Investigation on Streptomycin Inhibitors with the Aid of a Streptomycin-dependent *Vibrio comma*

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SUMMARY: The growth and amylase activity of a streptomycin-dependent *Vibrio comma* on starch agar plates was gradually decreased with decrease of the streptomycin-concentration. The concentrations of streptomycin and the streptomycin-antagonizing properties of bacterial products also could be assayed by a cup plate method. The diameters of the zones of decomposed starch around the cups were proportional to the logarithms of streptomycin concentration. In the presence of a constant concentration of streptomycin they were inversely proportional to the concentration of bacterial antagonist. The following species and genera of micro-organisms produced antagonists (in decreasing order of their activity): *Pseudomonas* sp., *Proteus morganii*, *Clostridium botulinum*, *C. parabotulinum*, *C. welchii*, *C. septicum*, *Bacillus megaterium*, *B. subtilis*, *Rhodotorula* sp., and some strains of the genera *Escherichia*, *Shigella* and *Klebsiella*. No inhibitory effects were observed with products of *Salmonella paratyphi* A and B, *Serratia marcescens*, *Staphylococcus aureus*, *S. albus*, *Streptococcus faecalis*, *S. lactis*, *Brucella abortus*, *B. melitensis*, *Pasteurella pestis*, *P. pseudotuberculosis*, *Alkaligenes faecalis*, *Saccharomyces* sp., *Candida albicans*.

Recent investigations by Olitzki & Olitzki (1957) revealed that at low concentrations of streptomycin the amylase activity of a streptomycin-dependent strain of *Vibrio comma* was not developed. This inhibition was, therefore, used by them as an indicator in assaying streptomycin in liquid media. In the experiments reported this method was simplified by introducing a cup plate method which permitted the quantitative determination of concentrations of streptomycin and the antagonistic activities of different preparations against a fixed standard concentration of streptomycin.

The first investigations on antagonists of streptomycin were carried out by Lightbown (1950) who described a rapid method of demonstrating streptomycin antagonist activity in bacterial cultures. As indicator organisms *Bacillus subtilis* or *Staphylococcus aureus* were used. By incorporating both antibiotic and indicator organisms in nutrient agar, the antagonistic power of culture filtrates was assayed by a cup-plate method. The diameter of the zone of growth around the cup was proportional to the logarithm of the concentration of the antagonist, which was formed during the growth of *Pseudomonas aeruginosa* in chemically-defined medium and in ordinary culture medium. Lightbown (1954) isolated material produced by *Pseudomonas aeruginosa* which antagonized the activity of streptomycin, and which itself inhibited bacterial growth in low concentrations. The antagonist was produced by most strains of *P. aeruginosa* under favourable conditions. Strains of *Staphylococcus aureus*, *Escherichia coli*, *Serratia marcescens*, *Bacterium bodenheimeri*,

streptococci of Lancefield's group D, *Bacillus subtilis* and *B. pumilus* did not produce similar activity. The purpose of the work reported below was to determine whether the production of streptomycin antagonists is limited to the genus *Pseudomonas* or shared by other micro-organisms.

METHODS

Strains employed for production of inhibitors. Forty-five bacterial strains were investigated for their ability to produce antagonists to streptomycin. The vibrios used were described in detail elsewhere (Olitzki & Olitzki, 1955, 1957) and by Sulitzeanu & Olitzki (1955). The indicator organism was the streptomycin-dependent strain of *Vibrio comma*, strain Ogawa 40.

Growth of the organisms. The aerobic and facultative anaerobic strains were grown on the surface of nutrient agar in Roux bottles from an inoculum of 2×10^9 bacteria suspended in 5 ml. of saline.

The anaerobes were cultivated on a medium which contained (% w/v): 0.05, sodium mercaptoacetate; 0.025, cysteine; 2.5, enzymically-hydrolysed caseine; 0.05, tryptophan; 0.5, glucose; 0.0000005, biotin; 0.0000005, thiamine; 0.00001, *p*-aminobenzoic acid; (% v/v) 1.0, solution A; 0.1, solution B. Solution A contained (% w/v): 10.0, K_2HPO_4 ; 10.0, KH_2PO_4 . Solution B contained (% w/v): 4.0, $MgSO_4 \cdot 7H_2O$; 0.2, NaCl; 0.2, $FeSO_4 \cdot 7H_2O$; 0.2, $MnSO_4 \cdot 4H_2O$.

Production of the inhibitory substances. After 48 hr. of incubation at 37° the fluid which was still present in the bottles of the aerobic-growing organisms was removed and filtered through a Seitz pad. The bacteria which remained on the surface were suspended in a minimal volume of buffered saline solution (pH 7.4) which just permitted a complete removal of the whole bacterial growth. The bacterial suspensions were exposed for 30 min. to the action of a Raytheon Sonic Vibrator (9 keyc.), the debris centrifuged down and the supernatant fluids tested for their protein content. All extracts were brought to a dilution which contained 10 mg. protein/ml.

The anaerobic bacteria were removed by centrifugation from their medium after 24 hr. of incubation at 37°, resuspended in a small volume of the same medium to give a suspension of 10^9 bacteria/ml., incubated for 48 hr. at 37°, and the supernatant fluids examined for their inhibitory action.

Contact of streptomycin with inhibitors. One-tenth ml. of the final products (the supernatant fluids and the cell extracts) were incubated for 3 hr. with 0.9 ml. of a streptomycin solution (final streptomycin concentration of 20 µg./ml.).

Assay of activity of the resulting streptomycin solution. After a 3 hr. exposure at 37° 0.2 ml. portions of the solution were transferred to glass cups placed on the surface of a nutrient agar plate. The nutrient agar contained (% w/v): Bacto peptone, 1.0; NaCl, 0.5; Na_2HPO_4 , 0.25; Bovril, 0.3; starch 0.5; agar, 2.5; (the pH was adjusted to 8.5).

This medium was poured into plates of different sizes with a bacterial suspension to give 5×10^8 vibrios/ml. agar. After incubation for 24 hr. an

iodine solution was poured on the surface of the plates and the diameter of the white zone resulting from the decomposition of starch was measured. A concentration of 20 μg . streptomycin/ml. produced an average diameter of 20.8 mm. and by comparison with this standard the antagonist activity of the different preparations was determined (see Plate 1).

The reliability of the method used. Figure 1 shows the relationship between log concentration streptomycin in a range from 2.5 to 20.0 μg ./ml. and the diameters of the zones of decomposed starch which appeared as white zones

Fig. 1. The diameters of the zones of starch decomposition (mm.) in relation to the logarithms of the streptomycin concentrations.

by treatment of the plates with iodine. The average diameters, for 5, 10 and 20 μg . streptomycin/ml., determined from 28 repeated examinations, were 14.8, 18.3, and 20.8 mm., respectively, the respective standard deviations were ± 0.9 , ± 1.13 and ± 1.44 mm. Under these circumstances a decrease of the zone diameter from 20 to 18 mm. should indicate a 50 % and a decrease to about 15 mm. a 75 % loss in activity of streptomycin. On the other hand, there appeared in the presence of 20 μg . streptomycin/ml. and dilutions 1/32, 1/16, 1/8, 1/4, and 1/2 of an inhibitory preparation of *Pseudomonas aeruginosa* the following respective diameters of white zones (mm.): 20.8, 18.1, 14.7, 8.8, 0. According to Fig. 1 the respective concentrations of active streptomycin were exactly 20, 10, 5, 2.5 and 0 μg ./ml. Thus this experiment proved that in a certain range of dilutions the diameters of the zones of starch decomposition were inversely proportional to the logarithm of the concentration of antagonist.

RESULTS

Preparations of antagonists of different origins

Table 1 shows the effects of several antagonistic bacterial products revealed by this method. The most active strains were found in the genus *Pseudomonas*. Four out of 9 strains effected a complete inactivation, 4 others an inactivation of 75 %, and one strain of 50 %. The supernatant fluids resulting from

Table 1. *Effect of bacterial products on zone of starch decomposition produced by Vibrio comma (Ogawa 40, streptomycin-dependent) in the presence of a standard concentration of 20 µg. streptomycin/ml.*

Strain tested	Diameter of zone of decomposition (mm.) in the presence of	
	Supernatant fluids	Cell-free extracts
<i>Pseudomonas hydrophila</i>	13.0	14.0
<i>P. non-liquefaciens</i>	14.0	15.0
<i>P. fluorescens</i>	15.0	12.0
<i>Pseudomonas</i> sp. from eggs	15.0	15.0
<i>P. aeruginosa</i>	0	0
<i>Pseudomonas</i> sp.	17.0	19.0
<i>Pseudomonas</i> sp. 46/4	0	0
<i>Pseudomonas</i> sp. 56/1	0	0
<i>Pseudomonas</i> sp. 84/2	0	0
<i>Vibrio comma</i> (Inaba)	18.0	20.0
<i>V. comma</i> (Celebes)	18.5	20.0
<i>V. comma</i> (Ogawa 1)	19.5	20.0
<i>V. comma</i> (Ogawa 40)	19.0	20.0
<i>Vibrio</i> sp.	21.0	19.0
<i>Proteus vulgaris</i> V ₁	18.0	17.5
<i>P. vulgaris</i> V ₂	18.5	18.0
<i>P. morganii</i> M ₁	15.0	16.5
<i>P. morganii</i> M ₂	19.5	18.5
<i>P. rettgeri</i> R ₁	20.0	18.0
<i>P. rettgeri</i> R ₂	18.0	18.5
<i>Escherichia coli</i>	18.5	15.0
<i>Klebsiella</i> sp.	19.0	17.0
<i>Shigella flexneri</i>	20.0	16.5
<i>Clostridium parbotulinum</i> 3732	—	14.4
<i>C. welchii</i> 8798	—	13.5
<i>C. botulinum</i>	—	13.5
<i>C. septicum</i>	—	14.5
<i>C. histolyticum</i> 503	—	18.0
<i>Bacillus megaterium</i>	21.0	15.0
<i>B. subtilis</i>	20.0	17.0
<i>Rhodotorula</i> sp.	15.5	20.0

Diameter of the zone of decomposition in the presence of streptomycin 20 µg./ml. without inhibitor was 20.8 mm.

centrifugation of culture fluids showed almost the same degrees of activities as the cell-free extracts obtained after disruption of the organisms by sonic waves. However, the antagonistic actions were not limited to the genus *Pseudomonas*. Two out of 5 vibrios (Inaba and Celebes) showed some

antagonistic effects, decreasing the activity of streptomycin to about 50 % of its original value. Another group of micro-organisms able to produce inhibitor was found in the genus *Proteus*. The cell-free extracts of 6 strains decreased the activity of streptomycin to about 50 % and one strain, *Proteus morganii* even to about 75 %.

The extracts obtained by autolysing of 4 clostridia decreased the activity of streptomycin to about 75 % and of *Clostridium histolyticum* to 50 % of its original value. The supernatant fluids of 3 strains of Enterobacteriaceae (*Escherichia coli*, *Klebsiella* sp., *Shigella flexneri*) exerted a relatively weak effect. However, in the presence of their sonic extracts nearly 75 % of streptomycin was inactivated. The supernatant fluids of two cultures of the genus *Bacillus* were inactive. However, the cell-free extracts of the same strains decreased the activity of streptomycin by more than 50 %. On the other hand, the supernatant fluid of a *Rhodotorula* sp. showed some antagonistic activity, while its sonic extract was inactive. Table 2 shows results obtained with the products of other micro-organisms which were almost inactive in this test.

Table 2. Starch decomposition by the streptomycin-dependent strain Ogawa 40 in the presence of different inactive or slightly active bacterial products at a standard concentration of 20 µg. streptomycin/ml.

Strain tested	Diameter of zone of decomposition (mm.) in the presence of	
	Supernatant fluids	Cell-free extracts
<i>Salmonella paratyphi</i> A	19.0	21.0
<i>S. paratyphi</i> B	21.5	20.0
<i>Serratia marcescens</i>	18.5	22.0
<i>Staphylococcus albus</i>	20.0	18.0
<i>S. aureus</i>	21.0	18.0
<i>Streptococcus faecalis</i>	17.5	20.0
<i>S. lactis</i>	20.5	20.0
<i>Brucella abortus</i>	18.5	18.5
<i>B. melitensis</i>	18.5	19.5
<i>Pasteurella pestis</i>	20.0	18.5
<i>P. pseudotuberculosis</i>	18.0	19.0
<i>Alkaligenes faecalis</i>	18.0	18.0
<i>Saccharomyces</i> sp.	18.0	20.0
<i>Candida albicans</i>	18.0	20.0

Control of the bacterial products for growth-inhibitory activity

In order to avoid false interpretation of the results caused by growth-inhibitory bacterial products, we tested the antagonistic products for their antibiotic activity in the absence of streptomycin against the following bacteria: *Vibrio comma* (Ogawa 1, streptomycin-sensitive) which was the parent strain of the dependent strain Ogawa 40, *Bacillus subtilis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Neisseria catarrhalis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella typhi*, and *Streptococcus pyogenes*. In addition to the plate method antagonists were added to cultures of Ogawa 40 on the semi-defined medium described by Olitzki & Olitzki (1956), but no inhibition was

noted when at least 100 μg . streptomycin/ml. was present. To avoid false interpretation by an inhibitory effect of the anaerobic medium, streptomycin was brought in contact with this medium before the clostridia were inoculated. No inhibitory effect was observed.

In a final experiment (Table 3) it was shown that under the same experimental conditions as used for demonstrating the antagonism, an increase in concentration of streptomycin above the standard concentration of 20 μg ./ml.

Table 3. *Effect of increasing streptomycin concentrations on the streptomycin-inhibiting effect of different bacterial preparations*

Extract prepared from strain	Diameter of zone of decomposition (mm.) in the presence of streptomycin (μg ./ml.)				
	20	40	80	160	640
<i>Pseudomonas hydrophila</i>	14.2	18	21	—	—
<i>P. non-liquefaciens</i>	15	18	20.5	—	—
<i>P. fluorescens</i>	12.2	15	18.5	21	—
<i>Pseudomonas</i> sp. from eggs	15	18.5	21	—	—
<i>P. aeruginosa</i>	0	0	8.5	15	21
<i>Pseudomonas</i> sp. 56/1	0	0	9	15.5	21
<i>Vibrio comma</i> (Ogawa 1)	19.7	20.5	20.5	—	—
<i>Proteus vulgaris</i> V ₁	17	20	20.5	—	—
<i>P. morganii</i> M ₁	10	16	19	21	—
<i>P. rettgeri</i> R ₁	18	20	21	—	—
<i>Escherichia coli</i>	15.5	18	21	—	—
<i>Klebsiella</i> sp.	19	20.5	21	—	—
<i>Shigella flexneri</i>	17	20	21	—	—
<i>Clostridium parbotulinum</i> 3732	15	18.5	20	—	—
<i>C. welchii</i> 8798	15	19	20.5	—	—
<i>C. histolyticum</i> 503	18	19	20	—	—
<i>Bacillus megaterium</i>	15	18.5	21	—	—
<i>B. subtilis</i>	16.5	19	21	—	—

Remarks. Diameter of the zone of decomposition in the presence of 20 μg . streptomycin/ml. without inhibition was 20.7 mm.

produced less antagonism than at the lower streptomycin concentration. The highly active preparations of *Pseudomonas aeruginosa* required for the complete disappearance of the antagonism 640 μg . streptomycin/ml., while with relatively weak inhibitors from Enterobacteriaceae and Bacillaceae the inhibiting effect disappeared at 40–80 μg streptomycin/ml. These results confirm the observations made in the former experiments where a preparation of *Pseudomonas aeruginosa* diluted 1/16 inactivated 10 μg . streptomycin/ml. It was therefore expected that the undiluted preparation should inactivate at least 160 μg . streptomycin/ml.; Table 3 confirms this expectation. In the presence of the preparation from *Pseudomonas aeruginosa* and 160 μg . streptomycin/ml. there appeared a zone of diameter of 15 mm., indicating that only 5 μg . streptomycin/ml. remained active, and that 155 μg ./ml. had been inactivated. On the other hand, it was expected that preparations which even when undiluted inactivated only 10 or 15 μg . streptomycin/ml. would not give any inhibitory effect in the presence of 80 μg . streptomycin/ml. Table 3 confirms this expectation for all the weakly active preparations.

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EXPLANATION OF PLATE

PLATE 1. [Natural size]

- (1) 2.5 μ g. streptomycin/ml. (2) 5.0 μ g. streptomycin/ml. (3) 10.0 μ g. streptomycin/ml. (4) 20.0 μ g. streptomycin/ml. (5) 20.0 μ g. streptomycin/ml. + extract of *Pseudomonas* sp. (6) 20.0 μ g. streptomycin/ml. + extract of *Bacillus subtilis*. (7) 20.0 μ g. streptomycin/ml. + extract of *Proteus morganii*. (8) 20.0 μ g. streptomycin/ml. + extract of *Vibrio comma*, strain Ogawa 1.

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A. L. OLITZKI & D. GODINGER—STREPTOMYCIN INHIBITORS. PLATE 1

(Facing p. 60)