traub et al. (31). Final concentrations in the 150- $\mu\text{l}$  natural messenger RNA-directed reaction mixture were the following: 4.8 mM Tris-hydrochloride (pH 7.4); 20 mM  $\text{NH}_4\text{Cl}$ ; 4.8 mM magnesium acetate; 0.03 mM guanosine 5'-triphosphate; 1 mM adenosine 5'-triphosphate (neutralized); 3.6 mM 2-mercaptoethanol; 0.03 mM dithiothreitol; all amino acids (valine and cystine omitted; 0.048 mM each); 0.0019  $\mu\text{mol}$  of [ $^{14}\text{C}$ ]valine (252 mCi/mmol); 0.150 mg of tRNA; optimal amounts of crude initiation factors (8) and S-100 enzymes (24) purified from a streptomycin-sensitive strain; 10  $\mu\text{g}$  of calcium leucovorin per ml; 1.7  $A_{260}$  units of 70S 468d ribosomes or 0.8  $A_{260}$  units of 70S 468 ribosomes; with or without 100  $\mu\text{g}$  of streptomycin per ml. After a 40-min preincubation period at 40 C, phage R 17 RNA (7) (3.5  $A_{260}$  units for 468d; 1.8  $A_{260}$  units for 468) was added to the reaction mixture. After a 30-min incubation period at 40 C, reactions were terminated by the addition of 3 ml of 5% trichloroacetic acid containing 0.6% Casamino Acids. After heating for 20 min at 90 C, precipitates were collected on membrane filters (Millipore Corp.) and washed four times with 5% trichloroacetic acid. Filters were dried and counted. Background, as determined by incubation of identical reaction mixtures without messenger RNA, was subtracted in each case.

**Reagents.** Isotopes were purchased from New England Nuclear. [ $^{14}\text{C}$ ]phenylalanine was used to charge *E. coli* K-12 tRNA (General Biochemicals) by a modified procedure of Sokoloff and Rappaport (28).

## RESULTS

**Effect of streptomycin deprivation on growth rate.** The streptomycin-dependent strain AB-468d grows optimally in the presence of 100  $\mu\text{g}$  of streptomycin per ml at 37 C. When logarithmically growing cells are washed free from streptomycin and suspended in medium containing antibiotic, the cells resume logarithmic growth without lag. If they are suspended in a medium without the antibiotic (Fig. 1), the streptomycin-starved culture goes through the following stages: (i) growth continues at the exponential rate of optimally growing (with

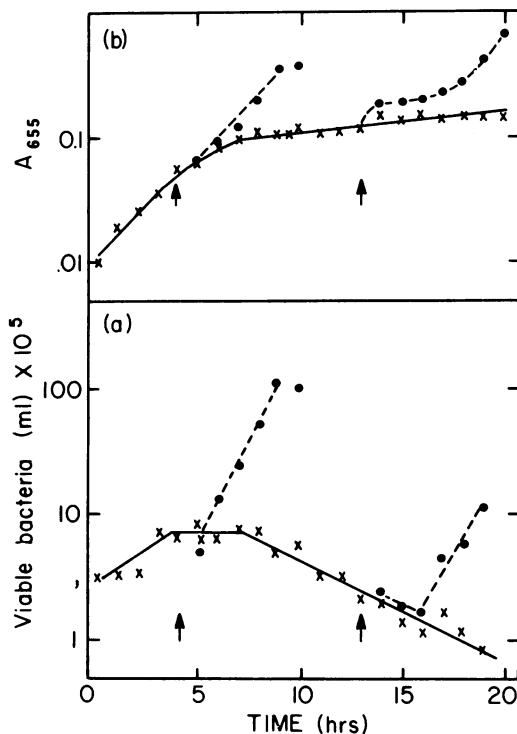


FIG. 1. Effect of streptomycin deprivation on the growth of AB-468d. (a) Viable cell count; (b) increase in mass. Symbols:  $\times$ , no streptomycin (antibiotic removed at time zero);  $\bullet$ , streptomycin (100  $\mu\text{g}/\text{ml}$ ) added at times indicated by arrows.

streptomycin) 468d for 45 to 60 min; (ii) a period (approximately 6 h in duration) with a progressively declining growth rate follows; (iii) the streptomycin-starved culture then enters stationary phase with no further increase in the mass of the culture.

The increase in number of viable cells (Fig. 1a) ends during the period of declining mass increase, and an increasing loss in the number of viable cells occurs during the stationary phase (Fig. 1b).

Microscope examination of the cells indicates that under streptomycin starvation conditions they fail to divide successfully. These streptomycin-deprived cells appear as long filaments with lengths 2 to 10 times that of AB-468d growing under optimal conditions. If streptomycin is returned to the medium, the viable snake-like bodies successfully form septae and there is an increase in the quantity of "controlled" cells.

Both optical density and viable cell measurements indicate that as the length of starvation time increases there is a progressively longer lag

period upon streptomycin addition before normal growth rates resume (Fig. 1). After 4 and 13 h of streptomycin deprivation, there are 1- and 3-h lags, respectively, before the surviving cells resume exponential growth.

**Effect of streptomycin deprivation on macromolecule synthesis.** To determine whether the lethal effect of antibiotic deprivation on streptomycin-dependent *E. coli* was the result of unbalanced growth, RNA and protein synthesis were measured in streptomycin-deprived cultures. [ $^3\text{H}$ ]uracil and [ $^{14}\text{C}$ ]arginine were added to optimally growing and streptomycin-starved AB-468d. The data (Fig. 2) show that the depressed rates of RNA and protein synthesis parallel the depressed increase in mass. In terms of RNA and protein synthesized per unit mass density, there is no quantitative difference between the streptomycin-starved and nonstarved cells (Fig. 3). There appears to be no imbalance in the synthesis of macromolecules during antibiotic deprivation (Fig. 3a) as had been previously reported (29, 30). An analysis of rates of synthesis of RNA and protein at different times after removal of

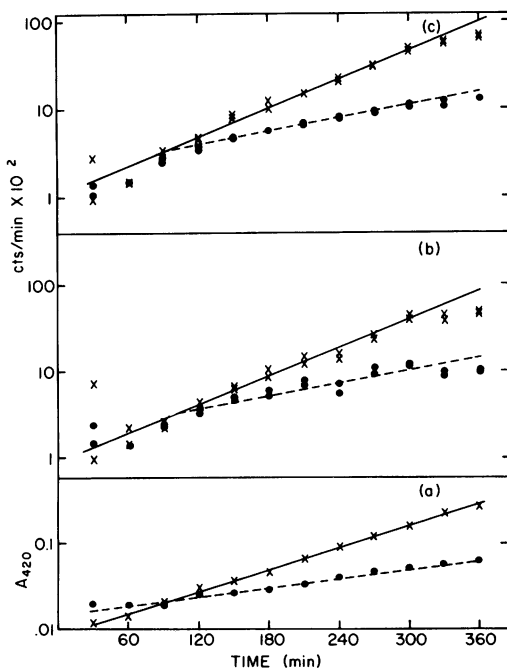


Fig. 2. Effect of streptomycin starvation on RNA and protein synthesis. (a) Mass density measurements; (b) incorporation of [ $^3\text{H}$ ]uracil; (c) incorporation of [ $^{14}\text{C}$ ]arginine. Symbols:  $\times$ , 100  $\mu\text{g}$  of streptomycin per ml;  $\bullet$ , without streptomycin. (Streptomycin removed at time zero.) [ $^3\text{H}$ ]uracil and [ $^{14}\text{C}$ ]arginine were added at time zero.

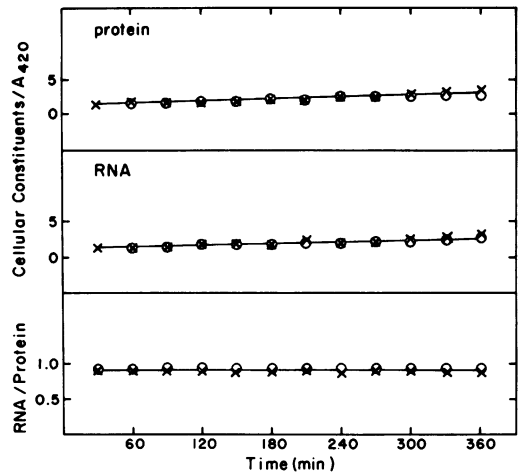


Fig. 3. Effect of streptomycin deprivation on the RNA/protein ratio per cell. Data are from Fig. 2.

the antibiotics in these cultures confirmed these observations. The constant RNA to protein ratio suggests that most cellular control mechanisms are operative during streptomycin deprivation.

**Effect of streptomycin deprivation on ribosome assembly and composition.** Evidence exists indicating that ribosomes extracted from streptomycin-sensitive, -resistant, and -dependent cells can express their respective phenotypes in vitro (3, 6, 15, 16, 18, 22, 25). Therefore, it was of interest to determine whether aberrant ribosomes or some alteration in the assembly process could be detected that might be responsible for the depressed rate of cellular metabolism in streptomycin-starved, dependent cells. The assembly of ribosomal subunits during streptomycin deprivation was followed by means of a sedimentation profile of the 30S and 50S subunits on sucrose gradients. Cultures were exposed to short pulses of [ $^3\text{H}$ ]uracil immediately and 6 h after removal of streptomycin. The profiles in Fig. 4a and b indicate that upon streptomycin deprivation progressively fewer subunits are made and there is an increased proportion of what appear to be precursor particles. When the [ $^3\text{H}$ ]uracil pulse given after 6 h of starvation is followed by an excess of unlabeled uracil for 1 h, the "precursor particles" chase into particles cosedimenting with the 30S and 50S subunits (Fig. 4c), indicating that the particles are precursors and not degradation products. No unusual species of ribosomes were found in the starved dependent cells. The ratio of 30S to 50S subunits of ribosomes labeled with  $^{14}\text{C}$  before streptomycin deprivation appeared constant throughout these

experiments, indicating no degradation of completed ribosomes synthesized either before or after streptomycin removal. The molecular weight, charge, and number of ribosomal proteins, as determined by two-dimensional gel electrophoresis, were not altered by at least 7 h of streptomycin starvation in the dependent cell (D. I. Viceps, Ph.D. thesis, Temple University, Philadelphia, Pa., 1973). At the time of cell harvesting, 85 to 90% of the ribosomes in these cells had been synthesized during starvation conditions. The absence of compositional differences in ribosomes from streptomycin-deprived dependent cells led us to investigate possible alterations in their functional capacity to account for the physiological changes reported above.

**Ribosomal conformational interconversions in vitro.** Ribosomes from streptomycin-sensitive and -resistant bacterial strains exist

in different conformational states with respect to their ability to bind aminoacyl-tRNA nonenzymatically (20, 21, 32, 33). Based on the conclusion of Miskin and Zamir (20, 21), our work predicted that the ribosomes from streptomycin-dependent cells deprived of the antibiotic would be different from those of dependent cells grown in the presence of streptomycin or from wild-type ribosomes in their ability either to inactivate or reactivate.

The possibility that streptomycin may affect the inactivation of dependent ribosomes was investigated. After reactivation in a medium containing a high concentration of magnesium, ribosomes were diluted into a low-magnesium inactivation buffer either in the presence or absence of the antibiotic and subsequently tested for their tRNA-binding activity. In the presence of 2 or 20  $\mu\text{g}$  of streptomycin per ml, ribosomes from starved and nonstarved depend-

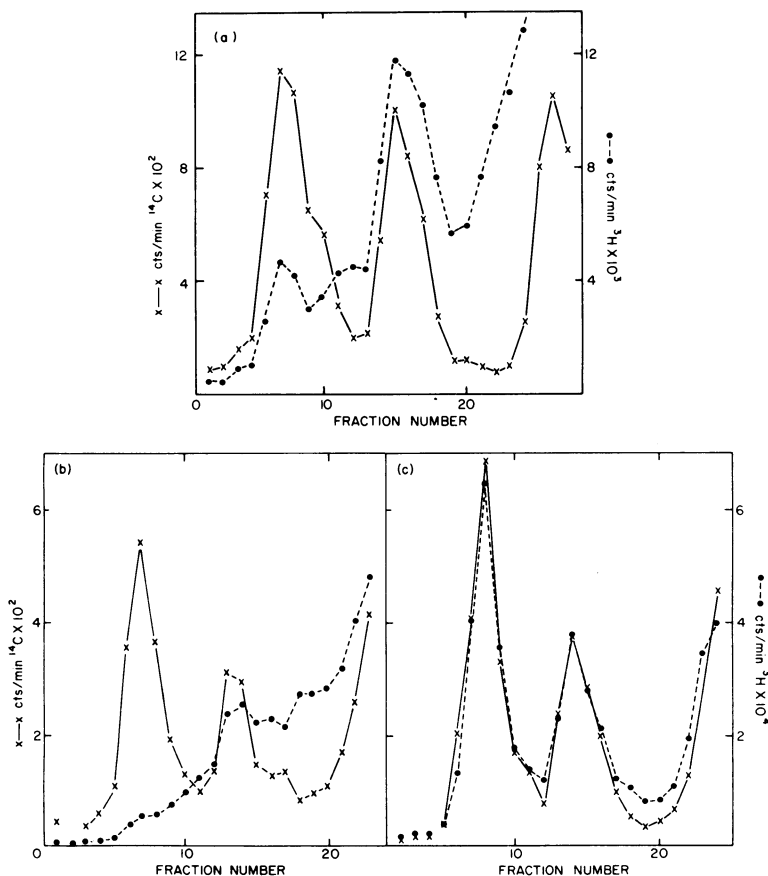


FIG. 4. Effect of streptomycin deprivation on ribosome assembly. Sedimentation on the 5 to 20% sucrose gradients is from right to left.  $^{14}\text{C}$ -labeled particles were labeled during exponential growth and subsequently pulse labeled with [ $^3\text{H}$ ]uracil. (a) A 10-min pulse with no streptomycin deprivation. (b) A 10-min pulse after 6 h of streptomycin deprivation. (c) A 10-min pulse after 6 h of streptomycin deprivation and a 1-h exposure to excess cold uracil.

ent cells appear only slightly inhibited in their inactivation (Fig. 5a,b). Similarly, streptomycin appears to slightly inhibit the inactivation of ribosomes from 468 (Fig. 5c). The binding of various concentrations of tRNA to

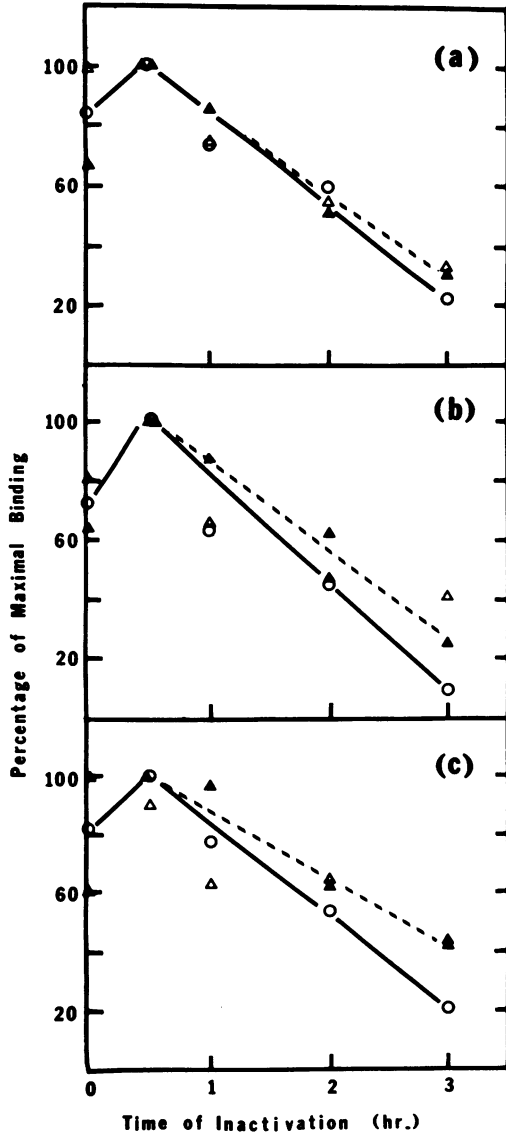


FIG. 5. Effect of streptomycin on inactivation of AB-468d and AB-468. Maximal binding is the percentage of  $f$ , where  $f$  is moles of tRNA bound per mole of ribosome. All points on the figure are the average of duplicate samples. (a) Dependent ribosome: (O) no streptomycin, at 100% activity  $f$  (100%  $f$ ) = 0.035; (▲) 2  $\mu$ g/ml, 100%  $f$  = 0.046; ( $\Delta$ ) 20  $\mu$ g/ml, 100%  $f$  = 0.60. (b) Starved dependent ribosome: (O) no streptomycin, 100%  $f$  = 0.035; (▲) 2  $\mu$ g/ml, 100%  $f$  = 0.043; ( $\Delta$ ) 20  $\mu$ g/ml, 100%  $f$  = 0.067. (c) Sensitive ribosome: (O) no streptomycin, 100%  $f$  = 0.032; (▲) 2  $\mu$ g/ml, 100%  $f$  = 0.027; ( $\Delta$ ), 20  $\mu$ g/ml, 100%  $f$  = 0.039.

reactivated ribosomes in the presence of polyuridylic acid indicated that the streptomycin starvation in vivo does not alter the tRNA-binding affinity of these ribosomes (D. I. Viceps, Ph.D. thesis). The ribosomes from the resistant strain inactivate to the same degree regardless of the drug concentration. These findings suggest that the site of action of streptomycin in sensitive and dependent cells is not the prevention of this conformational transition of the ribosome from an active to an inactive state.

The possibility that this antibiotic may facilitate the reciprocal conversion of the ribosome from the inactive to the active state was also pursued. Inactivated ribosomes were incubated at 40 C in a high-magnesium reactivation medium in the presence or absence of streptomycin and tested for their tRNA binding activity. The reactivation of ribosomes appears to be increasingly inhibited with greater concentrations of the antibiotic (Fig. 6a,b) regardless of whether they are from streptomycin-deprived or from optimally grown dependent cells. By this type of assay, no difference was observed between the reactivation of ribosomes from dependent and starved dependent cells. Six hours of streptomycin starvation, while dramatically affecting the growth rate of cells, does not appear to be altering the fraction of ribosomes able to bind phenylalanyl-tRNA in vitro. The ribosomes of the sensitive parent strain, 468, appear more severely affected by the drug than those from the dependent cells (Fig. 6c). Reactivation of the sensitive ribosomes is almost completely inhibited in the presence of 20  $\mu$ g of streptomycin per ml, in agreement with the results of Misikin and Zamir (20).

**Effect of streptomycin on protein synthesis in vitro.** Since streptomycin did not appear to facilitate phenylalanyl-tRNA binding to ribosomes from dependent strains, we investigated other steps in an in vitro protein-synthesizing system to identify possible effects of streptomycin starvation on the function of mature ribosomes. Streptomycin stimulates protein synthesis in vitro when natural messenger RNA is used (Table 1) as has been reported previously by Kreider and Brownstein (15, 16). The ribosomes from dependent and starved dependent 468d appear to function identically in vitro (Table 1). Preliminary experiments have failed to localize the site of this stimulation in the initiation step ( $f$ -met-tRNA binding) or first peptide bond formation ( $f$ -met-puromycin formation). Not only do both starved and nonstarved ribosomes respond to streptomycin, but the ribosomes have similar tRNA-binding affinities. (The  $f$  values in Table 1 indicate the quantity of tRNA bound per ribosome.) Ribosomes from

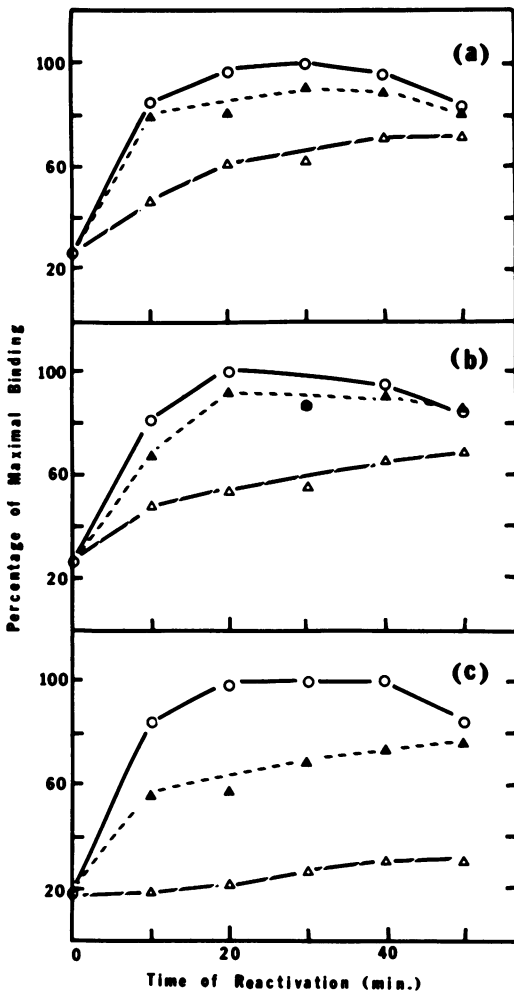


FIG. 6. Effect of streptomycin on the reactivation of AB-468d and AB468. Details are identical to those described in the legend for Fig. 5. (a) Dependent ribosome: at 100% activity  $f$  (100%  $f$ ) = 0.028. (b) Starved dependent ribosome: 100%  $f$  = 0.028. (c) Sensitive ribosome: 100%  $f$  = 0.023. Symbols: O, no streptomycin; ▲, 2 µg/ml; Δ, 20 µg/ml.

streptomycin-sensitive cells are inhibited by the antibiotic in their in vitro protein-synthesizing activity. These experiments provide further evidence that the functional capacity of the mature ribosomes obtained from dependent cells is not altered by streptomycin deprivation conditions.

**DISCUSSION**

The growth properties exhibited by the streptomycin-dependent strain of *E. coli*, AB-468d, in response to streptomycin starvation are consistent with the observations of Bertani (1) and Spotts (29, 30). The growth rate declines, ulti-

mately leading to cell death, with the bacteria forming long filaments as they fail to divide successfully. The longer the culture has been deprived of the antibiotic, the longer the lag time before it recovers to its exponential rate upon subsequent streptomycin addition. This lag time and the resumption of growth at an exponential rate suggest that some time-consuming event(s) must occur in all the cells before they are capable of dividing. Such events may be the degradation of some faulty cellular component(s) made during the starvation period and/or the synthesis of some cellular component(s) to a critical level necessary for cell division that was not synthesized, was synthesized improperly, or was degraded during starvation. Upon recovery, the number of viable cells increases more rapidly than cell mass (Fig. 1). This phenomenon has also been observed in some division-defective temperature-sensitive mutants (11), where the more rapid increase in cell number is due to the more immediate reduction in filament size rather than an increase in mass, which occurs at a slower rate.

The macromolecular analysis during streptomycin deprivation indicates that the synthesis of RNA and protein appeared in balance with the depressed growth rate (Fig. 2, 3). On a gross measurement basis, all of the cellular control mechanisms remain operative during streptomycin deprivation. Therefore, the cause of cell death in this strain of starved dependent bacteria, as well as in several other spontaneous dependent mutants isolated from other strains (not shown), does not appear to be due to an imbalance of macromolecule synthesis as had been reported by Spotts (29, 30). He may have been observing the imbalance resulting from diminished protein synthesis in a strain relaxed in RNA control. The strain chosen for this study is wild type (stringent) in its RNA control. These findings indicate that the previously reported loss of cellular control upon streptomycin deprivation does not occur in all strep-

TABLE 1. Effect of streptomycin (Str) on amino acid incorporation directed by R-17 RNA

Ribosomes	Str (µg/ml)	Counts/min per reaction mixture	$f^a$	+Str/Str
Dependent	100	3,304	0.135	2.6
		1,279	0.052	
Starved dependent	100	3,259	0.160	2.3
		1,434	0.070	
Sensitive	100	29	0.002	0.04
		514	0.051	

<sup>a</sup> Moles of tRNA bound per mole of ribosome.

tomycin-dependent strains of *E. coli*. Therefore, such a proposed imbalance of macromolecule synthesis cannot be the factor responsible for cell death.

The sucrose gradient studies indicate that ribosomal subunits with normal sedimentation values are made even under conditions of streptomycin deprivation, but their rate of synthesis and/or assembly is depressed. No degradation of these subunits is evident, whether they are made prior to the onset ( $^{14}\text{C}$  labeled) or during starvation ( $^3\text{H}$  labeled). Streptomycin starvation results in a decreased rate of subunit formation and a simultaneous accumulation of precursor particles. This may result from the following situations. (i) The depressed rate of ribosome assembly may just be a function of the slower growth rate under these starvation conditions. (ii) The precursor forms of the subunits are hindered at some point of assembly (e.g., precursor RNA is not cleaved; lack of methylation and/or acetylation; improper conformation) and cannot form mature particles at a normal rate. (iii) A specific component(s) (perhaps streptomycin itself) necessary for facilitation of ribosome assembly or proper conformational configuration is not present in sufficient quantity. Although the present results cannot distinguish between these possibilities, they do indicate that the depressed growth rate of streptomycin-starved dependent cells is not due to the synthesis of aberrant ribosomal species or the degradation of ribosomes as measured by their behavior in sucrose gradients. Using the criterion of reassociation of 50S and 30S subunits to 70S monomers, preliminary findings of Zitomer and Flaks (R. S. Zitomer and J. G. Flaks, *Fed. Proc.* 31:456, 1972) indicate some change in the stability of the 30S subunits in streptomycin-dependent strains.

The absence of any shift in protein patterns on two-dimensional electrophoretic gels substantiates that complete ribosomes are synthesized even under streptomycin starvation conditions. In addition to the compositional analyses, all of the functional activities tested (interconversion between active and inactive states; tRNA binding in response to polyuridylic acid and natural messenger RNA; ability to synthesize protein in vitro) indicate that the mature ribosomes in cells growing in the presence and absence of streptomycin are identical. However, it is possible that the isolation procedure confers a particular conformation on the ribosomes, thus eliminating any differences that may have existed in situ.

We do not eliminate the possibility that

streptomycin is required, at least transiently, to facilitate some conformational change related to translation in vivo. Our results do show that conformational transitions required for nonenzymatic tRNA binding occur in ribosomes from dependent as well as sensitive cells. However, this in vitro interconversion phenomenon is not dependent on streptomycin. It is not clear that these in vitro measurements of ribosomal activity, as described by Miskin and Zamir (20), are indicative of physiological phenomena. In contrast, streptomycin stimulates protein synthesis when a natural messenger RNA is available (Table 1), suggesting that the antibiotic does affect some event in the translational process.

In vivo streptomycin deprivation results in a reduced rate of protein synthesis accompanied by a slower growth rate. Kjeldgaard (14, 19) observed that cells maintain a "constant efficiency" such that any change in the rate of protein synthesis is strictly proportional to the number of mature ribosomes. The depletion of streptomycin from the media of dependent cells may represent an analogous process. If the rate of translation is handicapped, decreasing the amount of ribosomes synthesized and available for the translation process, the rate of protein synthesis in the cell will diminish proportionally to maintain constant efficiency.

Although the rate of protein synthesis decreases, the degradation of proteins, as has been reported in cases of nutritional depletion (23, 26, 27), may continue at a normal rate. Therefore, labile proteins would be rapidly depleted since their diminished rate of synthesis would not be able to keep pace with their normal rate of degradation. Nath and Koch (23) have identified a rapidly degraded class of proteins in *E. coli*, whose degradation rate is unaffected by nutritional deficiencies. The physiological observations reported here suggest that under streptomycin deprivation conditions, a labile protein(s) which is responsible for triggering cell division, as proposed by Jones and Donachie (13), is degraded faster than the dependent cell can synthesize it. In such a case, the streptomycin-deprived cells fail to form cell wall septae and become long filaments, as is observed with 468d. Upon subsequent streptomycin supplementation, similar to nutritional shift-up (14), the cell preferentially increases its rate of synthesis of ribosomes to optimal levels; the synthesis of other cellular proteins is then resumed. When the cells have replenished their supply of labile proteins, such as one to trigger cell division, the cells resume their exponential growth rate. Recovery time after streptomycin



supplementation appears to be proportional to the extent of the starvation period. During nutritional depletion, Pine (26, 27) has observed that, as the more labile substances are exhausted, degradation of secondary substrates (species with slower turnover rates) may be accelerated to provide raw material (amino acids) for the synthesis of the more labile proteins. Therefore, it appears reasonable that with longer streptomycin starvation times intracellular degradation becomes so extensive that recovery becomes impossible.

Nutritional shift-up and shift-down experiments have yielded much information on the interrelationships of cellular regulatory mechanisms. These similarities to the physiological response of streptomycin-dependent cells to depletion and supplementation of the antibiotic suggests to us that similar intracellular events may occur. Since previously published data indicate that the streptomycin-dependent mutation involves an alteration in the S12 ribosomal protein (3, 12), we have assumed that the antibiotic has a role with respect to the functional and/or structural capacity of the ribosome. We propose that the role of streptomycin is to facilitate proper conformation of one (S12) or more ribosomal proteins for rapid ribosome assembly and/or for efficient translation of messenger RNA.

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