MECHANISM OF STREPTOMYCIN ACTION IN *MYCOBACTERIUM TUBERCULOSIS* H37Rv*

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ABSTRACT

70S ribosomes isolated from a streptomycin (Sm) sensitive strain of M. tuberculosis *H37Rv bind labelled streptomycin rapidly. This binding depends on the drug concentration. At low concentration, there is one binding site on the ribosome, which increases to two as the concentration is increased. Sm causes a small amount of miscoding in a cell-free, poly U-directed system from* M. tuberculosis *H37Rv containing Sm-sensitive ribosomes. There are no differences in the uptake of* ^{3}*H-dihydrostreptomycin (DH Sm) by whole cells of Sm-sensitive and resistant strains; the uptake decreases after* 1 *hour and again increases upto about* 8 *hours. Sm causes the slow release of fMet-tRNA$_f$ from a preformed initiation complex,* in vitro.

Key words: Streptomycin action; Mycobacteria; *Mycobacterium tuberculosis* H37Rv.

1. INTRODUCTION

In a previous paper [1], we have described an efficient *in vitro* amino acid incorporating system from *Mycobacterium tuberculosis* H37Rv, and demonstrated that streptomycin (Sm) sensitivity and high-level resistance reside on the ribosome in a manner similar to that in *E. coli* and other systems. In spite of the extensive work on the mechanism of Sm action on *E. coli*, the killing action of the drug is still debated (for reviews *see* ref. 2, 3, 4); very much less is known in the *M. tuberculosis* system which is highly sensitive to this antibiotic (for review, *see* ref. 5). Two opposing conclusions have been drawn as a result of several studies on mechanism of inhibition of protein synthesis by Sm in *E. coli*: (i) Sm inhibits polypeptide chain initiation, and Sm-killed cells accumulate 70 S ribosomes blocked as aberrant

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initiation complexes [6, 7, 8], (ii) Sm inhibits polypeptide chain elongation *in vitro*; it acts immediately after the formation of initiation complexes [9–12]. *In vivo* also Sm blocks chain elongation, but this block differs from that on initiating systems in that it is reversible and incomplete [13]. In addition, it has also been shown by Wallace and Davis [14] that Sm causes a cycle of ribosomal initiation, blockage of chain extension, gradual release and reinitiation; the dominance of Sm sensitivity over resistance in Sm^s/Sm^r heterozygotes has been attributed to the resulting cyclic blockade.

In the present communication, we report studies on the action of Sm on the polypeptide initiation complex in *M. tuberculosis* system; the results indicate that *in vitro* Sm causes breakdown of preformed initiation complexes. In addition, we have carried out experiments designed to study the differences in the binding of labelled DH Sm to isolated ribosomes from Sm-sensitive and -resistant cells, to study the uptake of labelled drug by these strains and, thereby, to distinguish the nature of Sm-resistance from Sm-sensitivity. The capacity of Sm to cause misreading in an *in vitro* protein synthesizing system from Sm-sensitive *M. tuberculosis* has also been examined.

2. Materials and Methods

Chemicals.—GTP, and salmon sperm DNA were from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; MS2 viral RNA and trinucleotide codon AUG were from Miles Laboratories, Inc., Kankakee, Illinois, U.S.A.; Met-$tRNA_F$ was a generous gift from Dr. G. D. Novelli, Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A.; ^{14}C-methyl-methionine (59 mc/mmole), ^{3}H–dihydrosteptomycin sesquisulphate (3 C/mmole), ^{14}C-leucine (U) (338 mc/mmole), ^{14}C–L–isoleucine (U) (338 mc/mmole), ^{14}C–L–serine (U) (160 mc/mmole) and ^{14}C–tyrosine (U) (405 mc/mmole) were obtained from the Radiochemical Centre, Amersham, England. ^{14}C-streptomycin (Sm) sulphate-$CaCl_2$ complex (1·1 μc/20 mg) was a gift from M/s. Merck, Sharp & Dohme Ltd., Rahway, New Jersey, U.S.A.; Dihydrostreptomycin (DH Sm) sulphate was from Calbiochem Ag., Lucerne, Switzerland; ^{14}C-chlorella protein hydrolysate (13 mCi/m atom) was from Bhabha Atomic Research Centre, Bombay, India.

Organism.—Mycobacterium tuberculosis var. *hominis* H37Rv (strain No. 7416) was obtained from the National Collection of Type Cultures (England) and maintained by regular subculture on Petrik's solid medium [15]. The organism was grown on the synthetic liquid medium of Youmans and Karlson [16]. *Mycobacterium smegmatis* SN_2 was obtained from Dr. R. Bónicke, Institute for Experimental Biology and Medicine, Borstel, Germany. It

was routinely maintained on agar medium, which is yeast extract-tryptone broth]17] solidified with 2% agar. For experimental purposes, the organism was grown on synthetic medium [16], in a laboratory fermentor.

The mycobacteriophage I3, which is a transducing phage was isolated in this laboratory [18, 19]. The isolation of various Sm-resistant mutants of *M. tuberculosis*, and their classification into low-level and high-level resistant mutants according to their *in vivo* and *in vitro* levels of resistance have been described in the previous paper [1]. High-level resistance is due to altered ribosomes, while low-level resistance is because of altered permeability to the drug, as shown by the growth inhibitory effect of Sm on these mutants under certain conditions [1].

Preparation of cell-free extracts.—The isolation of S-100 and washed ribosome preparations from *E. coli* and *M. tuberculosis* is similar to that described earlier [1]. The S–100 from *E. coli* was used as a source of methionyl-tRNA synthetase and transformylase in the preparations of Met-$tRNA_F$.

Uptake of labelled Sm by whole cells.—M. tuberculosis H37Rv was grown as submerged culture at 37° in Youmans and Karlson medium [16] containing 0·02% (v/v) Tween–80. To 10 ml batches of rapidly growing cells (6–7 day old), labelled Sm (^{14}C–^{S}m or ^{3}H–DH Sm) was added and at specified intervals, the bacilli in 1·0 ml samples (2–3 mg dry weight) were collected on Millipore filters (0·45 μ). The membrane filters were washed twice with 2·0 ml of ice-cold Youmans and Karlson medium, placed in scintillation vials and dried. To each vial, 10 ml of a toluene-based scintillation fluid was added and the radioactivity was measured in a Beckman LS–100 liquid scintillation spectrometer.

RNA from phage I3–infected *M. smegmatis* cells was prepared as described earlier [1].

Preparation of formylated ^{14}C–Met–$tRNA_F$.—Formylated ^{14}C–Met–RNA was prepared according to the method of Hershey *et al.* [20]. Binding of formylated ^{14}C–Met–$tRNA_F$ to ribosomes was done according to Lelong *et al.* [9].

^{3}H–DH Sm binding to ribosomes.—The ability of 70 S ribosomes to bind ^{3}H–DH Sm was measured by the Millipore filtration technique as described by Lelong *et al.* [21].

The assay of poly U-dependent incorporation of ^{14}C-phenylalanine into polyphenylalanine is similar to that described earlier [1]. For misreading experiments, the assay systems contained ^{14}C–labelled leucine, tyro-

sine, isoleucine or serine or a ^{14}C–amino acid mixture instead of ^{14}C–phenyl alanine; 10 μg/tube of denatured salmon sperm DNA was also included in the assay mixture, as suggested by Likover and Kurland [22].

3. RESULTS

Misreading by Sm in an Sm-sensitive extract.—The capacity of Sm to cause coding ambiguity in a mycobacterial system was studied using a poly-U-directed amino acid incorporating system; the ability of the drug to increase the incorporation of ^{14}C-amino acid mixtures was measured (measure of total misreading). The results are shown in Table I. It is evident from Table I that in sensitive extracts, Sm at 5×10^{-6} M concentration, is able to induce the incorporation of amino acids not specifically coded by poly U, *i.e.*, there is a stimulation of incorporation of amino acids other than phenylalanine. However, this experiment does not give any idea as

TABLE I

Misreading of poly U by Sm-sensitive S-20 extract in the presence of Sm[a]

Concentration of Sm (M)	Incorporation of ^{14}C-amino acids (cpm)	Net stimulation	Stimulation corrected for ^{14}C-Phe inhibition[b]
..	1540	..	..
1×10^{-7}	1179	..	871
5×10^{-7}	1159	..	851
1×10^{-6}	1266	..	958
5×10^{-6}	2154	610	1856

[a] The assay system for the poly U dependent polyphenylalanine synthesizing system was the same as described by us (Ref. 1) except that in place of ^{14}C-phenylalanine, ^{14}C-chlorella protein hydrolysate (25,000 cpm) was used as the source of ^{14}C-amino acids; the assay mixture without poly U was preincubated with the drug for 10 minutes. The incubations were carried out for 20 minutes at 37°.

[b] Inhibition of ^{14}C–Phe incorporation was taken as 80% at concentrations of 10^{-7} M and above of Sm. (This is taken from several, separate experiments, not shown here and Ref. 1). Therefore the maximum incorporation, which may still be due to Phe will be 308, in presence of Sm concentrations 10^{-7} M and above. This value is subtracted from the total incorporation (given in column 2) to calculate the stimulation.

to which are the amino acids stimulated and also whether there is simultaneous inhibition of phenylalanine incorporation. When the amino acids which are normally incorporated due to miscoding in the presence of Sm in *E. coli* were used individually (^{14}C-Leu, -Ile, -Tyr, -Ser), there was no incorporation; however, when the experiment was carried out with a mixture of ^{14}C-isoleucine and serine, Sm did cause coding ambiguity (Table II). The simulation of incorporation of ^{14}C-Ile and -Ser is low when compared to the *E. coli* system even in the presence of denatured DNA. The addition of denatured DNA has been shown to enhance the miscoding effect caused by Sm leading to increased incorporation of these amino acids [22]. Sm at 10^{-5} M concentration, increases the ambiguity ratio from 0·28% to 4·87% (the ambiguity ratio being defined as the ratio of total amount of Ile and Ser incorporated to the amount of the Phe incorporated). In the *E. coli* system [22], Sm at the same concentration, increases the ambiguity ratio from 0·3 to 21%. In the absence of denatured DNA even the limited miscoding with Ser + Ile was greatly reduced (incorporation was only about 15% of that observed in presence of DNA).

TABLE II

Sm-induced ambiguity in Sm-sensitive S-20 extract[a]

Presence of Sm (10^{-5} M)	Amount of *aa* incorporated (p-mole)		Ambiguity ratio[b]
	Ile+Ser	Phe	
—	0·10	36·68	0·28
+	1·28	26·22	4·87

[a] The experimental procedures were similar to those described under Table I. A mixture of ^{14}C-Ile (338 mc/mmole; 0·05 μc/tube) and ^{14}C-Serine (160 mc/mmole; 0·05 μc/tube) was added and their incorporation into polypeptide was compared with that of ^{14}C-Phe. Cold Phe was added in the same amount as all other non-radioactive amino acids (10^{-4} M). In addition, heat-denatured salmon sperm DNA (10 μg/assay) was added to stimulate the Sm-induced misreading, as suggested by Likover and Kurland (22).

[b] Ambiguity ratio $= \dfrac{\text{(Total amount of Ile + Ser incorporated)}}{\text{Amount of Phe incorporated}} \times 100$

Specific activity of Ile was used to calculate values for the numerator.

Uptake of ^{3}H–DH Sm by whole cells of M. tuberculosis.—The uptake of ^{3}H-DH Sm by whole cells of Sm-sensitive and various Sm-resistant strains

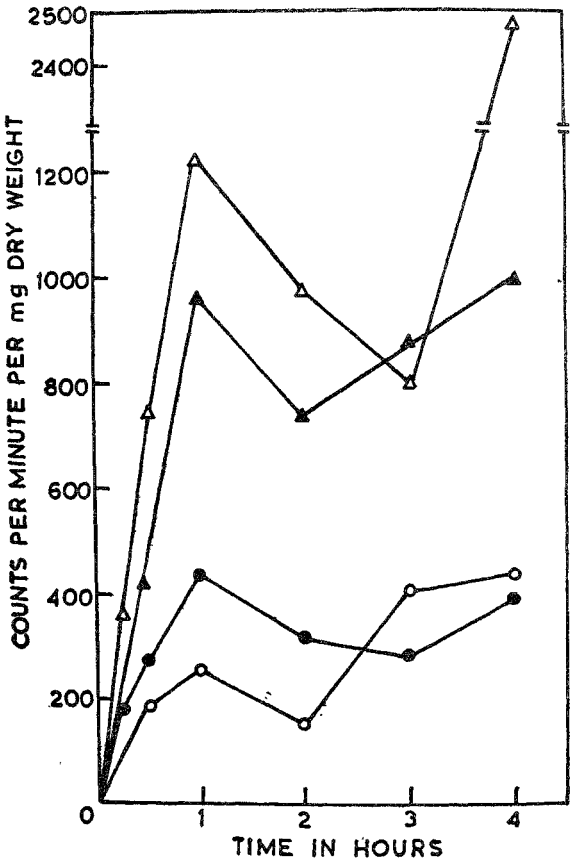

FIG. 1. Uptake of ^{3}H–DH Sm by Sm-sensitive and Sm-resistant *M. tuberculosis* cells: Uptake was carried out as described in methods; the concentration of ^{3}H–DH Sm was 0·243 μg/ml. ● Sm^s; ○ Sm^R Rv–5 (high level); ▲ Sm^R Rv–1 (low level); △ Sm^R_{10} (N)–1 (high level)

is shown in Figure 1. Irrespective of the Sm-phenotype, the uptake of ^{3}H–DH Sm by whole cells of *M. tuberculosis* follow a single pattern, *i.e.*, an initial increase at least upto one hour, a decrease, and a secondary increase. In the case of Sm-sensitive strain and one high-level Sm-resistant mutant, the secondary increase starts after 2 hours, whereas in the case of other mutants, the secondary increase begins after 3 hours. The uptake by two mutants (one high-level and one low-level resistant) was higher than that of the sensitive parent at any given time. The uptake of ^{14}C-Sm also followed more or less the same trend (not shown); however, the concentration of ^{14}C-Sm used was very high (400 μg/ml) since the specific radioactivity of the compound was extremely low.

The uptake of ^{3}H–DH Sm by Sm-sensitive cells depends on the external concentration of DH–Sm (Figure 2 *a*). The Lineweaver-Burk plot for Sm uptake is shown in Figure 2 *b*. The K_m for uptake is $5{\cdot}8 \times 10^{-7}$ M.

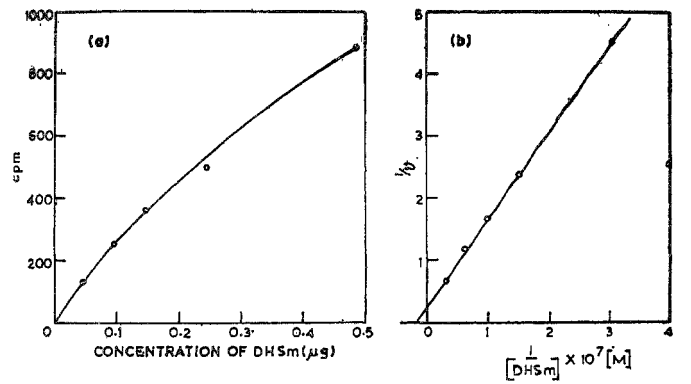

FIG. 2. Uptake of ^{3}H–DH Sm as a function of DH Sm concentration: Sm-sensitive *M tuberculosis* cells (2–3 mg dry wt.) in 1·0 ml, were treated with different concentrations of ^{3}HDH Sm, incubated for 1 hour at 37° C and processed as described in ' Methods'.

Binding of ^{3}H–DH Sm by isolated ribosomes.—The kinetics of binding of ^{3}H–DH Sm to isolated 70S ribosomes from Sm-sensitive strain is given in Figure 3. There is rapid initial binding of the drug by ribosomes which reaches a maximum by 10 minutes.

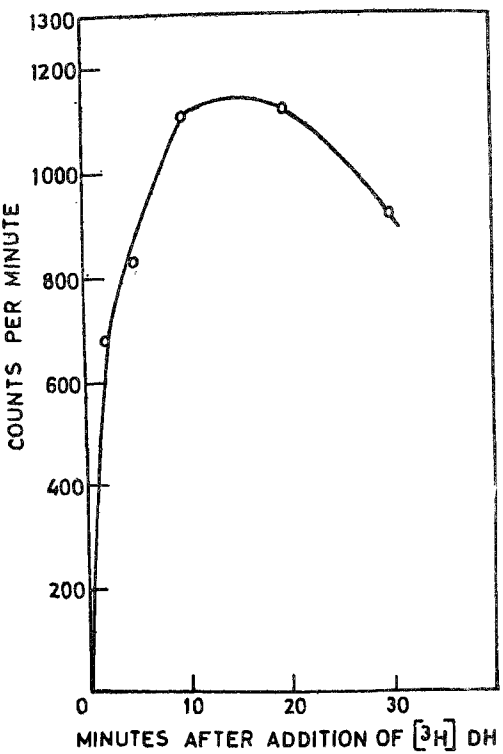

FIG. 3. Kinetics of binding of ^{3}H–DH Sm to isolated ribosomes: The ribosomes were prepared from Sm-sensitive strain. Assay mixtures (50 μl) contained: 20 mM Tris–HCl, pH 7·8 magnesium acetate, 5 mM; KCl, 60 mM; 2-mercaptoethanol, 7 mM; $7{\cdot}7 \times 10^{-7}$ M 70S ribosomes (1·7 A_{260} units), and $6{\cdot}6 \times 10^{-7}$ M ^{3}H–DH Sm ($2{\cdot}9 \times 10^{7}$ cpm). The reaction mixtures were incubated at 25° C for the times indicated, diluted with 4·0 ml cold buffer (same as that used for the assay) and immediately filtered through the Millipore membranes. The filters were washed four times with 4·0 ml ice cold buffer, dried and the amount of ^{3}H–DH Sm retained was counted.

The concentration-dependence of ^{3}H–DH Sm for its binding is given in Figure 4 for ribosomes from a Sm-sensitive strain. As shown in the figure, upto a concentration range of 0·5 μg, the binding increased rapidly and above this concentration the extent of binding decreased. Additional molecules of the antibiotic were bound to the ribosomes at concentration above 0·7 μg but these were far less than the expected one molecule per

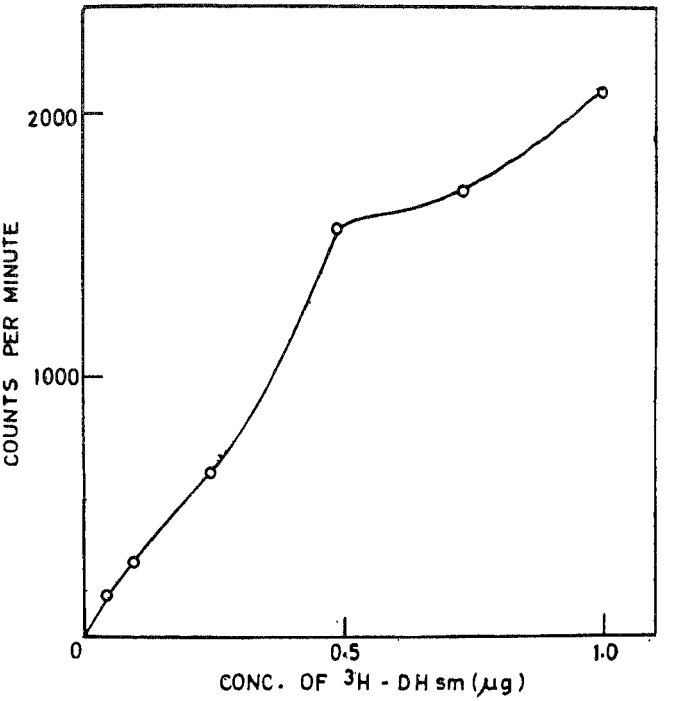

FIG 4. Concentration dependence of ^{3}H–DH Sm for binding to 70S ribosomes: Ribosomes were from Sm-sensitive strain. Standard assay conditions as described under Fig. 3 were used, except that the ^{3}H-DH Sm concentrations were varied. Incubations were carried out for 10 minutes at 25° C. Every point is an average of triplicate determinations, and each point did not show more than 5% deviation from the mean.

ribosome. This concentration-dependence of binding is very close to that given for *E. coli* ribosomes by Chang and Flaks [23]. However, there is a notable difference between the two; the maximum number of Sm molecules bound per ribosomes does not approach 1·0 as reported for *E. coli*. The above data were analysed by using the empirical Hill equation, as described by Monod *et al.* [24]. At lower drug concentration, the '*n*' value (Hill coefficient) was 1·0 and reached 2·4 at higher concentrations. This appears to indicate that there is one binding site at lower concentration which may increase at higher drug concentrations.

The binding of *^{3}H–DH* Sm by ribosomes isolated from various Sm-resistant strains was studied and the results are given in Table III. The ribosomes from high level Sm-resistant mutants exhibit very limited capacity to bind the drug (7–20% of the binding obtained with Sm-sensitive ribosomes), in contrast to the binding of low level Sm-resistant ribosomes (83%).

Binding of ^{14}C-fMet-tRNA to ribosomes and the effect of Sm.—The effect of Sm on the formation of the initiation complex, *i.e.*, on the binding of ^{14}C-fMet-tRNA$_F$ to isolated ribosomes in the presence of initiator codon or natural mRNA and the initiation factors was studied and the results are

TABLE III

Binding of ^{3}H–DH S_m to isolated ribosomes from various strains[a]

Ribosome source	cpm	Amount of DH Sm bound (mg)	Percentage binding[b]
Sm^s ..	625	1·05	100·0
Sm^R–Rv–1 ..	519	0·87	83·0
Sm^R–Rv–5 ..	131	0·22	21·0
Sm^R_{10} (N)–1 ..	50	0·08	7·6

[a] The assay mixtures (50 μl) contained 1·5 A_{260} units of 70S ribosomes ($7{\cdot}7 \times 10^{-7}$ M) prepared from various strains, $6{\cdot}6 \times 10^{-6}$ M ^{3}H–DH Sm ($2{\cdot}9 \times 10^5$ cpm) and assay buffer (*see* under Fig. 3). The reaction mixtures were incubated at 25° for 10 minutes, diluted with ice-cold assay buffer and processed as described in methods. Sm^R–Rv–1 is a low level Sm resistant strain (resistant to 10 μg/ml. of Sm). Sm^R–Rv–5 and Sm^R_{10}(N)–1 are high level resistant strains, resistant to 2,500 μg/ml and 10,000 μg/ml of Sm, respectively.

[b] The binding by Sm sensitive ribosomes was taken as 100%.

given in Table IV. Thes table clearly demonstrates that regardless of the messenger added to the system, inhibition of fMet-tRNA$_F$ binding to ribosomes is observable. This phenomenon is specific for Sm-sensitive ribosomes.

In order to test the possibility that the drug is inhibiting the binding of initiator tRNA by releasing it from the initiation complex, the effect of Sm on the stability of initiation complex was studied (Figure 5). It can be seen from this figure that the addition of Sm to preformed initiation complex causes a gradual reduction in the amount of bound initiator tRNA. Thus,

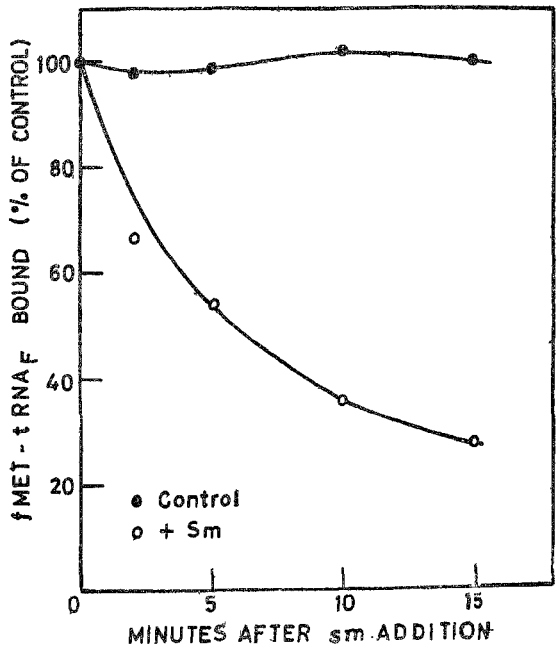

FIG. 5. Sm-induced release of fMet-tRNA$_F$ from initiator complex: The formation of the initiation complex with I3 RNA, fMet-tRNA$_F$ and ribosomes was carried out as given under Table IV on a larger but proportionate scale. After 15 minutes of incubation at 37°, Sm ($5{\cdot}48 \times 10^{-6}$ M) was added to the complex and at various times, 0·05 ml samples were assayed for ribosome-bound radioactivity.

it is clear that this slow release is reflected in the drug's effect on initiation complex formation.

TABLE IV

Inhibition of ^{14}C-fMet-tRNA$_F$ binding to ribosomes by Sm[a]

Experiment	Messenger	Ribosomes	^{14}C–fMet–tRNA bound (p-moles/A_{260} unit)	
			Control	Sm
1	AUG	Sensitive	0·470	0 (100)[b]
2	MS2 RNA	do.	0·386	0·210 (45)
3	I3 RNA[c]	do.	0·762	0 (100)
4	AUG	Resistant	1·080	0·772 (28·5)
5	I3 RNA	do.	0·700	0·600 (14·28)

[a] The reaction mixture, in a total volume of 50 μl, contained the following: Tris–HCl, pH 7·5, 50 mM; NH_4Cl, 80 mM; magnesium acetate, 5 mM (for AUG) or 8 mM (for natural mRNA); 2-mercaptoethanol, 7 mM; GTP, 1 mM; washed ribosomes, 2·0 A_{260} Units, ^{14}C-fMet-tRNA$_F$, 50 pmoles (5540 cpm); AUG, 0·15 A_{260} Units or I3 RNA, 1·3 A260 Units or MS2 RNA, 1·0 A_{260} Units; concentration of Sm, when present, $2·74 \times 10^{-6}$ M; incubation was carried out for 15 minutes at 25° (AUG) or 37° (I3 RNA or MS2 RNA) ^{14}C–fMet–tRNA$_F$ bound to ribosomes was measured after filtration through Millipore membranes. Each sample was counted for 100 min, and the background counts without messengers were subtracted. The Sm-resistant ribosomes were prepared from the high-level Sm-resistant mutant Sm^R_{10} $(N)-1$.

[b] Values in the parentheses indicate percent inhibition.

[c] I3 RNA denotes RNA isolated from phage I3-infected *M. smegmatis*.

4. DISCUSSION

In *Mycobacterium tuberculosis* H37Rv, Sm causes misreading to a small extent. Sm stimulates the incorporation of a mixture of Ile and Ser in the poly U-directed system by Sm-sensitive ribosomes. The misreading, as judged by the ambiguity ratio in *M. tuberculosis* system, is small when compared to *E. coli* results [22]. However, these results are in contrast to the report that there is no misreading on 70S ribosomes from *M. smegmatis* [25]. The intrinsic ambiguity associated with the sensitive ribosomes of *M. tuberculosis* is also low (Table II).

There are two reports [26, 27] concerning the uptake of Sm by mycobacteria. Linz [27] working with non-labelled Sm, reported that the primary phase of uptake of Sm in *M. tuberculosis* extends up to 2 days which is reversible, (similar to the uptake by other bacteria) followed by a lag of two days and becomes irreversible after 4 days. Beggs and Williams [26], however, working with *M. tuberculosis* H37Rv, found that the uptake of ^{14}C–Sm follows two phases similar to *E. coli* [28]. The results presented here show that uptake of labelled drug by *M. tuberculosis* H37Rv does not precisely follow the above pattern. If the uptake after 2 hours is considered as the secondary phase, then this should not occur in the case of resistant mutants, particularly in the permeability mutant Sm^{A}Rv–1 [1]. Contrary to expectations, the uptake by the above mutant and other mutants follow the same pattern. The reasons for this anomaly remain to be explained. Mere uptake of drug by cells might not give a true picture about the nature of resistance of the organism to Sm, as even in *E. coli*, there is little difference in the uptake between sensitive and resistant strains (J. Davies, personal communications).

The binding of ^{3}H–DH Sm by isolated ribosomes shows differences depending on the ribosome source—with high-level Sm-resistant ribosomes, the binding is greatly reduced; the ribosomes from a low-level Sm-resistant strain which is believed to be a permeability mutant [1] bind as much as 83% of labelled drug bound by sensitive ribosomes. Hence, this is additional evidence in favour of the hypothesis that one form of low-level resistance in *M. tuberculosis* in non-ribosomal.

With the sensitive ribosomes, the binding of ^{3}H–DH Sm is dependent on the concentration of DH Sm, and this exhibited two phases, similar to that reported by Chang and Flaks [23]. Since the plot of concentration of ^{3}H–DH Sm *versus* binding was different from the normal linear relationship, the kinetic data were analysed using Hill plots. Upto a concentration of 10^{-6} M, the 'n' value was more than 1·0 indicating that the ribosomes have the potentiality for more binding sites of similar affinity. However, this binding of more than one Sm molecule might represent nonspecific binding which may be associated with aggregation phenomena as has been suggested for *E. coli* [23]. Hence, similar to *E. coli* system, there is only one distinctive site for the tight binding of Sm in the ribosomes from Sm-sensitive strain which is related to the inhibition of protein synthesis. Although Kaji and Tanaka [29] have reported that in *E. coli* there is tight binding of two molecules of Sm per ribosome, they have used higher concentrations (more

than 5×10^{-5} M); hence, binding of two molecules of Sm to ribosomes might represent a non-specific binding resulting from aggregation.

When the effect of Sm on a specific stage in protein synthesis was examined, results identical to that reported for *E. coli* [9, 12] were obtained. The results presented in Table IV and Figure 5 demonstrate that in *M. tuberculosis* H37Rv *in vitro* system, Sm inhibits the formation of initiation complex and this inhibitory effect observed may be due to the release of fMet-$tRNA_F$ from the initiation complex. Hence, it is possible that Sm inhibits chain elongation (the formation of the first peptide bond in this case) as hypothesized by Modolell and Davis [12], rather than the formation of the initiation complex itself.

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6. ABBREVIATIONS

Sm., Streptomycin, DH Sm., Dihydrostreptomycin; AUG, the trinucleotide Ap Up Gp; Fmet-$tRNA_F$: Formylmethionyl tRNA.

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