ISOLATION OF STREPTOMYCIN FROM A PINK VARIANT OF *S. GRISEUS*.

BY HAMAO UMEZAWA, YASUO OGATA, TOMIO TAKEUCHI
AND TAIZO TABATA.

National Institute of Health of Japan, Tokyo

As one of the most important antibiotics, streptomycin has been studied in detail by many workers after the discovery of Waksman and his colleagues (1), but it has been a very difficult work to isolate a streptomycin-producing strain, until recently the above author (2) found a rapid isolation method. Thereafter several active strains were isolated also by us (3) and afterwards two strains by other Japanese workers in obedience to our publication (3). The fact that all active strains obtained both in America and in Japan belong to *S. griseus* seems to suggest the existence of a specific relation between streptomycin and *S. griseus*. But hitherto an antibiotic substance has been usually obtained from more than one species, such as penicillin from *P. chrysogenum notatum*, *A. flavus* and etc, Patulin from *P. patulum*, *P. expansum*, *P. leucopus*, *P. claviforme* and *A. clavatus*. While we were classifying numerous antibiotic strains of streptomycetes according to their antibacterial spectra, we found several strains producing a pink pigment and possessing the streptomycin-producing ability. Waksman and others (4) recently observed *Streptomyces bikiniensis* to produce a streptomycin-like substance, however these strains seem to be different from this strain. The antibiotic obtained from our strains conforms to streptomycin in its antibacterial spectrum, toxicity, solubility in various solvents and colour of Sakaguchi's reaction.

Present paper describes the isolation of the strains, and various foundations to decide it to be streptomycin. The characters of the strain in detail will be published elsewhere.

Isolation and Characters of the strains.

Isolation: About one gram of soil was suspended in about 2 cc sterile water and its one loop was streaked on the nutrient agar containing 0.03 mg streptomycin hydrochloride (6000u/mg) in 1 cc and incubated for about four days at 28° C. The colonies of streptomycetes were picked up and each of them was implanted on the agar plate in a spot and incubated for four days at 28° C. Then after the organism had well developed, ten times diluted broth culture of *E. coli* was sprayed on the plate which was afterwards incubated for about twenty hours at 37° C. The colonies, showing the inhibition zones around themselves, were picked up and afterwards the antibacterial spectrum of each strain was tested by streaking *E. coli*, streptomycin-fast *E. coli*, streptothricin-fast *E. coli*, *B. anthracis*, and *B. subtilis* N.R.R.L B-558 across the growing line of these strains.

In the above-mentioned process, three strains, SN-1, SN-2, SN-14, were found to produce an antibiotic substance showing the same antibacterial spectrum as streptomycin. The results are the same as the antibacterial spectrum of their cultured broth being shown in Table 1.

Table 1. Isolation of the strains and the antibacterial spectrum of the cultured broth

Origin		Strain		
		SN 1	SN 2	SN 14
		Garden soil at Itabashiku	Garden soil at Itabashiku	Garden soil at Itabashiku
Growth on the nutrient agar added with streptomycin hydrochloride (600 u/mg)	0.1 mg/cc	±	±	±
	0.05 mg/cc	++	++	++
	0.025 mg/cc	+++	+++	+++
The diameters of the growth inhibition zone (against <i>E. coli</i>) around the colony.		37.5 mm	37.0 mm	36.5 mm
Antibacterial spectrum (diameters of the length of the inhibition zone)	<i>E. coli</i>	12.0 mm	7.0 mm	9.0 mm
	Streptomycin-fast <i>E. coli</i>	0	0	0
	Streptothricin-fast <i>E. coli</i>	0	0	0
	<i>B. subtilis</i> N. R.R.L., B 558.	10.0 mm	8.5 mm	9.0 mm
	<i>B. anthracis</i>	14.0 mm	11.5 mm	12.5 mm
	<i>S. aureus</i>	10.0 mm	7.5 mm	7.5 mm

These three strains are more resistant to streptomycin than ordinary strains of streptomycetes, but if their resistance are compared with streptomycin-producing strains of *S. griseus* they are more susceptible to streptomycin than the latter. They grow well on the nutrient agar added with streptomycin in 0.025 mg/cc, but their growth is slightly inhibited on the agar with streptomycin in 0.05 mg/cc and almost completely in 0.1 mg/cc (the potency of streptomycin employed was 600u/mg). Characters of the strain: All four strains have the same characters. They grow well on the asparagine-glucose agar, and after three days incubation the surface is covered with light green spores and the reverse is pinkish red, which does not diffuse in the agar. The submerged mycelium, which breadth is 0.4–0.9 μ , is almost white and produces pinkish red colour substance near the mycelium. The three strains are same in their morphological and biological characters, and besides their antibacterial spectra, so the following studies were performed only by using the strongest strain SN-1.

Production of the antibiotics and their antibacterial spectrum.

Culture: 50 cc of the glucose broth (0.5% beef extract, 0.5% peptone, 0.5% NaCl, 1% glucose, 0.35% CaCO₃, pH 7.0) was put in a 350 cc long neck flask and after sterilization one loop of spores on the asparagine-glucose agar was implanted and incubated at 28° C, being placed on the shaking machine going and returning between 6 cm amplitude 120 times per minute.

The potency of the broth was not so high as in the case of the streptomycin-producing strain of *S. griseus*, and too low to be measured by the cup method using *S. aureus*

209-p strain, so it was measured by the broth dilution method. The cultivated broth was diluted in the nutrient broth without sodium chloride, because it inhibited the effect of streptomycin, and added with one drop of ten times diluted broth of *E. coli* (20 hours culture) and incubated for twenty hours at 37° C. Then the dilution of the broth showing almost complete inhibition was read.

The potency and pH of the broth during the shaking culture are indicated in Table 2. The streptomycetes began to grow well after twenty-four hours and afterwards produced pinkish red pigment, turning the broth from brown to pinkish brown. pH increased from the beginning and reached about 8.5 after four days, when the potency reached its maximum. The broth inhibited almost completely the growth of *E. coli* in the nutrient broth without NaCl at the dilution 1:20-160.

Table 2. The production of the antibiotic substance in the nutrient glucose broth* by shaking culture.

Days	pH	Highest dilution of the broth	
		for complete inhibition	for about 50% inhibition
3	7.0	1:20	1:80
4	7.6	1:40	1:160
5	7.8	1:20	1:160
6	8.2	1:20	1:160

* inoculated with SN 1 strain.

Anti-bacterial spectrum: When the potency of the broth reached its maximum, its anti-bacterial spectrum was tested. 20 cc of the agar was poured in a Petri dish and after the coagulation the cylinders usually used for penicillin cup assay were placed and filled with the cultured broth of the streptomycetes.

Then it was placed for 48 hours at 5° C in order to diffuse the antibiotic substance sufficiently. Thereafter *E. coli*, streptomycin-fast strain of *E. coli*, streptothricin-fast strain of *E. coli*, *B. anthracis* and *B. subtilis* N.R.R.L.-B558 were streaked radially from the cup and the plate was incubated for twenty hours at 37° C. As seen in Table 1, both the streptomycin-fast and the streptothricin-fast strain of *E. coli* were much more resistant to the antibiotic substance produced in the broth than the normal strain, and *B. anthracis* was more susceptible than *B. subtilis*. This relation of susceptibilities of the five bacteria is completely same as that against streptomycin.

Extraction and some chemical characters.

The antibiotic substance is not adsorbed by activated carbon at pH 2.0, but easily at pH 6.0-8.0, and eluted from the carbon into methanol, ethanol and 15% acetone water all at pH 2.0, but not at neutral reaction. And it is precipitated from the solutions of the above-mentioned solvents by the addition of acetone or ether. In Table 3 an example of the extraction is shown.

The brownish white powder obtained by the precipitation from acid methanol solution with ether is soluble in water and methanol, and slightly in ethanol and not in the

Table 3. Extraction process. (*potency was indicated in the highest dilution enough for almost complete growth inhibition of *E. coli*).

Its potency was measured by cup method using streptomycin reineckate as the standard and found to be 224 u/mg.

other organic solvents. The extraction process of the antibiotic substance and the solubility in various solvents conform completely to those of streptomycin, so the powder obtained from acid methanol solution seems to be a mixture of the hydrochloride of the antibiotic substance and the contaminating substance. Moreover the powder shows the beautiful red colour by Sakaguchi's reaction, just same as in the case of streptomycin. As previously reported, the streptothricin group substances in the form of the powder obtained by the precipitation from methanol solution with ether indicate a brownish red colour by Sakaguchi's reaction.

Antibacterial spectrum and toxicity of the antibiotic substance

Sixteen mg hydrochloride of the antibiotic substance, which potency was found to be 224 u/mg by cup method using crystalline streptomycin reineckate as the standard, was dissolved in 3 cc sterile water and this solution was put in the cylinders standing on the agar plate, as in the case of the penicillin assay. Then the plate was placed in ice box for twenty-four hours in order to make the antibiotic substance diffuse in the agar. Then various kinds of bacteria were streaked radially from the cylinder and the plate

was incubated at 37° C for about twenty hours. The length of the inhibition zones is indicated in Fig. 1 and besides for the sake of comparison the antibacterial spectrum of streptomycin is described in the same figure. The antibacterial spectrum of the antibiotic substance in the extracted powder conforms almost completely to that of streptomycin.

Toxicity: Various amounts of the hydrochloride of the antibiotic substance which potency was 224 u/mg were injected intravenously into mice which body weight is 9-10 g. Ten mg did not induce any toxic reactions and all mice survived, but in the case of 20 mg all mice died immediately after the injection. Through the all experiment, the

Fig. 1. Antibacterial spectrum the antibiotic substance of SN1 strain, and streptomycin

later appearing toxicity, which is common to the streptothricin-group substances, was never observed.

Development of the resistance of bacteria against the antibiotic substance

Growth of a strain of *E. coli* was at first completely inhibited in the nutrient broth (from which sodium chloride was removed) with the antibiotic substance in such a dilution as 1:160,000 but it increased its resistance to this antibiotic substance, after it had been successively transferred in the nutrient broth containing the antibiotic substance, as

seen in Table 4. This resistant strain grew well in the nutrient broth (without sodium chloride) containing the antibiotic substance in the dilution 1:1000.

Table 4. Development of tolerance of *E. coli* to the antibiotic substance of SN 1 strain.

The growth of *E. coli* in the nutrient broth added with the antibiotic substance in the following dilution:

Then it was tested whether the strain which resistance to the antibiotic substance of SN-1 strain was increased became also more resistant to streptomycin and streptothricin. As seen in Table 4, growth of the normal strain of *E. coli* was inhibited in the nutrient broth (without sodium chloride) with streptomycin in such a dilution as 1:640,000, but the resistant strain grew well even in the broth added with streptomycin in the dilution 1:1000. Among streptothricin-group substances, the hydrochloride of the antibiotic substance produced by No. 9 strain described in the previous report was used in this experiment. Growth of the normal strain of *E. coli* was completely inhibited in the nutrient broth (without sodium chloride) added with this streptothricin-group substance in dilution 1:256,000. As seen in Table 4, the strain which became much more resistant against the antibiotic substance of SN-1 strain was as susceptible to the streptothricin-group substance as the normal culture. In the previous report, we inferred that resistance of *E. coli* increased much more rapidly to streptomycin than streptothricin group substances, and a streptomycin-fast strain was as susceptible to streptothricin as the normal culture,

Table 5. The tolerance of a strain of *E. coli* which became more resistant to the antibiotic substance of SN 1 strain, and the normal strain against streptomycin and streptothricin-group substance.

Strain	Highest dilution necessary for complete inhibition	
	Streptomycin	Streptothricin
The normal strain of <i>E. coli</i>	1:640,000	1:256,000
The strain of <i>E. coli</i> resistant to the antibiotic of SN 1 strain	1:<1,000	1:256,000

though a streptothricin-group-fast strain was more resistant not only to a streptothricin-group substance but also to streptomycin. In the case of the antibiotic substance of SN-1 strain, *E. coli* increases rapidly its resistance just as in the case of streptomycin, and the resistant strain becomes simultaneously more resistant to streptomycin, but not to a streptothricin-group substance. In regards to these characters the antibiotic substance of SN-1 is just same as streptomycin.

DISCUSSION

Hitherto streptomycin has been extracted only from the culture liquid of streptomyces belonging to typical *S. griseus* and seemed to be a special antibiotic product of this species. But the antibiotic substance described here seems to be identical with streptomycin, in spite of the strain producing a pink pigment.

The strains, SN-1 SN-14, SN-2, produce always a pinkish red colour substance in the asparagine-glucose agar near the submerged mycelium, though their other characters conform to *S. griseus*. According to Dr. Waksman, they must be a variant of *S. griseus*.

The antibiotic substance produced by these strains could be extracted by the same process as in the case of streptomycin or streptothricin-group substances. In the previous report we noticed that streptomycin is different from the streptothricin-group substances in the following point: (1) a streptomycin-fast strain does not increase its resistance to streptothricin-group substances, though a streptothricin-fast strain becomes more resistant not only to the other streptothricin-group substances but also to streptomycin. (2) the powder obtained from the methanol eluate by addition of ether shows a beautiful red colour in Sakaguchi's reaction in the case of streptomycin, but a brownish red colour in the case of streptothricin-group substances, (3) the lately appearing toxicity is never observed in the case of streptomycin, but usually in the case of streptothricin-group substances.

The antibiotic substance here obtained has the same character as streptomycin in the above-mentioned three points. Moreover the fact that a strain of *E. coli* which acquired the resistance to the antibiotic substance became simultaneously resistant to streptomycin, and the relation of the growth inhibiting effects against the other bacteria was same as that observed in the case of streptomycin justifies its identity with streptomycin.

Recently Waksman (5) noticed the nomenclature of streptomycin and infers to apply the term streptomycin to the chemically pure substance such as N-methyl-glucosamido-streptoside-streptomycin and to call its derivatives in their chemical names. The preparations isolated from organisms other than *S. griseus*, and showing an antibiotic spectrum and other biological and chemical characters similar to those of streptomycin is being called as streptomycin-like substance. Then the antibiotic substance presented in this paper must belong to streptomycin-like substance in his classification.

SUMMARY

- (1) The antibiotic substance of a strain of streptomyces which is a pink variant of *S. griseus* was confirmed to be identical with streptomycin.

- (2) The strain produces pinkish red pigments near the submerged mycelium in the asparagine-glucose agar.
- (3) Its identity with streptomycin was confirmed in the following points:
 - (1) It can be extracted in the same manner as in the case of streptomycin.
 - (2) Its antibacterial spectrum is same as streptomycin.
 - (3) A streptomycin-fast strain and A streptothricin-fast strain is more resistant to it than the normal strain, and if *E. coli* becomes more resistant to this antibiotic substance, then it becomes simultoneously to streptomycin, but not to streptothricin-group substances.
 - (4) The extracted hydrochloride of the antibiotic substance shows beautiful pink colour, by Sakaguchi's reaction just as in the case of streptomycin.
 - (5) Its toxicity is the same order as streptomycin, and lately appearing toxicity is never observed.

Here we should like to express our sincere thanks to Dr. Waksman for his kind and valuable advices.

REFERENCES

- 1) Waksman, S. A.: Microbial antagonisms and antibiotic substances. New York the Commonwealth Fund 1945.
 - 2) Waksman, S. A., Reilly, H. C., and Harris, D. A.: A rapid method for demonstrating the identity of streptomycin-producing strains of *S. griseus*.
 - 3) Umezawa, H., Hayano, S., and Ogata, Y.: On the differentiation of streptomycin and other resembling substance (streptothricin-group) and rapid isolation of streptomycin-producing strain. Jap. Med. Journ., 1 1948.
 - 4) Waksman, S. A.: Description of *S. bikiniensis*. J. Bact., 55, 1948.
 - 5) Waksman, S. A.: Nomenclature of streptomycin preparations. Science, 107, 233, 1948.
-