Streptomycin as an Antiviral Agent: Mode of Action

Abstract. *In host bacteria resistant to the antibiotic, streptomycin inhibits phage replication by inhibiting the process of injection. This effect is competitively reversed by certain divalent cations, polyamines, and streptidine. It is proposed that streptomycin inhibits injection by attaching to the phage DNA while it is still folded within the phage head, and in this way it prevents the unfolding which is essential for the injection process. The reversal agents probably function by displacing the antibiotic from the phage, but they also promote injection themselves.*

The antibiotic streptomycin acts as a specific antiviral agent for certain bacterial viruses, since it is inhibitory to the viruses even when the host bacteria are resistant to the antibiotic. Of *Escherichia coli* phages, only an RNA virus is completely sensitive (*1*) but a variety of DNA viruses which attack group D streptococci are affected (*2*). We have concentrated particularly on eight bacteriophages which attack a single strain of *Streptococcus faecium* (ATCC 8043). Since six of these phages are streptomycin-sensitive and the other two are streptomycin-resistant, this suggests that the antibiotic is acting only on the phages, since the host was identical in all cases. Tritium-labeled streptomycin has been used to show that the streptomycin-resistant host binds essentially no antibiotic (around 400 molecules per coccus), while the sensitive parent binds about 2×10^4 molecules per coccus.

In our experiments we used streptomycin-sensitive phage P9, and the host was a mutant, resistant to 1000 μg of antibiotic per milliliter. The phage could be inhibited by 100 to 125 μg of streptomycin per milliliter, but we used 1000 μg per milliliter unless otherwise stated. The methods of infection and phage assay have been described (*2*).

The phage adsorbs to the host cell in one of two ways. Reversibly adsorbed phage is firmly attached to the cell but is released by chloroform treatment and is then able to infect other cells. Reversible adsorption can occur in either the presence or absence of streptomycin. Reversibly adsorbed phage eventually becomes converted to irreversibly adsorbed phage, which is altered in some way so that it is chloroform sensitive, and one assumes that the tail components have been altered so that the particle is no longer infectious when released from the cell by chloroform. Irreversibly adsorbed phage has not injected its DNA, however, as shown by shearing experiments. Irreversible adsorption occurs in the presence of streptomycin. If crude phage preparations are used, about 30 percent of the phage is initially reversibly adsorbed, but less than 0.1 percent is reversibly adsorbed if purified phage is used. Since streptomycin does not affect irreversible adsorption, this indicates that the antibiotic does not inhibit the processes (which may require host participation) altering the phage tail so that the particle will no longer be infective if released from the cell by chloroform. Mature intracellular phage particles are resistant to chloroform, as are free phage particles.

The latent period of the phage is 30 minutes. When streptomycin was added more than 5 minutes after infection, it had no effect on virus replication. During the first 5 minutes the antibiotic had two effects. If the antibiotic was added before infection, the adsorbed virus was completely inhibited but was not inactivated, and replication could begin normally if the antibiotic was merely removed by hundredfold dilution. If the antibiotic was added between 1 and 5 minutes after infection, the virus was rapidly inactivated. It was proposed (*2*) that streptomycin added before infection inhibited injection, so that the virus nucleic acid remained intact inside the phage head, but when the antibiotic was added between 1 and 5 minutes, injection was proceeding but the nucleic acid was in a site still accessible to the antibiotic (possibly the cell membrane), and that at this time the nucleic acid was in such a condition that the antibiotic could bring about its inactivation.

To study the reversible inhibition of the injection process, a modification of the method of Hershey and Chase (*3*) was used.

If injection has not occurred, the phage DNA is still outside the cell, and if this infected cell is subjected to the extensive shearing forces produced in a fluid which is rapidly stirred or shaken, the phage particle may be stripped from the cell. An infected cell, when placed on an agar plate with a suitable indicator, will give rise to a single plaque, representing the subsequent replication of the virus attached to the cell and the release of virus particles which will attack the indicator cells and produce a plaque. If, before injection has occurred, the phage has been stripped off the cell by a shearing force, the cell when deposited on the plate will not give rise to a plaque. A cell in which injection has occurred will still give rise to a plaque even after shearing, since the virus nucleic acid has penetrated the cell and is protected from the forces which strip off the phage head. Thus, if a shearing force is applied to the infected population before injection has occurred, there will be a sharp decrease in the number of infected cells, whereas, if the shearing force is applied after injection, this decrease will not occur. By measuring the time course of the appearance of shear-resistant infected cells, one can measure indirectly the time course of injection. This reasoning was used by Luria and Steiner (*4*) to show that calcium ions were required for injection of T5 bacteriophage.

In preliminary experiments, phage was stripped off the infected cells in a Virtis homogenizer operating at 10,000 rev/min for 3 minutes. It was evident that streptomycin did inhibit injection, but it was not possible to make careful measurements because of the long blending time needed. For more precise experiments a Nossal vibrator (*5*) was used; the infected cells were mixed with an equal volume of glass beads (*6*) and subjected to reciprocal vibration at 8000 rev/min for 10 sec, while liquid CO_2 cooled the sample quickly from the incubation temperature of 37°C to less than 20°C. In this apparatus the shearing forces are created by the rapid movement of the glass beads through the liquid. In control experiments neither the host bacteria nor the phage were inactivated when vibrated separately for as long as 30 sec, whether in the presence or absence of streptomycin, but the plaque-forming capacity of 90 percent of the infected cells could be eliminated in 10 seconds.

With this procedure the kinetics of the injection process were measured. The cells were infected in the presence of streptomycin, filtered on membrane filters to remove unadsorbed phage, and resuspended in streptomycin-containing broth. They were then diluted 1:100 in broth either with or without streptomycin, and incubated at 37°C; samples were periodically subjected to shearing forces and the percentage of shear-resistant infected cells was determined (Fig. 1). If streptomycin was

Table 1. Effect of streptomycin, Mg^{++}, Ca^{++}, and spermine on the injection process of P9 bacteriophage. Infected cells, at a concentration of 4 to 5 $\times$ 10^5 cells per milliliter, were incubated for 15 minutes at 37°C in Todd-Hewitt broth or in tris buffer, with additions, then blended 10 seconds in a Nossal vibrator.

Addition	Shear-resistant infected cells (%)
Experiment 1—in Todd-Hewitt broth	
None	58
Streptomycin SO_4, 1 mg/ml	3.3
Spermine 4 HCl, 1 mg/ml	87
$MgCl_2$, 0.01*M*	80
Experiment 2—in tris buffer	
None	7
$MgCl_2$, 0.01*M*	37
$CaCl_2$, 0.01*M*	25
Spermine, 1 mg/ml	35
Todd-Hewitt control	65
Experiment 3—in Todd-Hewitt broth + streptomycin SO_4, 1 mg/ml	
None	1.4
$MgCl_2$, 0.01*M*	10
Spermine, 1 mg/ml	12
Spermine, 5 mg/ml	21

present throughout the incubation period, the infected cells remained sensitive to shear. If the antibiotic was removed by dilution, shear resistance quickly developed and was at a maximum within 5 minutes. It was shown in previous work that when streptomycin was added later than 5 minutes after infection, it had no effect on subsequent replication and the fact that shear resistance is complete within this same time period suggests that resistance to streptomycin, and development of shear resistance represent two aspects of the same process, namely, injection. The whole time period represented by Fig. 1 is within the latent period and the number of infected cells in unsheared populations remained constant throughout, either in the presence or the absence of the antibiotic.

Shear resistance never developed in more than 60 percent of the infected population, and this was true even if the whole process of adsorption and injection occurred in the complete absence of streptomycin. However, if $MgCl_2$ or the polyamine, spermine, was added to the Todd-Hewitt broth, 80 to 90 percent of the infected population became shear resistant (Table 1, experiment 1). When 0.01*M* $MgCl_2$ or 0.001*M* spermine tetrahydrochloride was added to the agar used for plaque assays, the number of plaques obtained per standard dilution of phage was approximately doubled, and the plaques were larger, clearer, and had more distinct margins. Since the same agents which stimulated injection also affected the plaque assays, Todd-Hewitt medium may be partially deficient in magnesium ions or polyamines, and when these substances were added to the agar they increased the efficiency of plating because they promoted injection. When infected cells were suspended in tris buffer instead of in Todd-Hewitt broth, injection was very poor even in the absence of streptomycin, and under these conditions injection was strongly stimulated by magnesium ions, calcium ions, or spermine (Table 1, experiment 2).

The possible reversal by metal ions or polyamines of the streptomycin inhibition of injection was next studied. Both magnesium ions and spermine reverse the streptomycin inhibition of injection (Table 1, experiment 3). Since streptomycin inhibits only the injection process, it was reasonable to expect that substances which reversed injection inhibition would also reverse the inhibition of plaque formation by the antibiotic. This prediction has been borne out; Table 2 shows the results of antagonism by magnesium ions, and indicates that streptomycin and magnesium ions interact in a competitive manner.

Calcium ions also reversed plaque formation inhibition, but were less effective than magnesium ions, whereas sodium, potassium, and ammonium ions were ineffective at 0.01*M* concentrations, and divalent copper, cobalt, and nickel ions were ineffective at 0.001*M* concentrations. Manganese and zinc ions presented an anomalous picture, since they themselves inhibited plaque formation. This inhibition was also reversed by magnesium ions, indicating that manganese and zinc ions may compete with the ingredients in Todd-Hewitt medium which are required for injection.

A study of the reversal by diamines and polyamines of streptomycin inhibition of plaque formation revealed that a variety of these substances reversed to varying degrees. Testing in the presence of 400 μg of streptomycin sulfate per milliliter ($5.5 \times 10^{-4}M$) revealed that spermine reversed at $5 \times 10^{-4}M$, spermidine at $5 \times 10^{-3}M$, and cadaverine at $1 \times 10^{-2}M$. A homologous series of diamines was tested from diaminopropane through diaminooctane and the best reversal occurred with the C_3-, C_4-, and C_5-compounds.

The question may be asked: What features of the streptomycin molecule are responsible for its activity? One portion of the molecule is the substance streptidine, which is 1,3-diguanido-2,4,5,6-cyclohexanetriol. This substance has two guanido groups in *cis* configuration separated by a distance of approximately 7.7 Å. Deguanidodihydrostreptomycin and monoaminomonoguanidodihydrostreptomycin did not inhibit plaque formation of P9 even at 10 mg per ml. Since the phosphate groups in a strand of the DNA chain are 7.65 Å apart (*7*), it seems possible that the two guanido groups of the streptomycin molecule could combine with two phosphate groups in one of the strands of the DNA double helix. Of the diamines which will stabilize phage DNA (*8*), cadaverine (C_5) is the most active, and this diamine has its two amino groups separated by approximately the same distance as the phosphate groups of the DNA chain (*7*). Cadaverine was also one of the most effective diamines in reversing streptomycin. Since guanido groups are more basic than primary amino groups, streptomycin would probably bind more tightly than a diamine, and this may explain how the antibiotic is able to compete effectively with the

Table 2. Reversal of streptomycin inhibition of plaque formation by $MgCl_2$. Phage P9 was assayed by the soft agar layer method on Todd-Hewitt agar base plates containing the $MgCl_2$ and streptomycin concentrations indicated. The same volume of phage suspension was put on each plate. Plaques were counted after 18 hours' incubation at 37°C. The figures are numbers of plaques per plate.

Streptomycin concentration (μg/ml)	$MgCl_2$ concentration				
	0.1*M*	0.01*M*	0.001*M*	0.0001*M*	0
0	243	230	112	109	106
100	228	183	0	68	66
200	239	202	27	13	14
400	204	127	0	0	0
600	208	44	0	0	0
800	164	0	0	0	0
1000	225	0	0	0	0

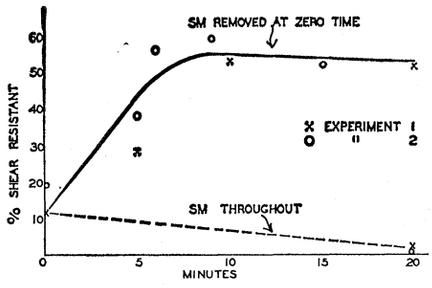

Fig. 1. Development of shear-resistance in infected cells after removal of streptomycin by dilution. Cells infected with P9 at 5 to 7 × 10^8 plaque-forming units per ml were diluted into Todd-Hewitt broth either with or without streptomycin and incubated at 37°C. SM, streptomycin.

polyamines. Since secondary amines like spermine and spermidine are more basic than the primary amines, these former compounds would probably bind to DNA more tightly and would thus be more effective than the simple diamines in reversing streptomycin activity.

Streptomycin is known to precipitate DNA, and Cohen (*9*) has shown that the streptidine portion of the molecule alone does not cause precipitation. From titration studies, Cohen concluded that the secondary amino group on the *N*-methyl-L-glucosamine portion of the molecule participated in DNA precipitation and that streptomycin probably precipitated by forming cross linkages in which the guanido group associates with one DNA molecule and the secondary amino group associates with a second molecule. Streptidine (*10*) had no inhibitory effect on phage P9 at concentrations as high as 1 × $10^{-2}M$ but competitively antagonized the inhibition induced by streptomycin. About seven streptidine molecules were required to reverse one streptomycin molecule. This difference may result from the tighter binding of the complete streptomycin molecule. The competitive relationship between these two substances suggests that at least one portion of the streptomycin molecule essential for activity is the streptidine portion.

We propose that streptomycin inhibits injection of phage P9 either by entering the phage head and combining with the coiled DNA in such a way that various portions of the molecule are cross-linked, so that the uncoiling which is essential for injection cannot occur, or by linking the DNA to the protein coat. Divalent ions, polyamines, and streptidine would then reverse streptomycin inhibition by preventing the binding of the antibiotics or by displacing the streptomycin from the nucleic acid.

As yet we have no idea why two of the streptococcus phages are resistant to the antibiotic. Permeability of the phage head to the antibiotic does not seem to be an explanation, since both resistant and sensitive phages of streptococcus undergo photoinactivation by methylene blue without a lag and are resistant to osmotic shock, implying free permeability. Also, a permeable mutant of phage T4 (*11*) of *E. coli* is completely resistant to streptomycin as is wild type T4.

Since it is possible that the simple system used here has isolated a single molecular aspect of streptomycin action, it is pertinent to ask whether our results will provide any insight into the mode of action of streptomycin as an antibacterial agent. Streptomycin inhibits protein synthesis in a cell-free ribosomal system (*12*) and alters the integrity of the cell membrane (*13*). Both ribosomes and cell membranes are stabilized by magnesium ions or by polyamines (*14*). Mager *et al.* (*15*) have suggested a common site for the action of polyamines, magnesium ions, and streptomycin in *E. coli* ribosomes. Cations reverse the antibacterial action of streptomycin and they are also able to inhibit the precipitation of DNA by the antibiotic (*16*). It thus seems possible that streptomycin acts on all these bacterial structures by competing for magnesium ions or polyamines and precipitating or altering the structure of the sensitive sites. This "unitary" hypothesis has the dual virtues both of explaining a confusing variety of antibacterial effects of streptomycin, and at the same time suggesting a number of possible experiments.

Recent work has shown that the DNA of purified phage P3 (streptomycin-resistant) and phage P9 (streptomycin-sensitive) do not contain any unusual bases, so that streptomycin sensitivity cannot be due to a peculiarity of base composition. The importance of these observations for an understanding of approaches to antiviral chemotherapy seems clear. Curiously, study of the injection process has been neglected in recent years, perhaps because of the greater fascination with how the nucleic acid works after it has entered the cell (*17*).

THOMAS D. BROCK
SUSAN O. WOOLEY
Department of Bacteriology,
Indiana University, Bloomington

References and Notes

1. T. D. Brock, *Biochem. Biophys. Res. Commun.* **9**, 184 (1962).
2. ———, J. Mosser, B. Peacher, *J. Gen. Microbiol.*, in press.
3. A. D. Hershey and M. Chase, *J. Gen. Physiol.* **36**, 39 (1952).
4. S. E. Luria and D. L. Steiner, *J. Bacteriol.* **67**, 635 (1954).
5. McDonald Engineering Co., 27105 Knickerbocker Road, Bay Village, Ohio.
6. Pavement Marking Beads, Minnesota Mining and Manufacturing Co., Type 110.
7. D. Fraser and H. R. Mahler, *J. Am. Chem. Soc.* **80**, 6456 (1958).
8. H. R. Mahler, B. D. Mehrotra, C. W. Sharp, *Biochem. Biophys. Res. Commun.* **4**, 79 (1961).
9. S. S. Cohen, *J. Biol. Chem.* **168**, 511 (1947).
10. Streptidine was provided by Merck, Sharp and Dohme.
11. Bacteriophage T4 $r^{+}o_5$ was provided by Dr. S. Champe.
12. J. G. Flaks, E. C. Cox, J. R. White, *Biochem. Biophys. Res. Commun.* **7**, 385 (1962).
13. N. Anand, B. D. Davis, A. K. Armitage, *Nature* **185**, 23 (1960).
14. H. Tabor, C. W. Tabor, S. M. Rosenthal, *Ann. Rev. Biochem.* **30**, 579 (1961).
15. J. Mager, M. Benedict, M. Artman, *Biochim. Biophys. Acta* **62**, 202 (1962).
16. R. Donovick, A. P. Bayan, P. Canales, F. Pansy, *J. Bacteriol.* **56**, 125 (1948).
17. One of us (T.D.B.) is a research career development awardee of the U.S. Public Health Service (grant No. AI-K3-18,403). This work was supported in part by grants from the U.S. Public Health Service (AM-04630-03) and U.S. Army (DA-CML-18-064-61-G13). We thank W. B. Deville for help with certain experiments.

22 May 1963

Information Storage Requirements for the Contents of the World's Libraries

Abstract. *Three calculations of the number of different things stored in the world's libraries yield estimates from 7.5 × 10^7 to 7.7 × 10^8. At 10^5 words per volume, five letters per word, and 12 bits per letter, the information capacity used for storage is between 4.6 × 10^{14} and 4.6 × 10^{15} bits, and is increasing at about 2 × 10^6 bits per second.*

In the course of a project aimed at understanding the problems and possibilities of the library (or the successor of libraries) of the next century, we have collected and reorganized information about the "size" of the world's literature. The information is useful as a basis for estimating the magnitude of the storage problem. Particularly if we are to consider the use of computers and computer memory systems as replacements for many present functions of a library, we wish to have estimates of the amount of storage that is needed and the rate at which it is growing. With that information we can judge whether presently available computers can serve as a major library, and, if not, what kinds of developments are needed.