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The Effects of Streptomycin or Dihydrostreptomycin Binding to 16S RNA or to 30S Ribosomal Subunits

(misreading and drug binding/30S subunit reconstitution and drug binding)

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ABSTRACT **Evidence is presented suggesting that streptomycin binds to 16S RNA or to 30S ribosomal subunits at the same topographical site located on the RNA chain. The equally bactericidal dihydrostreptomycin binds to the same site as streptomycin but with lower affinity. The effect of drug binding to 16S RNA (measured by reconstitution inhibition) is readily reversible, while that of drug binding to 30S subunits (measured by misreading) persists after removal of the drug. Binding of the drug is not a necessary and sufficient reason for killing.**

The earlier publication (1) that the site of streptomycin (Sm) binding to the 30S ribosomal subunit could be the 16S RNA may be criticized from several viewpoints. On the one hand, according to stoichiometry established for dihydrostreptomycin (H_2Sm)–30S binding (2, 3), too much Sm was bound; on the other hand, in view of the potential of nucleic acid for binding the drug, too little Sm was bound. Furthermore, it is unexpected that Sm should have any specificity at all (for example, stable binding to 16S and no binding to 23S RNA).

Experiments to answer these criticisms were designed with every attempt being made to relate *binding* and *physiology*. We report here the results of these experiments, which (*1*) confirm the role of 16S RNA as drug binding site; (*2*) demonstrate that Sm and H_2Sm are quantitatively different with regard to binding (nevertheless they remain indistinguishable with respect to bactericidal action); (*3*) confirm that misreading is a hysteretic effect of either Sm or H_2Sm and does not require the physical presence of the drug; and (*4*) show that binding of the drug is not a necessary and sufficient reason for killing.

MATERIALS AND METHODS

Bacterial Strains. Four *Escherichia coli* B strains were used. Two carry the wild-type (Sm sensitive) *strA*$^+$ allele; they are B wild type and L1 (*argF40 argR11*) selected as an arginine derepressed mutant from B40, an *argF* amber mutant of strain B (4). The other two are Sm resistant derivatives of L1, differing only by the *strA* allele which has been introduced by P1 transduction. *strA40* (carried by L1-431) is a moderately restrictive allele that responds to phenotypic suppression; *strA1* (carried by L1-401) is a very restrictive allele, irresponsive to phenotypic suppression (5). Four *Escherichia coli* K12 strains were used. One, X7029, obtained from J. Beckwith, is an F$^-$ carrying *lac* deletion X74. Two, A19 and Q13, are derivatives of Hfr AB301 carrying a defective RNase I (6). Finally, A19-3 is a spontaneous Sm resistant mutant of A19.

Growth Conditions. Cultures were grown in medium L (7) [Bacto-tryptone 1%, yeast extract 0.5%, sodium chloride 0.5%, glucose 0.2%] at 37° with vigorous aeration. Cells were harvested near the end of the log phase (10^9 cells per ml), washed, and frozen. For the preparation of radioactive 16S RNA, 1 mCi of [^{3}H]uridine was added to 16 liters of medium L.

Preparation of Ribosomal Components. 70S ribosomes were prepared by sedimentation (270,000 × *g*) of the DNase-treated lysate obtained by grinding frozen cells with alumina and removing cell debris. Subunits were dissociated by dialysis in low Mg buffer (B2$^+$) followed by large scale zonal centrifugation in a Beckman TI-15 rotor. 16S RNA was isolated, by phenol extraction, directly from the 30S subunits obtained through the zonal centrifugation. The 30S subunits were absorbed on a DEAE-cellulose column; washed with 0.25 M NH_4Cl (2 column volumes), and eluted with 0.5 M NH_4Cl. These subunits, which fail to produce ethanol-soluble radioactivity when tested for RNase activity on [^{3}H]poly(uridylic acid), were the source of the total 30S protein fraction, prepared by mixing with an equal volume of 4 M LiCl–8 M urea solution. 30S subunits from the isolated 16S RNA (^{3}H-labeled 16S RNA extracted from a [^{3}H]uridine grown culture, was used whenever possible) and total protein components were reconstituted by incubation in RC$^+$ buffer following the usual procedure. For details of all manipulations concerning the ribosomal components see refs. 1 and 8.

The physical characteristics of each 30S subunit or 16S RNA component preparation, and of pellets obtained from each reconstitution experiment, were determined by absorbance and/or radioactivity measurements made upon each aliquot of a fractionated sucrose density gradient (5–20% sucrose in B2$^+$) prepared by centrifugation (Beckman SW41 Ti rotor, 40,000 rpm in a Beckman L2-65B centrifuge) for 6–6.5 hr. Biological activity of reconstituted ribosomes was tested by measurement of poly(U)-directed phenylalanine incorporation and, when necessary, by determination of misreading (9). For calculations: molecular weights of 16S RNA and 30S subunits are 5.5×10^5, and 8.5×10^5, respectively; A_{260} of 1 mg/ml of 16S RNA = 25; of 30S = 15.

Abbreviations: Sm, streptomycin; H_2Sm, dihydrostreptomycin; Sm^S, Sm^R, streptomycin-sensitive and resistant.

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TABLE 1. *[^{3}H]H_2Sm bound to 30S subunits and 16S RNA during equilibrium dialysis*

Ribosomal components				^{3}H cpm in 0.2 ml		
(mg)	Component	Phenotype	Genotype	Inside	Outside	Corrected Δ*
2.0	30S subunit	Sm^S	*strA*$^+$	18,203	6,951	5,626
1.3	30S subunit	Sm^R	*strA40*	9,165	7,340	1,404
1.5	30S subunit	Sm^R	*strA1*	7,822	7,778	29
0.6	16S RNA	Sm^S	*strA*$^+$	8,268	7,495	852
0.5	16S RNA	Sm^R	*strA40*	8,171	7,546	824
0.6	16S RNA	Sm^R	*strA1*	8,587	7,781	888
—	Buffer	—	—	7,491	7,501	(−10)

Outside the dialysis bag: 20 ml of TM buffer containing 25 μg/liter of [^{3}H]H_2Sm sulfate, 3000 Ci/mol. Inside the dialysis bag: the indicated amount of ribosomal component dissolved in 1 ml of TM buffer. Molecular ratio of ribosomal component to total H_2Sm is about 2. The dialysis lasts 18 hr. Three separate runs, each of duplicate trials, are performed and all six found values are averaged (range = ±200). Buffer N^+ and RC are also tested; while binding to 30S subunits is not influenced by buffer, 16S RNA binds the best in TM buffer, less in N^+, and the least in RC.

* The Δ is corrected for equimolecular amount of ribosomal component.

Drug-Binding Conditions. Two conditions were employed. (*a*) Exhaustive dialysis: Sm or H_2Sm sulfate or [^{14}C]Sm chloride (0.052 μCi/mg; a gift of Merck Sharp & Dohme, Rahway, N.J.) or [^{3}H]H_2Sm sulfate (2.1 mCi/mg; Amersham/Searle, Arlington Heights, Ill.) were added at the desired concentration and specific activity to 1 ml of a solution of the particular sample of 16S RNA or 30S subunit in the appropriate buffer. After incubation at 37° for 15 min, the mixture was dialyzed at 4° for 22 hr against 20 volumes of buffer with five changes. Aliquots of the dialyzed mixture and of the buffer changes were dissolved in Aquasol (New England Nuclear Corp., Boston) and radioactivity was measured in a Packard model 3375 liquid scintillation counter. (*b*) Equilibrium dialysis: 1 ml of a solution of the particular sample of 16S RNA or 30S subunit contained in a dialysis bag was equilibrated with 20 ml of buffer containing the radioactive drug at the desired concentration and specific activity. After 20 hr at 4°, radioactivity inside and outside the bag was measured in aliquots dissolved in Aquasol as in (*a*).

Chemical Purity of Labeled Drugs. The purity of [^{14}C]Sm and [^{3}H]H_2Sm has been checked by thin-layer chromatography. Clean glass plates of de-activated silica gel were prepared (10). Two microliters of 5 mg/ml of [^{14}C]Sm or 20 μl 0.5 mg/ml of [^{3}H]H_2Sm were applied to the plate. The dried plates were developed in distilled water containing 0.25% (w/w) Na_2SO_4 and the indicator employed was sulfuric acid. The plates were then heated at 260° in an oven for 30 min. R_F values for Sm and H_2Sm were 0.84 and 0.56, respectively. Half-cm sections were scraped from the plate (20 to 25 sections per plate); the silica powder was added to 15 ml of Aquasol (New England Nuclear Corp.) scintillation fluid, and its radioactivity was measured in a Beckman LS230 scintillation counter. The [^{14}C]Sm from Merck Sharp & Dohme and [^{3}H]H_2Sm from Amersham/Searle used each gave only one char spot, at the expected R_F, and the only concentrated radioactivity (five to six times higher than background) on the plate corresponded to this char spot. Furthermore, the bactericidal effect (measured by a zone of inhibition on *E. coli* plates) was that expected for the same amount of unlabeled pure Sm and H_2Sm.

Buffers Employed. N (0.08 M NH_4Cl, 0.011 M $MgCl_2 \cdot 6H_2O$, 0.01 M Tris, pH 7.8 with HCl); *B2* (0.03 M NH_4Cl, 0.3 M $MgCl_2 \cdot 6H_2O$, 0.01 M Tris, pH 7.8 with HCl); *BM3* (1.0 M NH_4Cl, 0.01 M $MgCl_2 \cdot 6H_2O$, 0.01 M Tris, pH 7.5 with HCl); *BM4* (0.25 M NH_4Cl, 0.01 M $MgCl_2 \cdot 6H_2O$, 0.01 M Tris, pH 7.5 with HCl); *RC* (1000 ml of BM4 + 60 ml of BM3); *TM* (0.1 M NH_4Cl, 0.01 M $MgCl_2 \cdot 6H_2O$, 0.01 M Tris, pH 7.5 with HCl) [In the *text*, when the name of the buffer is followed by a $^+$ (e.g., N^+), this denotes the addition of 6 mM 2-mercaptoethanol.]

RESULTS

Initially, Sm and H_2Sm were compared for their ability to form stable complexes with different ribosomal components. Mixtures containing 100 μmol of [^{14}C]Sm or of [^{3}H]H_2Sm and 1 μmol equivalent of 30S subunits or of 16S RNA from different *strA* ribosomal mutants were exhaustively dialyzed under identical conditions. As expected from previous results (1), an excess of ^{14}C was found in all samples containing 16S RNA, irrespective of origin (whether from *strA*$^+$ or *strA* mutated ribosomes), but an excess of ^{14}C was found only in those samples containing 30S subunits extracted from the *strA*$^+$ wild-type streptomycin-sensitive (Sm^S) cells. This excess of ^{14}C corresponded to 2 μmol of Sm per μmol of 16S RNA, and about 2.7 per μmol of 30S subunits. Surprisingly, however, H_2Sm (as determined by measuring excess ^{3}H) did not bind to *any* sample of either 30S subunits or 16S RNA in any meaningful amount (less than 0.01 mol of drug per mol of ribosomal material).

These results were interpreted to be a consequence of reduced affinity of H_2Sm for the drug-binding site(s), so that the H_2Sm complexes, in contrast to the Sm complexes, were unable to withstand the drastic conditions of exhaustive dialysis. Accordingly, an equilibrium dialysis experiment was performed in which H_2Sm binding to ribosomal material was determined in the presence of free H_2Sm. The drug was allowed to diffuse into a dialysis bag containing 30S subunits or 16S RNA from different ribosomal mutants, experimental conditions being such that when equilibrium was reached, the concentration of ribosomal material relative to that of free H_2Sm was 100 to 1. The surplus ^{3}H per unit volume found inside the bag is an index of the affinity for H_2Sm of the ribosomal material tested. The results, presented in Table 1, show that the expected drug-binding pattern for H_2Sm is observed under equilibrium dialysis conditions. The *strA*$^+$ 30S subunits bound the most drug, in quantitative agreement with results reported by others (11). In contrast, no binding by

TABLE 2. *Chase of [^{14}C]Sm bound to 16S RNA by Sm or H_2Sm*

Initial (cpm)	cpm after 2 hr of dialysis: Control (no drug)	In the presence of [^{12}C]drug: H_2Sm	In the presence of [^{12}C]drug: Sm	Molar excess of [^{12}C]drug per mol of 16S RNA
267	245	176	95	10
		163	44	100
		49	40	1,000

The [^{14}C]Sm–16S RNA complex was prepared by mixing 20 mg of 16S RNA dissolved in 10 ml of RC buffer with [^{14}C]Sm sulfate (0.081 Ci/mol) such that the molar ratio of RNA to drug was 1 to 100. At the end of the exhaustive dialysis (see *Materials and Methods* for dialysis details), the amount of Sm bound corresponded to about 2 mol of Sm per mol of 16S RNA. Aliquots (1-ml) of a solution of this complex (2 mg of RNA per ml) were separately dialyzed for two additional hours against 20 ml of RC buffer, either alone or containing different amounts of Sm or H_2Sm, according to the table. The ^{14}C inside the bag was determined on 200 μl of each sample.

strA1 30S is seen. Equilibrium dialysis reveals, in addition, a difference between *strA40* and *strA1* which was not observed in exhaustive dialysis experiments. It is seen that of the *strA* mutants, indistinguishable by their resistance to the bactericidal action of Sm (or H_2Sm), one, *strA1*, yields subunits that do not bind H_2Sm at all, while the 30S extracted from the other, *strA40*, possesses a remarkable affinity for H_2Sm, as high as 25% of that of the Sm-sensitive wild type.

The binding of H_2Sm to 16S RNA in equilibrium dialysis is poor and not comparable to that found for Sm in exhaustive dialysis. Furthermore, it is very sensitive to the buffer used: with RC buffer, it is about nil, and even when it is at its maximum, with TM buffer, it is only $^1/_7$ of that with *strA*$^+$ 30S subunits. By contrast the binding of Sm to 16S RNA is not affected by the buffer used, and is close, on a molar basis, to that found for *strA*$^+$ 30S subunits (1). These differences leave open to question the meaning of the ^{14}C found associated with the 16S RNA samples in the exhaustive dialysis experiment.

We have, therefore, performed the following chase experiment. The supposed [^{14}C]Sm–16S RNA complex was prepared (1). At the end of the exhaustive dialysis, the ^{14}C left associated with the material contained in the dialysis bag was determined, and was found to account for 2 mol of Sm per mol of 16S RNA. The dialysis was now protracted for an additional 2 hr in the same buffer, either alone or containing an excess of 10, 100, or 1000 mol of [^{12}C]Sm or [^{12}C]H_2Sm. The results, presented in Table 2, show that the ^{14}C radioactivity, which decreases only 8% in the control with buffer alone, is readily chased by [^{12}C]Sm and by a higher molar ratio of [^{12}C]H_2Sm. This indicates, first, that the ^{14}C found in the bag really is [^{14}C]Sm; and, second, that H_2Sm binds at the same site(s) as Sm, but with lower affinity.

We reported earlier (1) that Sm bound to 16S RNA prevented reconstitution of active 30S particles. We now find that this inhibition is limited only to reconstitutions using 30S proteins derived from *E. coli* strain B. We have studied two B strains (wild type and L1) and three K12 strains (X7029, A19, and Q13), performing homologous and heterologous reconstitution experiments. In all cases we have found the same pattern, i.e., with B proteins, Sm and H_2Sm prevent reconstitution, while with K12 proteins, reconstitution is indifferent to the presence of the drug.

The results of a typical experiment are presented in Table 3. The experiment consists of mixing L1 or Q13 RNA with either

TABLE 3. *Effect of Sm and H_2Sm on 30S reconstitution*

Strain donating the 30S components: 16S RNA*	30S protein	Phenylalanine cpm incorporated by reconstituted 30S subunits: Control without drug	Drug added to 16S RNA followed by exhaustive dialysis: Sm	Drug added to 16S RNA followed by exhaustive dialysis: H_2Sm	Drug directly added to the reconstitution mix: Sm	Drug directly added to the reconstitution mix: H_2Sm
L1	L1	5,229	77	5,290	(−27)	10
Q13	L1	3,572	15	3,670	0	4
Q13	Q13	5,525	4,394	4,431	4,487	4,342
L1	Q13	1,792	2,092	2,017	1,982	2,062

Reconstitutions were performed by mixing stoichiometric amounts† of 30S protein with pre-warmed 16S RNA (both in RC$^+$ buffer) in the presence or absence of drug (usually each sample contained 1–3 mg of 16S RNA). The drug was added either directly to the 16S RNA during pre-warming at a molar ratio of 10:1, drug to RNA, or before exhaustive dialysis at an initial molar ratio of 100:1, drug to RNA. Two controls (without drug), one dialyzed as in exhaustive dialysis, and a second un-dialyzed, gave essentially identical results, and their average value is therefore reported here. At the conclusion of the reconstitution incubation, the mixture was immediately cooled and centrifuged. The pellet obtained by this procedure (when the reconstitution is successful, 1.5–4 mg of 30S subunit are recovered) was dissolved in N$^+$ buffer, its concentration was adjusted to 2.5 mg/ml, and incorporation of phenylalanine directed by poly(uridylic acid) was measured in an aliquot of this solution. The amount of 50S subunit for each incorporation was 50 μg from a standard preparation of L1 ribosomes. This is the stoichiometric amount needed for 25 μg of reconstituted 30 S, which is the maximum obtainable from our experiment. The values in the table should therefore be interpreted as a titration measurement of the amount of 30S present under each condition. (Note that reconstitutions performed with heterologous components give a low yield of 30S subunit, as expected.)

* L1 16S RNA was ^{3}H labeled in order to facilitate interpretation of analytical sucrose gradients which were performed upon the pelleted material recovered from each attempted reconstitution. When biological activity was recovered from a reconstitution attempt, gradient analysis showed 30S material to be present; when biological activity was absent, analysis showed 16S RNA to be present, no evidence for hydrolysis being seen.

† Stoichiometry is based upon the assumption that the 30S protein recovered from a given amount of 30S subunit is "equivalent" to that same amount of reconstituted 30S subunit.

Sm or H_2Sm and exhaustively dialyzing the mixture, following the usual procedure (1). With this RNA material and 30S proteins obtained from RNase-free 30S subunits derived either from strain L1 or from Q13, a standard reconstitution is then attempted. Controls are performed in parallel in which either (*a*) 16S RNA not exposed to the drugs is used, or (*b*) the drugs are present during reconstitution. The reconstituted particles are tested for biological activity in promoting poly(U)-directed incorporation of phenylalanine into acid insoluble material, and for their physical characteristics in an analytical sucrose gradient test. In the section of Table 3 presenting the experiments with L1 proteins, it is seen that both Sm and H_2Sm are equally inhibitory when added to the RNA and protein mixture during the reconstitution reaction. Yet when the RNA is exposed to either drug previously and reconstitution is attempted after exhaustive dialysis, the inhibition is still complete only when the previous exposure was to Sm, while it is completely lost if the drug was H_2Sm. This result confirms that Sm, but not H_2Sm, firmly binds to 16S RNA. In the section of the table presenting the experiments with Q13 proteins, it is seen that neither Sm nor H_2Sm have any effect upon reconstitution under any conditions.

The K12 reconstitution system, which is indifferent to the presence of antibiotic, was utilized to determine the stability of the 16S RNA-Sm binding during the reconstitution process. The 16S RNA-Sm complex, stable to exhaustive dialysis, was prepared using A19-3 16S RNA and [^{14}C]Sm. It was shown to have 2 mol of Sm bound per mol of RNA, as usual (1). The complex was divided in two, one half being used for *strA*$^+$ K12 protein reconstitution (from A19), the other half for *strA* K12 protein reconstitution (from A19-3). After recovery of the reconstituted 30S subunit by centrifugation, two measurements were made: biological activity [poly(U)-directed incorporation of phenylalanine]; and a sucrose density gradient analysis monitoring A_{260} and cpm of each fraction. All reconstitutions gave biologically active material, shown by sucrose density gradient analysis to be 30 S. However, only those 30S subunits formed from *strA*$^+$ proteins had an associated radioactivity, corresponding to 0.6–0.7 Sm:30S RNA. The remaining radioactivity in each case was found at the top of the gradient.

Drug binding to 30S subunits might be studied independently from direct measurement of bound radioactivity if one takes advantage of the misreading effect of Sm and H_2Sm on amino-acid incorporation directed by synthetic messenger. For such an experiment 30S subunits from *strA*$^+$, *strA40* or *strA1* strains (L1, L1-431, and L1-401, respectively) are exposed to Sm or H_2Sm and subsequently dialyzed exhaustively. Their ability to induce isoleucine misreading in a poly(U)-directed phenylalanine incorporating system is then determined, without or with further addition of drug to the incorporation mix. Table 4 gives the results of four different experiments performed with *strA*$^+$ 30S subunits exposed to four different concentrations of the two drugs, *strA40* and *strA1* subunits also being tested. It is found, as expected, that either drug induces the same level of misreading when added to *strA*$^+$ 30S subunits during incorporation, and that misreading is practically zero with *strA1* subunits and barely measurable with *strA40*. It was also expected, and it is found, that misreading is maximal with 30S subunits exposed to Sm prior to exhaustive dialysis, since the Sm–30S subunit complex is stable. Furthermore, on the basis that H_2Sm fails to bind stably to 30S subunit one could expect no effect on misreading by H_2Sm followed by exhaustive dialysis. However, it is seen in the table that misreading is high also with the 30S subunit exposed to H_2Sm (about 70% of that with Sm–30S subunit). This result suggests that contact with the drug produces an alteration in the 30S subunits which persists even after the drug has been removed, in agreement with published *in vivo* observations (12–14) cited in the *Discussion*. Obviously, this fact invalidates any attempt to use misreading as an indirect measurement of stable binding.

TABLE 4. *Sm and H_2Sm binding to 30S subunits detected by misreading*

	Isoleucine cpm misread per 1,000 phenylanine cpm incorporated				
	Drug added before exhaustive dialysis				Drug added during incorporation† (Sm or H_2Sm indifferently)
Strain donating 30S subunits	Molar ratio tested (drug/30S subunit)	Control* (no drug)	Sm	H_2Sm	
strA$^+$	100	13	148	140	147
strA$^+$	10	11	108	69	107
strA$^+$	5	14	148	100	150
strA$^+$	2	9	75	50	84
strA40	10	6	17	13	18
strA1	10	6	7	8	10

In each experiment (horizontal lines), 1 ml of 30S subunit solution of about 3 mg/ml was subjected to exhaustive dialysis against RC$^+$ buffer in the presence or absence of either Sm or H_2Sm at the molar ratios shown in the table. At the conclusion of exhaustive dialysis, the 30S subunit concentration was adjusted to 2.5 mg/ml, and *in vitro* poly(uridylic acid)-directed incorporation of phenylalanine and isoleucine was measured. The numbers in the table are, therefore, a direct index of misreading, since the amount of 30S subunit (25 μg) in each incorporation was kept constant, and limiting.

* A control without dialysis was also performed and the value reported here is the average of the two essentially identical numbers.

† In order to measure the maximum misreading index obtainable, 2 μg of either Sm or H_2Sm were added per incorporation to all samples, whether or not exhaustive dialysis was performed (in the case of the controls) and whether Sm or H_2Sm was present during exhaustive dialysis. The misreading index obtained from each of these four conditions was so similar that the average value is reported here.

DISCUSSION

We have unexpectedly found that [^{3}H]H_2Sm binding to 16S ribosomal RNA is unstable to exhaustive dialysis, and that binding can only be shown when [^{3}H]H_2Sm and 16S RNA are present in equilibrium. However, we have confirmed that 16S RNA firmly binds a limited amount of [^{14}C]Sm; under our experimental conditions the ^{14}C radioactivity retained after exhaustive dialysis accounts for about two molecules of drug per molecule of RNA, in agreement with previous results (1). Chasing experiments indicate that in spite of this difference, the two drugs bind to the same RNA site(s).

Confirmatory evidence of this difference in binding behavior between Sm and H_2Sm was obtained by contrasting Sm

and H_2Sm in a test independent from the use of labeled drugs. We had already shown (1) that Sm prevented 16S RNA and 30S proteins from *E. coli* B from reconstituting active 30S particles, and that this inhibition persisted even after exhaustive dialysis of the 16S RNA–Sm complex. We find now that H_2Sm has an identical effect when added directly to the reconstitution mixture, but that its inhibition is completely lost after exhaustive dialysis, a result consistent with the observed binding difference between Sm and H_2Sm.

The inhibitory effect of Sm upon reconstitution is only observed using B strains, and it is not seen when 30S components of strain K12 are used. Moreover, by criss-cross reconstitution experiments, it is shown that the inhibitory effect depends upon the protein source, and not on the 16S RNA source. If the 16S RNA–Sm binding were an artifact, blocking specifically the 16S RNA assembly sites, then an interference with assembly should be expected with the proteins of either strain, since in the absence of the drug they can easily produce an interstrain hybrid 30S particle.

If the [^{14}C]Sm–16S RNA complex is incubated with K12 proteins from either a *strA*$^+$ (Sm^S) or a *strA* mutated (Sm^R) strain, active 30S subunits are obtained in both cases, but only those from the *strA*$^+$ strain bear attached ^{14}C radioactivity. This result is consistent with the notion that the 16S RNA drug-binding site could not be an assembly site, since it must be open in the completed 30S particle to enable the sensitive ribosomes to bind Sm; and that the drug site in the naked 16S RNA is topographically the same as in the 16 S associated with the proteins to form the 30S subunit. Unfortunately, this experiment does not exclude the possibility that the Sm is dislodged from the 16S RNA in both cases by either *strA*$^+$ or *strA*-mutated proteins, a 30S particle is formed, and Sm rebound to the newly-formed *strA*$^+$ 30S particle; therefore, it should be considered circumstantial evidence at best.

Melting and re-annealing of 16S RNA largely affects the number of [^{14}C]Sm molecules bound (1), and we now find the binding of [^{3}H]H_2Sm in equilibrium dialysis to be dependent upon the buffer composition. The dependence of drug binding upon salt concentration has also been observed by others (15). Clearly, the availability of the drug-binding site(s) is largely dependent on the degree of secondary structure of the 16S RNA, which is easily modifiable when the molecule is naked, but is stabilized when the molecule and the 30S proteins have coalesced. This may account for the increased stability of the drug–30S subunit complex to agents (such as salt concentration) known to interfere with hydrogen bonding. Furthermore, the same difference in affinity between Sm and H_2Sm found for 16S RNA is also found for the 30S particle, i.e., when [^{3}H]-H_2Sm is tested, binding is demonstrable in equilibrium dialysis, but not in exhaustive dialysis. This is consistent with the assumption that binding to the 30 S is of the same chemical nature as binding to naked RNA.

The rigidity conferred upon the 16S RNA chain when embedded into the 30S structure might account for a drastic difference between 16S RNA and 30S subunits with respect to the *effects* of drug binding. Dialysis easily removes dHSm from either 16S RNA or 30S subunits, with the effect on 16S RNA being readily reversed (reconstitution inhibition is completely relieved after exhaustive dialysis), while those effects on the 30S subunit persist after removal of the drug ([^{3}H]H_2Sm is not detectable, yet the ribosomes still misread as if they were in the presence of the drug). A similar persistence of drug effects has also been observed *in vivo* in two instances: (*a*) pregrowth in the presence of Sm, H_2Sm, or paromomycin confers on a *strA40* strain the ability to suppress phage nonsense mutations by ambiguity, although the drug is no longer present at the moment of infection (12); and (*b*) as "phenotypic masking" in drug dependent strains that require either Sm or paromomycin for growth but are sensitive to these drugs used together or to either one after previous growth in the other (13, 14). In both cases, extracted ribosomes are shown: (*1*) to misread, although free of the drug (which was added during pregrowth); and (*2*) to be restored to normal accuracy by high salt treatment. This suggests a reversible structural modification of the ribosome brought about by the drug and persisting after its removal.

Finally, our results indicate that drugs binding is not a necessary and sufficient reason for killing since: (*a*) H_2Sm binding to both 30S subunits and 16S RNA is weaker than that of Sm, yet the two drugs are known to have equal bactericidal power; and (*b*) *strA1* 30S subunits do not bind H_2Sm in equilibrium dialysis while those of the *strA40* strain have a binding capacity as high as 25% of that of the *strA*$^+$ wild type. If one concedes that it should not be surprising to find a difference, since *strA40* is responsive to the misreading action of Sm (or H_2Sm) while *strA1* is not, the conspicuous binding difference between two equally resistant strains clearly indicates that killing is not a direct function of binding.

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