THE BINDING OF STREPTOMYCIN TO RIBONUCLEOTIDES*

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Incubation of streptomycin (SM)** with [^{32}P]5′-ribonucleotides at pH 7.0 produces fractions that migrate towards the cathode in high voltage electrophoresis (HVE) separations at pH 3.5. SM appears to interact with pG, pA and pC but not with pU. The appearance of these [^{32}P]-labeled fractions is dependent on incubation time and SM concentration. Incubation of nucleotides with dihydrostreptomycin (DSM) or SM reduced with sodium cyanoborohydride ($NaBH_3CN$) at pH 5.0, does not produce detectable changes in [^{32}P] nucleotide mobility on HVE; however, incubation with SM reduced with $NaBH_3CN$ at pH 7.0 does produce [^{32}P]-labeled fractions migrating with a net positive charge. Elution of [^{32}P]-labeled material migrating towards the cathode from SM—5′-nucleotide incubations and re-electrophoresis results in nucleotides migrating with pG, pA and pC markers. These data indicate a reversible interaction between the SM-streptose aldehyde and amino-group containing nucleotides. This type of interaction may form an additional binding site for SM to RNA, relative to DSM.

Both streptomycin (SM) and dihydrostreptomycin (DSM) bind to ribosomes[1~6], although SM appears to have a greater affinity towards *Escherichia coli* 30S sub-units[3] and 70S ribosomes[7] than DSM. SM also has a larger affinity than DSM for 16S RNA[8] and to be more efficient than DSM in precipitating RNA[9]. One possible mechanism of enhancement of binding of SM to RNA and ribosomes relative to DSM may be an interaction between the SM aldehyde and amines. In this paper, we show that SM, as opposed to DSM, interacts with RNA nucleotides that contain amino groups, but not with uridylic acid. We also find that the interaction between SM-streptose aldehyde and the amino-group containing nucleotides is reversible. This type of interaction may form an additional binding site for SM to RNA, relative to DSM and may play a role in the enhanced binding of SM relative to DSM to RNA, and possibly to ribosomes.

Materials and Methods

Streptomycin sulfate was a gift from Dr. H. BROWN, Merck Sharp and Dohme Research Laboratories, and dihydrostreptomycin sulfate was from Calbiochem. Sodium cyanoborohydride ($NaBH_3CN$) was from Eastman.

Preparation of [^{32}P] RNA Nucleotides

E. coli D 10, RNase I$^-$ was used for the isolation of ribosomal RNA. To label RNA, cells were grown in minimal phosphate media[10] with $H_3{}^{32}PO_4$ (100 μCi/ml, New England Nuclear). [^{32}P]-Phosphate was added to growing cells at $A_{550}=0.10$. Cells were harvested at $A_{550}=0.50$. After quick chilling to 0°C and centrifugation of cells, ribosomes were isolated according to TRAUB *et al.*[11]

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** Abbreviations: Streptomycin (SM); dihydrostreptomycin (DSM); sodium cyanoborohydride ($NaBH_3CN$); high voltage electrophoresis (HVE).

[^{32}P]-Ribosomal RNA was prepared by the phenol method and the high molecular weight RNA fraction was purified by precipitation in 3 M potassium acetate, at 0°C for 24 hours[12]. After resuspension, and ethanol precipitation, samples were vacuum dried. The specific activity was 3.1×10^7 CPM/A_{260}. To prepare ^{32}P-labeled nucleotides, purified RNA was digested in 5λ nuclease Pl (Boehringer-Mannheim) 5 mg/ml for 3 hours in H_2O. [^{32}P]-5′-Mononucleotides were isolated by HVE on cellulose Mn 300 (Brinkmann) thin-layer plates using 5% acetic acid adjusted to pH 3.5 with NH_4OH, as a tray buffer. Following electrophoresis, plates were exposed to Dupont Cronex 4 X-ray film for localization of [^{32}P]-nucleotides. Spots were eluted with distilled water and vacuum dried.

Antibiotic Binding

SM (or DSM) was incubated at 20°C, pH 7.0 (0.05 M citrate-phosphate buffer) with [^{32}P]-nucleotides (0.1 ~ 0.5 A_{260}, depending on the experiment) for 24 hours in 5 μl; antibiotic concentrations used are given in the text. After incubation, samples were applied to thin-layer cellulose plates and electrophoresed at pH 3.5. Separations were at 16°C, 1.2 KV, 15 mA for approximately 2 hours. After electrophoresis, radioautographs were obtained, spots were eluted with 1 ml of H_2O, the cellulose removed by centrifugation and filtration with Millipore filters; the samples were then counted in a counting mixture containing toluene, Triton X-100, PPO and POPOP. The percent migration towards the cathode was measured relative to [^{32}P] eluted from nucleotide spots migrating towards the anode.

Reduction Modification with $NaBH_3CN$

SM was reduced with $NaBH_3CN$ (2 mg/ml) at pH 4.7 (0.05 M citrate phosphate) or at pH 7.0 (0.05 M phosphate buffer) at 20°C for 24 hours. $NaBH_3CN$ solutions were filtered before use.

Results

SM—Nucleotide Interaction

[^{32}P]-labeled 5′-phosphate ribonucleotides incubated at pH 7.0 with SM have components that migrate towards the cathode in HVE separations at pH 3.5; these are absent in controls (Fig. 1). The extent of interaction is in the order pG > pA > pC (Table 1). No [^{32}P]-labeled fractions migrating towards the cathode from SM-pU incubated samples were detected, within the limits of sensitivity of our separation system (Table 1). Similar results were obtained using

Fig. 1. Electrophoresis of [^{32}P] mononucleotides, with and without preincubation with SM.

[^{32}P]-5′-Nucleotides were incubated with 10 mg/ml SM in 5 μl of 0.05 M citrate-phosphate buffer, pH 7.0 or, with buffer alone (controls) for 24 hours at 20°C.

After incubation, samples were applied to thin-layer cellulose plates and nucleotides were electrophoresed at pH 3.5. Electrophoresis was at 1.2 KV, 15 mA for 1.5 hours. After the run, plates were dried and radioautographs were obtained.

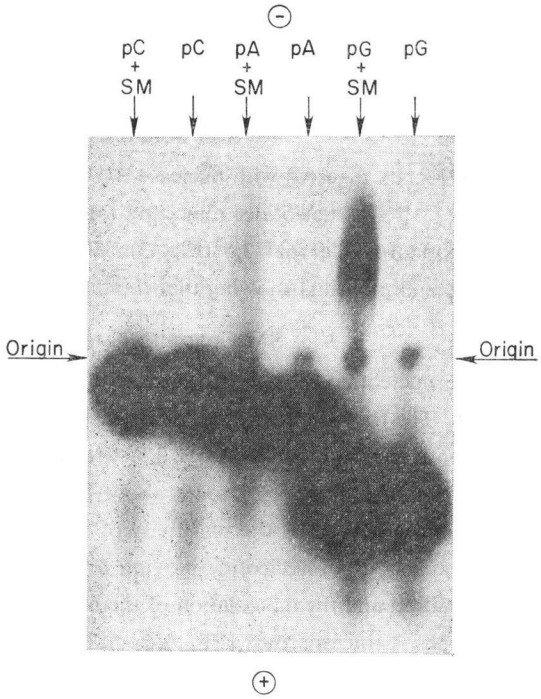


Table 1. % [^{32}P] Nucleotide moving towards the cathode in HVE separations.

Nucleotide	% [^{32}P] Migrating toward cathode	
	Control 0 mg/ml SM	10 mg/ml SM
pG	0.03	1.6
pA	0.02	0.66
pC	0.03	0.11
pU	0.02	0.03

Fig. 2. Electrophoresis of pG and pA with and without preincubation with SM (10 mg/ml).

Incubations were as described in Fig. 1. HVE separations were in 0.05 M acetic acid, 7 M urea (pH 3.5).

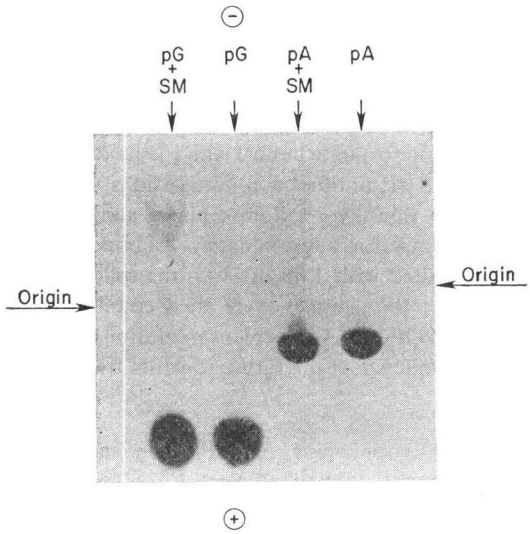
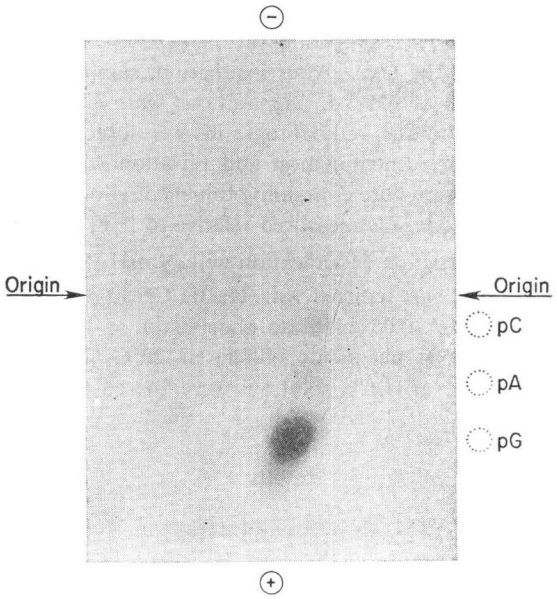


Fig. 3. Re-electrophoresis of [^{32}P] fractions that migrate towards the cathode.

Sample was from an incubation of SM (10 mg/ml) with [^{32}P] RNA digested to 5′-nucleotides with nuclease P_1. The [^{32}P]-labeled fraction migrating towards the cathode was eluted with H_2O (1 ml), dried under vacuum, resuspended in 5 μl H_2O and re-electrophoresed at pH 3.5. The migration of marker nucleotides is shown at the right. [^{32}P] spots correspond to pG, pA and pC.



3′-phosphate nucleotides from nuclease digests using T2, T1, and pancreatic RNase (data not shown).

[^{32}P]-Nucleotides incubated with SM also move towards the cathode when separated at pH 3.5 in 7 M urea. Compared with HVE separations without using 7 M urea, the bands are sharper and 2 components appear in the fractions that move towards the cathode (Fig. 2).

Re-electrophoresis of SM-interacting [^{32}P]-Nucleotides

[^{32}P]-RNA digested with nuclease P_1 to 5′-nucleotides was incubated with SM and electrophoresed at pH 3.5. [^{32}P]-Nucleotides migrating towards the cathode were eluted with H_2O, vacuum dried and re-electrophoresed at pH 3.5 (Fig. 3). [^{32}P]-Labeled nucleotides migrated with nucleotide markers (pG, pA and pC) thus showing that the binding of SM to these nucleotides is reversible.

SM Concentration Dependence and Time Dependence

The nucleotide mobility towards the cathode was measured as a function of SM concentration (Fig. 4). The fraction of [^{32}P]-pG migrating towards the cathode is linearly dependent on SM concentration with electrophoresis at pH 3.5, with and without 7 M urea. The lowest concentration of SM that gives a significant [^{32}P] count above background (40 cpm) is 50 μg/ml. Detection at lower concentrations is limited by the sensitivity of our separation system. Since the interactions are reversible, the amount of [^{32}P]-nucleotide moving towards the cathode that we detect is representative of both the formation and the dissociation of the interactions. The time course of interaction of SM with [^{32}P]-nucleotides, as measured by [^{32}P]-nucleotide migration towards the cathode is shown in Fig. 5.

Fig. 4. The percent [^{32}P] pG migrating towards the cathode with HVE, as a function of SM concentration.

pG was incubated with various concentrations of SM at pH 7.0 for 24 hours and electrophoresed at pH 3.5.

●---● electrophoresis in 7 M urea;
○—○ electrophoresis without 7 M urea.

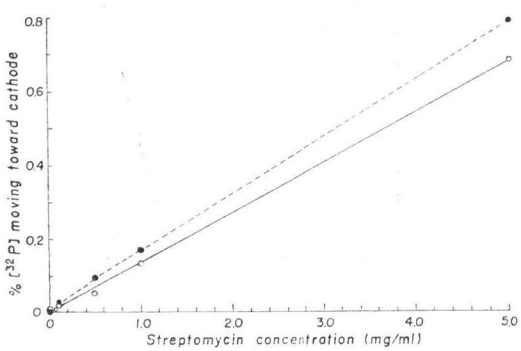
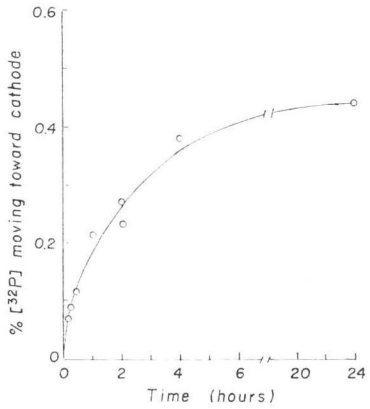


Fig. 5. The percent of [^{32}P] nucleotide migrating towards the cathode as a function of incubation time with SM.

[^{32}P] was digested with nuclease P_1 for 2 hours. Samples were incubated with SM (10 mg/ml) at pH 7.0 at given times and electrophoresed at pH 3.5 for 2 hours. [^{32}P] Material was eluted and counted.



Nucleotide Incubation with Dihydrostreptomycin

The incubation of [^{32}P]-nucleotides with dihydrostreptomycin (10 mg/ml) produced no detectable movement of nucleotides towards the cathode (Fig. 6). DSM is a commercial preparation synthesized by the calatytic reduction of SM with sodium borohydride. SM was also reduced with $NaBH_3CN$ in our laboratory and used for incubations with nucleotides. The cyanohydridoborate anion has been used in selective reductive procedures for various functional groups[13]. $NaBH_3CN$ reduces aldehydes at pH 3~5 but poorly at 6~7. We find no [^{32}P]-nucleotide movement towards the cathode using SM reduced at pH 5 but a significant amount of label migrating towards the cathode using SM reduced with $NaBH_3CN$ at pH 7.0 (Fig. 7). Sample 4 of Fig. 7 contained 257 cpm (incubation with SM reduced with $NaBH_3CN$ at pH 7.0) and sample 1 (SM reduced with $NaBH_3CN$ at pH 5.0) as well as the control, sample 2, were at background levels. These data strongly suggest that the streptose aldehyde of SM is the

Fig. 6. [^{32}P] Nucleotide mobility in HVE of RNA hydrolysates incubated with SM or DSM.

[^{32}P] RNA was digested with nuclease P_1 for 3 hours. Left, incubation of RNA hydrolysate with SM (10 mg/ml); center, incubation without antibiotic; right, incubation with DSM (10 mg/ml). All samples were incubated for 24 hours and electrophoresed at pH 3.5. The 4 major spots migrating towards the anode are pU, pG, pA and pC.

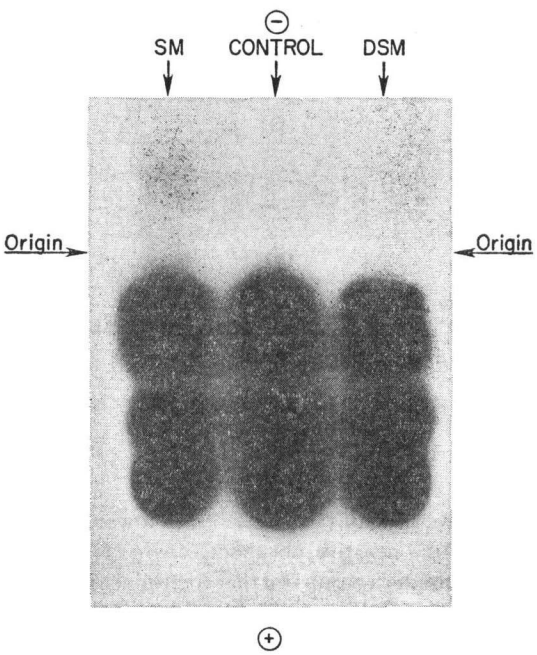


Fig. 7. The effect of incubation of [^{32}P] nucleotides with SM reduced with $NaBH_3CN$ at pH 5.0 and 7.0.

[^{32}P] RNA was digested with nuclease P_1 for 3 hours. Six samples were used as follows:

1. 10 μl of [^{32}P] RNA was incubated at pH 5.0 at 20°C, 24 hours with 5 μl of SM (previously reduced with $NaBH_3CN$, 2 mg/ml, at pH 5.0, 20°C, for 24 hours); final SM concentration, 1 mg/ml.
2. 10 μl of [^{32}P] was incubated with 5 λ of $NaBH_3CN$ (2 mg/ml) at pH 5.0, 20°C, for 24 hours.
3. 10 μl of [^{32}P] was incubated at pH 5.0, 20°C, 24 hours, with 5 μl of SM (pre-incubated at pH 5.0, 20°C for 24 hours in buffer alone, pH 5.0); final SM concentration, 1 mg/ml.
4. Same experimental conditions as 1, except for incubation at pH 7.0.
5. Same experimental conditions as 2, except for incubation at pH 7.0.
6. Same experimental conditions as 3, except for incubation at pH 7.0.

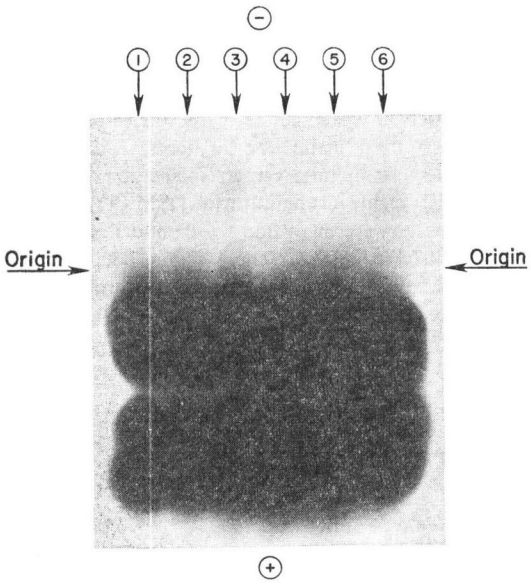
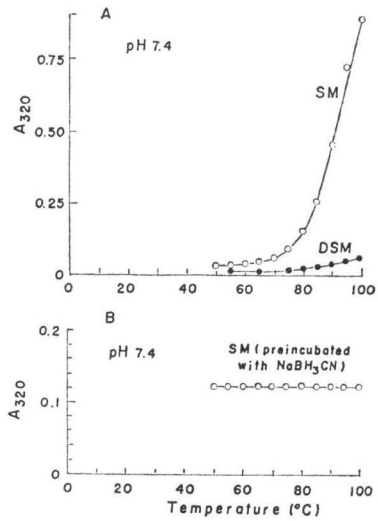


Fig. 8. Absorbance changes with heating of SM and DSM.

A. SM and DSM heated at pH 7.4.

B. SM heated at pH 7.4 (previously reduced with $NaBH_3CN$ at pH 4.7).

Buffers were 0.05 M citrate-phosphate, pH 4.7 and 0.05 M phosphate pH 7.4. Antibiotic concentrations were 100 μg/ml.



functional group interacting with RNA nucleotides.

Self-Interaction of SM

SM, which contains amino groups in the streptidine ring and the aldehyde on the streptose ring, has the potential for SCHIFF-base type interactions between 2 or more SM molecules. DSM, which has a reduced aldehyde, should not have the ability to interact in this fashion. To investigate this, SM was heated and the absorbance at 320 nm (A_{320}) was measured. SM, but not DSM or SM reduced with $NaBH_3CN$ at pH 4.7, produced a large increase in A_{320} with temperature, indicating the formation of aggregates of SM (Fig. 8). These data suggest a self-association of SM molecules *via* aldehyde reactions.

Discussion

Movement of [^{32}P]-nucleotides towards the cathode during electrophoresis at pH 3.5 could only occur if the negative phosphate charge is counterbalanced with more than one positive charge. SM has 2 guanidino groups on the streptidine ring with pKa values of ~12; these groups are thus protonated at the pH conditions used in the experiments described here. We conclude that the [^{32}P]-labeled fractions detected during electrophoresis of SM-incubated samples have a net positive charge due to bound

SM.

The experiments described in this paper suggest that the streptose ring aldehyde of SM interacts with nucleotide base amino groups. This conclusion is supported by the following:

1. Only the amino group containing nucleotides appear to form an interaction with SM.
2. DSM or, SM reduced at pH 5.0 with $NaBH_3CN$, do not appear to form interactions.
3. [^{32}P]-Nucleotide-SM interactions are stable in 7 M urea, thus minimizing any role of hydrogen bonding in these interactions.
4. Elution of [^{32}P]-labeled components that migrate towards the cathode and re-electrophoresis indicate a reversible interaction between SM and nucleotides pG, pA and pC.

It would appear that the nucleotide amino groups and the SM aldehyde are the crucial groups that are interacting and SCHIFF-base type reactions may be occurring.

SM interactions with [^{32}P]-nucleotides are both time dependent and concentration dependent. The extent of reaction that we detect is about $1 \sim 2\%$ for pG at a concentration of 10 mg/ml of SM; the minimum detectable interaction is with 50 μg/ml SM. Since the interaction is reversible, the extent of reaction may be much greater than what we are able to detect and the interaction may occur with much lower antibiotic concentrations.

SM appears to interact strongest with pG and the weakest with pC. The order of pG > pA > pC may represent the degree of nucleophilicity of the nucleotide base amino groups, the degree of stability of the interactions, or both of these factors.

Two electrophoretic bands are seen in 7 M urea after incubation of SM with pG and pA (Fig. 2). These may represent multiple interactions of the SM aldehydes with different nucleophilic sites; *i.e.*, on the purine base such as the N7 centers, in addition to the 2-amino group of guanine and the 6-amino of adenine.

The importance of the SM aldehyde group is exemplified in separate experiments by the extensive optical absorbance changes at 320 nm with temperature of SM as compared to DSM (Fig. 8). Presumably, aldehyde cross-linking to guanidino groups is occurring to produce aggregates of SM.

Our studies show that there is an interaction between amino group containing nucleotides and SM. The SM aldehyde may play a role in the binding of the drug to free RNA. Our results on SM-nucleotide interactions may form a rationale for the greater efficiency of SM, relative to DSM, in binding to 16S RNA[5] and in the precipitation of RNA[9], although the physiological significance of the binding to nucleotides is unclear.

What role does the aldehyde play in the binding of SM to ribosomes? Since SM appears to bind more efficiently than DSM to 30S ribosomal subunits[3] and 70S ribosomes[7], the aldehyde of SM may function in a secondary role in stabilizing the interaction of the drug with the ribosome. However, the nature of the binding site of SM and DSM to ribosomes is still unclear. There is indirect evidence, using reconstitution techniques[5] and affinity-labeling with SM-analogs[14,15] which shows that ribosomal proteins form part of the binding site, but the results from these studies are inconsistent. Reconstitution studies[5] indicate that proteins S3 and S5 are crucial for DSM binding to occur. However, results from affinity-labeling with SM-analogs are incongruous. GIRSHOVICH *et al.*[15] determined that proteins S7, S14, S16 and S17 are in or near the active site, and PONGS and ERDMANN[14] tentatively identified S3 and S4 as the reactive proteins. The ribosomal 16S RNA has also been considered as the binding center on the ribosomes for the drug[8] but no confirming evidence has been provided. In addition, binding constants have been determined only for DSM to ribosomes by different investigators[4,5] and with reasonable agreement on affinity constants, at least for the 30S ribosomal subunit, no quantitative data are available on affinity constants for SM.

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