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Protein Synthesis in *Mycobacterium tuberculosis* H37Rv and the Effect of Streptomycin in Streptomycin-Susceptible and -Resistant Strains

M. S. SHAILA, K. P. GOPINATHAN, AND T. RAMAKRISHNAN
Microbiology & Pharmacology Laboratory, Indian Institute of Science, Bangalore 560012, India

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An efficient in vitro amino acid-incorporating system from *Mycobacterium tuberculosis* H37Rv was standardized. Ribonucleic acid (RNA) isolated from phage-infected *M. smegmatis* cells served as natural messenger RNA and directed the incorporation of ^{14}C-amino acids into protein. The effects of various antitubercular drugs and "known inhibitors" of protein synthesis on amino acid incorporation were studied. Antibiotics like chloramphenicol and tetracycline inhibited mycobacterial protein synthesis, though they failed to prevent the growth of the organism. This failure was shown to be due to the impermeability of mycobacteria to these drugs by use of "membrane-active" agents along with the antibiotics in growth inhibition studies. Several independent streptomycin-resistant mutants of *M. tuberculosis* H37Rv were isolated. Streptomycin inhibited the incorporation of ^{14}C-amino acids into proteins by whole cells of a streptomycin-susceptible strain by more than 90%, whereas very little or no inhibition was observed in either high-level or low-level streptomycin-resistant strains.

In vitro, streptomycin was an effective inhibitor of susceptible strains, whereas in streptomycin-resistant strains the concentration of streptomycin at which half-maximal inhibition was produced varied according to the resistance of whole cells, and there was a correlation between the two. In one low-level streptomycin-resistant mutant, the in vitro amino acid-incorporating system was as sensitive to various concentrations of streptomycin as the parental type, and a possible involvement of a membrane site in the development of low-level resistance was indicated. Streptomycin susceptibility and high-level resistance were shown to be ribosomal in nature.

Although the mechanism of protein synthesis and the action of antibiotics such as streptomycin have been worked out in *Escherichia coli* and other organisms in great detail (10, 12), very little is known about in vitro protein synthesis and the mode of action of streptomycin in mycobacteria. This is all the more surprising since the chemotherapy of tuberculosis is getting more complicated as a result of the occurrence of bacilli resistant to streptomycin. The observation of Erdös and Ullmann (4, 5) about the inhibition of protein synthesis by streptomycin was made in an undefined system in which "pH 5 enzyme" source devoid of ribosomes was used. Efficient in vitro protein-synthesizing systems have so far been described only from *Mycobacterium smegmatis* (14) and BCG (21). The available information on protein synthesis in mycobacteria was summarized by Ramakrishnan et al. in a recent review (13). A systematic and detailed study of protein synthesis in the virulent strain of *M. tuberculosis* and the action of streptomycin was therefore desirable. In this report, we describe an efficient cell-free system from *M. tuberculosis* H37Rv capable of incorporating amino acids into protein and the inhibitory effect of streptomycin. The purpose of the present investigation was to compare the protein synthesis in susceptible and resistant strains of *M. tuberculosis* H37Rv and to localize the site of susceptibility or resistance to streptomycin. Some features of the polyuridylic acid (poly U)-dependent in vitro incorporating system and the effects of several antimycobacterial drugs and antibiotic inhibitors of protein synthesis are described.

MATERIALS AND METHODS

Poly-U (K salt), β-mercaptoethanol, phospho-(enol) pyruvate (K salt), pyruvate kinase, adenosine triphosphate (ATP, trisodium), guanosine triphosphate (GTP, trisodium), alumina type 303, erythromycin, puromycin, and yeast ribonucleic acid (RNA) were from the Sigma Chemical Co., St. Louis, Mo.; *E. coli* transfer RNA (tRNA) and yeast tRNA were from Schwarz-Mann Research Laboratories; streptomycin sulfate was from Merck, Sharp, Dohme of India Ltd.; DL-phenylalanine-*1*-^{14}C (26.6 mCi/mmol) and ^{14}C-chlorella protein hydrolysate (13 mCi/m-atom) were from Bhabha Atomic Research Centre, Bombay, India; rifampin and kanamycin were from Mann Research Laboratories, New York, N.Y.; neomycin was from the Unichem Laboratories, Bombay, India; *p*-amino salicylic acid (PAS), Na salt, was from Rhodia Chemicals, Paris, France; isonicotinic hydrazide (INH) was from Dumex (India) Private Ltd., Bombay, India; N^5, N^{10}-formyl tetrahydrofolate, Ca salt, was from the Lederle Laboratories, London, England; *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) was from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and ethyl methane sulfonate (EMS) was from Eastman Kodak Co., Rochester, N.Y.

Growth of the organism and preparation of cell-free extract. *M. tuberculosis* H37Rv strain 7416 NCTC, which is streptomycin-susceptible, and the various mutants derived from it were grown routinely on the liquid synthetic medium of Youmans and Karlson (23) as surface cultures. When submerged cultures were desired, 0.02% (vol/vol) Tween 80 was incorporated into this medium and the cultures were incubated at 37 C in a rotary shaker (125 rpm, at a throw of 3.8 cm).

Mycobacterial cells were harvested after 12 days of growth and ground with an equal weight of alumina or glass powder in a prechilled mortar for 10 min. The resulting paste was suspended in cold 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.8, containing 0.01 M magnesium acetate, 0.06 M ammonium chloride, and 0.006 M β-mercaptoethanol (2 to 3 ml of buffer/g of wet weight) and centrifuged for 45 min at 20,000 × *g* at 4 C; the supernatant fluid constitutes the S-20 extract. A 100,000 × *g* supernatant fraction (denoted as S-100) was prepared, when indicated, by centrifuging the S-20 extract in a Beckman model L2-50 ultracentrifuge at 100,000 × *g* for 90 min. The top two-third portion constituted S-100. The remaining supernatant fluid was discarded, and the ribosomes were washed with "standard buffer" (0.01 M Tris-hydrochloride, pH 7.8; 0.01 M magnesium acetate, 0.06 M ammonium chloride, and 0.006 M β-mercaptoethanol) and pelleted again by centrifuging for 90 min at 100,000 × *g*. The washed ribosomes were suspended in the same buffer and stored frozen at −20 C until use.

The RNA content of the ribosomes was determined by the method of Ceriotti (3) with yeast RNA as the standard; the number of ribosomes in the assay mixture was calculated by the method of Van Dijk-Salkinoja (22) based on the RNA content.

Protein content of the ribosomes and S-100 extract was determined by the method of Lowry et al. (9).

Isolation of streptomycin-resistant mutants by chemical mutagenesis. A 12-day-old culture of *M. tuberculosis* H37Rv was inoculated into tubes containing 5 ml of medium with 0.02% (vol/vol) Tween 80. NTG (final concentration, 500 μg/ml) was added to one of the tubes, and EMS (final concentration, 0.2%) was added to the other. The contents of the tubes were thoroughly mixed and incubated at 37 C with shaking. After 1 h, 2.0-ml portions from each tube were removed and centrifuged, and the cells were suspended in 2.0 ml of fresh medium. Samples of 0.2 ml were added to Petrik's egg medium (8) containing 1, 10, and 100 μg of streptomycin/ml (all in duplicates). The procedure was repeated at the end of 3 h except that the centrifuged cells were suspended in 1.0 ml of medium (to compensate for the cell death due to the action of mutagen). All of the tubes were incubated at 37 C for 3 weeks. Three steps of purification of single colonies were carried out to avoid errors resulting from clumping of mycobacteria. The purified mutants were maintained by regular subculture on Petrik medium.

Similarly, several spontaneous streptomycin-resistant mutants were isolated (by exposing the organisms to 1 and 2 μg of streptomycin/ml) and purified.

Incorporation of ^{14}C-amino acids into proteins by whole cells. Eight-day-old *M. tuberculosis* H37Rv cells (or a streptomycin-resistant mutant), grown as a submerged culture in Youman's medium containing 0.02% (vol/vol) Tween 80, were diluted with fresh medium so as to contain approximately 1 mg (dry weight) per ml, and 0.25 μCi of ^{14}C-chlorella protein hydrolysate/ml was added. At various time intervals, 1 ml of culture was removed into 1 ml of 10% trichloroacetic acid, heated at 90 C for 30 min, cooled, and filtered through glass-fiber filters (GF/A, 2.4-cm circles). The filters were washed with 5% trichloroacetic acid several times, then with a mixture of ethanol-ether (3:1), and finally with ether. They were dried and the radioactivity was measured in a Beckman LS-100 liquid scintillation spectrometer. The results were expressed as counts per minute per milligram of dry weight. This procedure was repeated in the presence of 100 μg of streptomycin/ml.

Preparation of RNA from phage-infected M. smegmatis cells. *M. smegmatis* SN_2 was grown in the liquid medium of Youmans and Karlson (23), with vigorous aeration, to a cell density of 10^8 cells/ml. The cells were infected with mycobacteriophage I_3, a transducing phage isolated in this laboratory (19, 20), at a multiplicity of infection of 0.7, and were allowed to grow until rise period (about 6 h). The culture (before lysis started) was chilled and centrifuged in a Sharples superspeed centrifuge. The cells were washed with 0.01 M Tris, pH 7.2, containing 0.01 M magnesium acetate, and RNA from these phage-infected cells was isolated according to Salser et al. (15).

Poly U-directed incorporation of ^{14}C-phenylalanine. The reaction mixture contained (in a total volume of 110 μliters) the following: Tris-hydrochloride buffer, pH 7.8, at 37 C, 50 mM; ammonium chloride, 3 mM; magnesium acetate, 15 mM; dithiothreitol, 2 mM; phospho (enol) pyruvate, 10 mM; pyru-

vate kinase, 5 μg; GTP, 0.2 mM; 19 L-amino acids (except L-phenylalanine), 0.1 mM; *E. coli* tRNA, 100 μg; poly U, 100 μg; S-20 protein, 40 to 80 μg; and DL-phenylalanine-*1*-^{14}C, 50,000 counts/min (1,000 counts/min = 21.7 pmol). All of the components except poly U and ^{14}C-phenylalanie were mixed and preincubated at 37 C for 10 min. Drugs or antibiotics were added, wherever indicated, to the reaction mixture before preincubation. The reaction mixtures were chilled, ^{14}C-phenylalanine and poly U were added, and incubation was continued for a further 30 min at 37 C. Samples (100 μliters) were layered onto Whatman 3 MM filter disks which had been treated for 5 min with 100 μliters of 1 N NaOH. This treatment was necessary to destroy the incorporation due to amino acyl-tRNA (11). The disks were allowed to stand at room temperature for about 30 min and then were dropped into 10% trichloroacetic acid. After the disks had been washed with 5% trichloroacetic acid, ethanol-ether (3:1) mixture, and ether, they were air-dried and counted in a Beckman LS-100 liquid scintillation spectrometer with the use of a toluene-based scintillation fluid.

I_3 RNA-directed incorporation. The conditions used were similar to those described above except that formyl tetrahydrofolic acid, 0.4 mM, was added to the reaction mixture and the Mg^{2+} concentration was reduced to 10 mM. In place of ^{14}C-phenylalanine, ^{14}C-chlorella protein hydrolysate or ^{14}C-leucine was added, and about 700 μg of I_3 RNA was used.

Charging of tRNA. The system contained (in a total volume of 125 μliters) the following: cacodylate buffer, pH 7.0, 100 mM; magnesium acetate, 10 mM; ATP (pH 7.0), 2 mM; ammonium chloride, 10 mM; DL-phenylalanine-*1*-^{14}C, 50,000 counts/min; S-20 protein, 40 to 80 μg; various amounts of *E. coli* tRNA (or tRNA from other sources), and water if necessary to make 125 μliters. After incubation at 37 C for 30 min, 100-μliter samples were removed and layered onto Whatman 3 MM filter disks. After 2 min at room temperature, they were dropped into 10% trichloroacetic acid, processed, and counted as described above.

RESULTS

The age of the culture had a marked influence on the proteosynthetic activity of S-20 extracts; maximal activity was obtained in extracts derived from 12-day-old cells. The activity was drastically reduced when S-20 extract from 14-day-old cells was used, and practically no activity could be detected with 18-day-old cells.

Kinetics of incorporation. ^{14}C-phenylalanine incorporation was linear up to 30 min (Fig. 1), and 5- to 10-fold stimulation was observed over the blank.

Concentration of tRNA. The system was limiting in endogenous tRNA. Since the amount of tRNA in *M. tuberculosis* H37Rv is one-twentieth of that in *E. coli*, and also the nuclease activity of the extract is very high (unpublished data), it was necessary to add high concentrations of tRNA to the system. A concentration of 100 μg of tRNA was suitable for optimal charging. Heterologous tRNA could be used in the system (Fig. 2), and good charging was observed with *E. coli* tRNA. The higher incorporation rates observed with *E. coli* tRNA may be due to the higher purity of this preparation as compared with the *M. smegmatis* tRNA preparation. In subsequent experiments, we

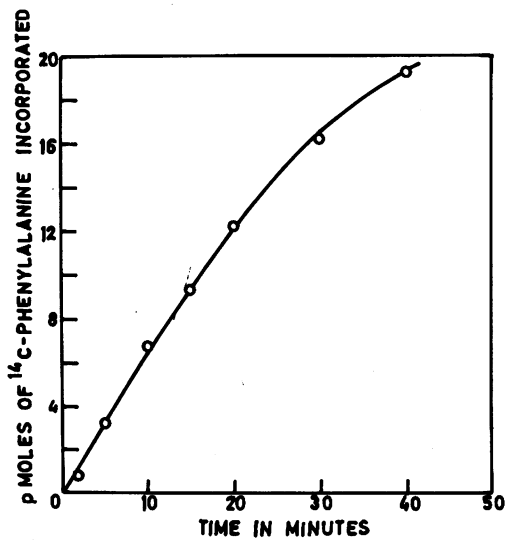

FIG. 1. *Kinetics of incorporation of ^{14}C-phenylalanine into protein. Assay conditions as described in Materials and Methods.*

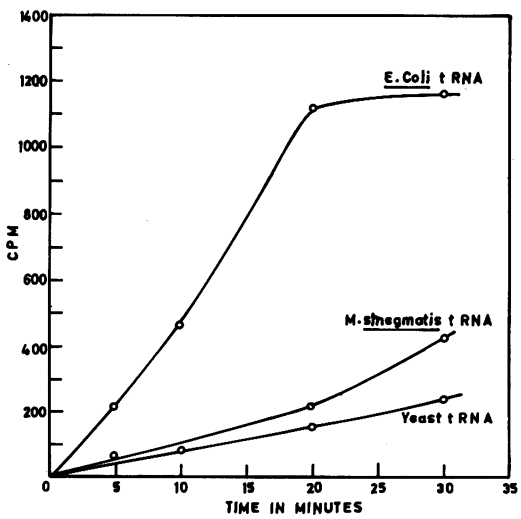

FIG. 2. *Amino acid activation of (i) E. Coli tRNA, (ii) M. smegmatis tRNA, and (iii) yeast tRNA by M. tuberculosis H37Rv aminoacyl tRNA synthetases. Assay conditions as described in the text. ^{14}C-chlorella protein hydrolysate was used for charging; 100 μg amounts of different tRNA were used.*

obtained comparable results using partially purified *M. smegmatis* tRNA.

A high concentration of poly U (100 μg/100 μliters) was necessary to produce considerable stimulation of ^{14}C-phenylalanine incorporation, which is in contrast to the very small amounts needed for stimulation of incorporation in other systems; optimal incorporating ability was observed at a Mg^{2+} concentration of 15 mM.

Incorporation directed by natural messenger RNA. RNA isolated from phage-infected *M. smegmatis* cells exhibited very little ability to stimulate the incorporation of a mixture of amino acids into proteins; the extent of stimulation, however, was dependent on the concentration of added RNA. A concentration of 0.73 mg per assay was found to be satisfactory. When ^{14}C-leucine was used in place of ^{14}C-algal protein hydrolysate, the system incorporated 15.8 pmol of ^{14}C-leucine per ml.

Effect of various antitubercular drugs on incorporation. The antitubercular drugs exhibited various degrees of inhibition of incorporation at the concentrations tested (Table 1). At high concentrations, certain antitubercular drugs like PAS, cycloserine, thiacetazone, and thiosemicarbazide inhibited the incorporation to a small extent, whereas others like INH, ethionamide, and pyrazinamide did not show any inhibitory effect. Aminoglycoside antibiotics inhibited the incorporation strongly even at low concentrations, streptomycin being most effective. Fusidic acid and capreomycin also exhibited marked inhibitory action. It is significant to observe that rifampin, which is an inhibitor of RNA polymerase, inhibited the poly U-dependent synthesis of polyphenylalanine even at low concentrations.

Various antibiotic inhibitors known to inhibit the synthesis of proteins in other systems were tested (Table 2). Since all of these antibiotics inhibited poly U-dependent incorporation of ^{14}C-phenylalanine to a certain extent, the effect of these inhibitors on the growth of the tubercle bacilli was evaluated. The site of action of most of these antibiotics has been established to be at the level of protein synthesis. Nevertheless, they did not show any bactericidal action on *M. tuberculosis* H37Rv even at a concentration of 10 μg/ml, suggesting that the lack of action of these antibiotics on whole cells may be due to their impermeability to these antibiotics. Hence, the action of these antibiotics in the presence of sodium dodecyl sulfate (SDS), a "membrane-active" agent which has been reported to alter the cellular permeability of other bacteria (1) as well as that of INH-resistant mutants of *M. tuberculosis* H37Rv (17), was studied. If the antibiotics enter the cell in the presence of SDS, they should prove to be bactericidal to *M. tuberculosis* also. The results presented in Table 3 indicate that these antibiotics (even at 1 μg/ml) were bactericidal in the presence of 0.01% SDS. SDS at this concentration is not inhibitory by itself.

Properties of streptomycin-resistant mutants. All of the mutants were found to be genetically stable and did not revert on repeated subculture in drug-free medium. The

TABLE 1. *Effect of antitubercular drugs on ^{14}C-phenylalanine incorporation*[a]

Drug	Percent inhibition				
	10^{-3} M	10^{-4} M	10^{-5} M	10^{-7} M	10^{-9} M
Streptomycin ..	80.00	80.00	80.00	70.00	55.00
Rifampin	—	—	63.30	63.50	29.00
Kanamycin	—	69.00	50.30	18.30	—
Fusidic acid	—	76.30	72.30	62.20	43.70
Thiosemicarbazide.....	22.50	18.60	13.60	—	—
Thiacetazone ..	32.80	29.90	0.00	—	—
Cycloserine	17.30	0.00	0.00	—	—
Ethambutol	37.10	31.50	29.80	—	—
p-Amino salicylic acid .	46.00	33.00	6.70	—	—
Isonicotinic acid hydrazide	0.00	0.00	0.00	—	—
Ethionamide ...	0.00	0.00	0.00	—	—
Pyrazinamide ..	—	20.00	0.00	—	—
Capreomycin ...	100.00	100.00	66.20	57.20	50.00

[a] Assay conditions were as described in the text. The drugs were added to the reaction mixture before the addition of poly U and preincubated for 20 min; poly U was then added and incubation was continued for a further 30 min.

TABLE 2. *Effect of antibiotics which are known to inhibit protein synthesis on ^{14}C-phenylalanine incorporation*[a]

Antibiotic[b]	Percent inhibition
Erythromycin	40.0
Tetracycline	37.0
Chloramphenicol	34.0
Lincomycin	58.4
Amicetin	67.2
Sparsomycin	40.0
Spectinomycin	69.4
Puromycin	83.1

[a] Assay conditions were as described in Materials and Methods.

[b] Each present at a concentration of 10^{-5} M.

growth of certain spontaneous mutants was slow in the presence of the antibiotic as compared with growth in streptomycin-free medium. The maximal levels of streptomycin which the mutants could tolerate were determined (Table 4). The mutants were classified into two categories according to the level of resistance of the cells: low-level resistant mutants were those which were susceptible to the antibiotic at concentrations above 100 μg/ml, and high-level resistant mutants were those which could tolerate streptomycin at concentrations above 100 μg/ml. None of the mutants, however, was dependent on streptomycin for growth. These mutants are presumably single-step mutants, as they were not exposed to graded concentrations of the drug.

TABLE 3. *Action of antibiotics on the growth of M. tuberculosis H37Rv*[a]

Antibiotic	Growth (mg of dry wt) expressed as percentage of control	
	No SDS	+ SDS
Erythromycin	100.0	0.0
Chloramphenicol	100.0	0.0
Tetracycline	69.0	0.0
Amicetin	50.0	0.0
Lincomycin	69.0	0.0
No antibiotic	100.0	—

[a] A 12-day-old *M. tuberculosis* H37Rv culture was inoculated into 5.0 ml of medium containing 1 μg of various antibiotics/ml, with or without 0.01% (wt/vol) SDS, and incubated at 37 C (all in duplicate tubes). Growth was judged visually every week for 21 days at the end of which dry weights were determined.

TABLE 4. *Tolerance of streptomycin-resistant mutants to streptomycin*[a]

Strain	Mutagen	Isolated as resistant to streptomycin (μg/ml)	Maximal tolerance to streptomycin (μg/ml)
Sm^R Rv-1	Spontaneous	1	10
Sm^R Rv-2	Spontaneous	1	10
Sm^R Rv-5	Spontaneous	2	2,500
Sm^R Rv-1/1	Spontaneous (second-step mutant, derived from Sm^R-Rv-1)	100	2,000
Sm_{10}^R (N)-1	NTG	10	10,000
Sm_{10}^R (N)-3	NTG	10	1,000

[a] Various concentrations of streptomycin were added to 5.0 ml of medium in tubes and incubated for 3 weeks. The growth was judged visually as well as by determining the dry weight after 3 weeks.

Inhibition of protein synthesis in whole cells by streptomycin. The incorporation of ^{14}C-amino acids into proteins by whole cells of streptomycin-susceptible strains was inhibited almost completely by 100 μg of streptomycin/ml, whereas in a high-level resistant mutant very little or no change in protein synthesis was observed (Fig. 3). The effect of streptomycin on the incorporation of labeled amino acids into proteins by a low-level mutant was also studied and very little inhibition was observed even at a streptomycin concentration of 100 μg/ml (Fig. 4).

Effect of streptomycin on poly U-dependent incorporation of ^{14}C-phenylalanine. The incorporation of ^{14}C-phenylalanine, directed by poly U, was drastically inhibited by streptomycin at low concentrations in the streptomycin-susceptible strain. Figure 5 shows the extent of inhibition of ^{14}C-phenylalanine incorporation as a function of streptomycin concentration. Half-maximal inhibition was observed at 2.5×10^{-9} M streptomycin. At the 50% inhibition point, the number of molecules of streptomycin was 2.71×10^{12}, and the number of ribosomes present in the reaction mixture (calculated from the ribosomal RNA content) was 4.8×10^{12}. Hence, the number of molecules of streptomycin per ribosome was 0.56.

In different streptomycin-resistant mutants, the extent of inhibition of ^{14}C-phenylalanine incorporation varied according to the resistance of the whole cells to streptomycin. The incorporation was not at all inhibited by streptomycin when Sm_{10}^R (N)-1 was used. With two other mutants, the concentration of streptomycin required for half-maximal inhibition was 10,000-fold higher than with the parental type (Fig. 5). With one low-level mutant (Sm^R Rv-1), the amino acid-incorporating system exhibited sensitivity to various concentrations of streptomycin equal to that of the susceptible parent.

As can be seen from Table 5, there was a good correlation between the levels of resistance of the whole cells and in vitro resistance of the system.

Ribosomal basis of streptomycin susceptibility and high-level resistance. The aminoacylation of tRNA, with the use of the mycobacterial enzyme and labeled amino acid, was completely insensitive to the action of streptomycin. Therefore, the target of streptomycin action may presumably be the ribosome, as in other well-characterized systems. To test this,

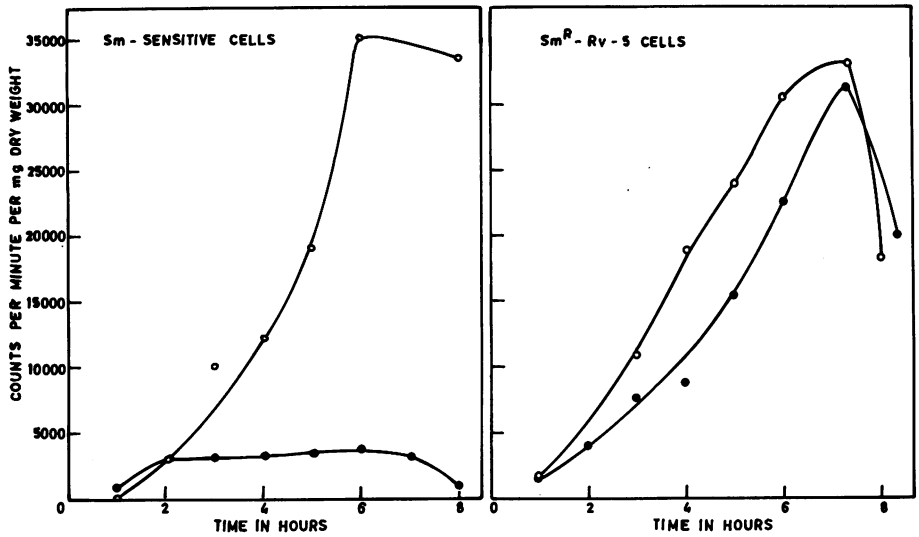

FIG. 3. *Effect of streptomycin on incorporation of ^{14}C-amino acids into proteins by whole cells of M. tuberculosis H37Rv and Sm^R-Rv-5 (streptomycin-resistant strain). Assay conditions as described in the text. Incorporation of ^{14}C-amino acids into proteins in the absence of streptomycin* (O) *and in the presence of 100 μg of streptomycin/ml* (●).

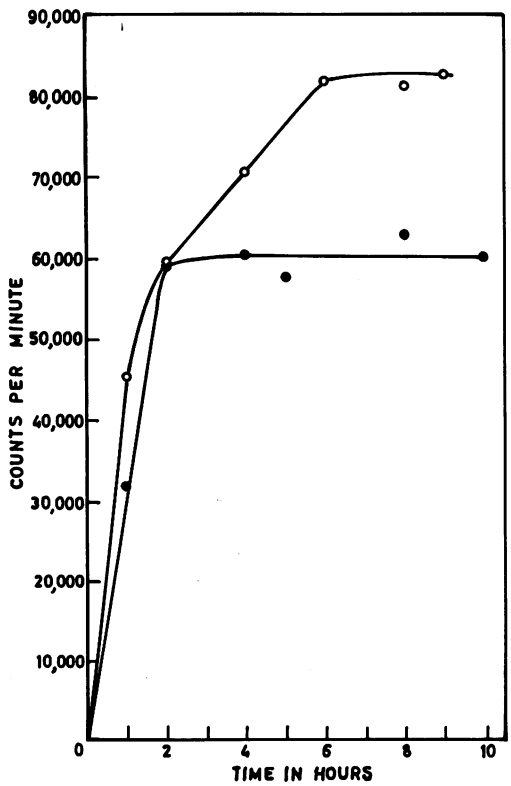

FIG. 4. *Incorporation of ^{14}C-amino acids into proteins by whole cells of Sm^R-Rv-1 (low-level streptomycin-resistant mutant) and the effect of streptomycin.* (O) *Without streptomycin;* (●) *100 μg of streptomycin/ml.*

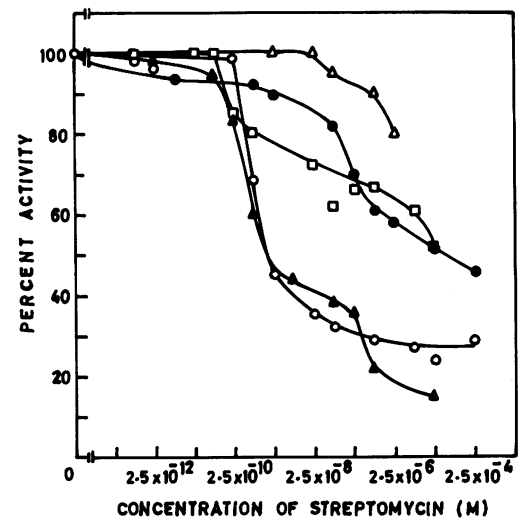

FIG. 5. *Effect of various concentrations of streptomycin on ^{14}C-phenylalanine incorporation in a poly U-dependent system from streptomycin-susceptible and streptomycin-resistant strains. Assay conditions as described in Materials and Methods* (O) *Streptomycin-susceptible strain;* (●) *Sm^R-Rv-5;* (△) *Sm$^R{}_{10}$(N)-1;* (□) *Sm^R-Rv-1/1;* (▲) *Sm^R-Rv-1.*

ribosomes from the susceptible and resistant strains were separated from the S-20 fraction, and a cross-mixing experiment was done to demonstrate that the ribosome is the target of streptomycin action. As can be seen in Table 6, the inhibition caused by streptomycin was dependent on the source of the ribosome whereas

the supernatant fluid had no effect.

Effect of streptomycin on the growth of low-level and high-level resistant mutants in the presence of SDS. The effect of streptomycin on the growth of Sm_{10}^R (N)-1 and Sm^R Rv-1 mutants in the presence of a membrane-active agent, SDS, which is known to alter the permeability in other bacteria (1) as well as in an INH-resistant mutant of *M. tuberculosis* H37Rv (17) was tested. Growth was judged visually for 21 days in the presence of 0.01% SDS, and later the dry weights were determined. In Sm_{10}^R (N)-1 (high-level mutant), streptomycin did not inhibit the growth of the cells at concentrations up to 6,000 μg/ml even after the permeability had been altered with SDS, whereas in Sm^R Rv-1 (low-level mutant) streptomycin completely inhibited the growth at 1 μg/ml in the presence of SDS.

TABLE 5. *Resistance of the in vitro amino acid-incorporating system from various streptomycin-resistant strains to inhibition by streptomycin and comparison with their in vivo resistance*[a]

Strain	Streptomycin concn for half-maximal inhibition (moles/liter)	Resistance (μg of streptomycin/ml)	
		In vitro amino acid-incorporating system	Whole cells
Sm^S	2.25×10^{-9}	0.0013	0
Sm^R Rv-5	5.00×10^{-5}	24.00	2,500
Sm_{10}^R (N)-1	No inhibition	Highly resistant	10,000
Sm^R Rv-1	2.25×10^{-9}	0.0013	10
Sm^R Rv-1/1 (second step mutant derived from Sm^R-Rv-1)	2.50×10^{-5}	11.70	2,000

[a] Assay procedure was as described in Materials and Methods.

DISCUSSION

A systematic description of the amino acid-incorporating system from mycobacteria was given for the first time by Rieber and Imaeda (14). Our system shows marked similarities to Reiber and Imaeda's system from *M. smegmatis* ATCC 607 (i) in its requirement for high Mg^{2+} for optimal incorporation (15 mM), (ii) in its requirement for a high poly U concentration (100 μg per assay), and (iii) in that both possess high nuclease activity.

RNA isolated from phage-infected *M. smegmatis* was able to stimulate the incorporation of ^{14}C-amino acids into protein, though the capacity to stimulate was low as compared with T4 RNA in *E. coli* (15). Hence, RNA from phage-infected cells can be used to program the in vitro synthesis of proteins in *M. tuberculosis* H37Rv, although the amount of RNA required is very high. The messenger RNA fraction in this RNA preparation may be low, and, therefore, high concentrations are required to saturate the amino acid-incorporating system.

The poly U-directed system was used to test the inhibitory activities of several antitubercular drugs which include mostly first and second

TABLE 6. *^{14}C-phenylalanine incorporation with ribosomes from susceptible and resistant strains of M. tuberculosis*[a]

Susceptible		Resistant		Streptomycin concn (M)	^{14}C-phenylalanine incorporation (pmol/0.1 ml)	Percent inhibition
Supernatant	Ribosome	Supernatant	Ribosome			
+	+	−	−	0	16.32	0
+	+	−	−	10^{-9}	7.40	54.78
+	+	−	−	10^{-7}	3.93	75.93
−	−	+	+	0	12.98	0
−	−	+	+	10^{-9}	13.82	0
−	+	+	−	0	15.37	0
−	+	+	−	10^{-9}	6.90	57.71
−	+	+	−	10^{-7}	5.25	66.82
+	−	−	+	0	13.82	0
+	−	−	+	10^{-9}	14.17	0
+	−	−	+	10^{-7}	14.78	0

[a] The incubation system was as described in Materials and Methods, with 50 μg of S-100 protein and 20 μg of ribosomal protein. S-100 and ribosomes were mixed separately and added to the reaction mixture. The resistant strain used was a high-level resistant mutant, Sm_{10}^R (N)-1.

line drugs and other inhibitors of protein synthesis. Among the drugs, streptomycin, kanamycin, rifampin, and capreomycin were highly effective at low concentrations. Maximal inhibition obtained with streptomycin was 80 to 85%, which is similar to the inhibition obtained in the *E. coli* system when poly U was used as messenger (6).

The inhibition of amino acid incorporation by rifampin indicates that in *M. tuberculosis* H37Rv the antibiotic may also act on the ribosome in addition to its action on RNA polymerase, as suggested for *Bacillus subtilis* (2). Similar results were obtained by Trnka and Smith (21) in a poly U-directed phenylalanine-incorporating system from mycobacteria. Experiments with rifampin in a poly U-dependent incorporating assay indicate that the drug may act on the translation process by interacting with the ribosomes, since an excess of poly U (molar concentration of poly U exceeding that of rifampin) partially reverses the inhibition of polyphenylalanine synthesis (M. S. Shaila, K. P. Gopinathan, and T. Ramakrishnan, Symposium on control mechanisms in cellular processes, Bhabha Atomic Research Centre, Bombay, India, 1-3 February 1973, in press). More experiments are needed to understand the primary site of rifampin action in mycobacteria.

Chloramphenicol and tetracycline at 10 μg/ml did not inhibit the growth of *M. tuberculosis* H37Rv, but they did inhibit in vitro protein synthesis, indicating that the cells may be impermeable to them. Altering the permeability barrier by using SDS enabled these antibiotics to enter the cells, leading to bactericidal action, presumably by blocking protein synthesis.

The incorporation of ^{14}C-amino acids into proteins by whole cells of streptomycin-susceptible *M. tuberculosis* H37Rv was almost completely inhibited by streptomycin, whereas in the resistant strains—both high-level and low-level—the incorporation was unaffected. This suggests that the mechanism of action of streptomycin in *M. tuberculosis* may be the same as in other systems.

In the susceptible strain, a very low concentration of streptomycin (2.5×10^{-9} M) was needed for 50% inhibition of in vitro protein synthesis, which is 200 times less than the amount required for 50 % inhibition of *E. coli* in vitro protein synthesis (6). This suggests that the number of ribosomes present in the reaction mixture may be less than those present in *E. coli* system. This view is supported by the observation that the number of ribosomes per cell is 2,700 (unpublished data) as compared with the value of 10,000 in *E. coli* (6). Further, the number of active ribosomes capable of participating in protein synthesis may be few, since this cell-free preparation was less active than that reported by Speyer et al. (16) when the polyphenylsynthesizing ability was compared. At 2.5×10^{-9} M, the ratio of streptomycin per ribosome is 0.56. Thus, for blocking the protein synthesis completely, one molecule of streptomycin per ribosome is needed.

In the high-level resistant strains, 10,000-fold more streptomycin is needed to cause half-maximal inhibition, indicating that these systems are resistant to the action of the drug. There is a good correlation between the levels of resistance of the cells and the in vitro resistance of the incorporating system. These results are similar to those obtained in pneumococci (18). Further, the results in Table 5 show that susceptibility and high-level resistance reside in the ribosome and not in the supernatant fluid, thus resembling the *E. coli* (7) and other systems.

From the results presented in this paper, it is clear that a low-level mutant shows the same in vitro susceptibility as the wild type, whereas the whole cells are not susceptible to the action of streptomycin. This suggests that, in this mutant, the site may be located elsewhere than on the ribosome, presumably on the membrane. To support this hypothesis, growth inhibition studies in the presence of a membrane-active agent, SDS, were carried out. Altering the permeability allows streptomycin to enter the low-level resistant cells, and once inside the cell it proves to be lethal, for the ribosomes are sensitive. In contrast, in the high-level resistant mutant, even though more streptomycin enters as a result of the action of SDS, the protein synthesis and hence the growth of the organism are not inhibited, since the ribosomes are resistant to the antibiotic. Thus, we conclude that in low-level resistant mutants the origin of resistance is on the membrane, whereas streptomycin susceptibility and high-level resistance reside on the ribosome.

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