NEOMYCIN-RELATED METABOLITES PRODUCED BY STREPTOMYCES FRADIAE MUTANTS

by

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I. INTRODUCTION

The neomycin complex produced by Streptomyces fradiae consists of two isomer substances, neomycins B and C: Neomycin B is composed of neamine and neobiosamine B, while neomycin C contains neobiosamine C and neamine (Rinehart, 1964).

In the early studies on the biosynthesis of the neomycins D-glucose, L-arabinose and maltose were found to be especially effective in promoting total neomycin production. When the chemically defined medium was supplemented with alanine, glutamic acid or lysine, either alone or with D-glucose, the amounts of neomycin activity produced were practically the same, and D-glucose was shown to be converted to neomycin (Sebek, 1955).

Rinehart and coworkers (1961,1964a,1964b and 1967) repeated Sebek's experiment and confirmed the incorporation of glucose-^{14}C into neomycin and found that the ribose, deoxystreptamine, and neosamines B and C contained similar radioactivity.

The same experiment was repeated with both glucose-1-^{14}C and glucose-6-^{14}C. The level of labeling of the three nitrogen-containing fragments was almost exactly the same as that from glucose-U-^{14}C, but the activity per carbon atom was lower for ribose.

The authors carried out similar experiments with glucosamine-1-^{14}C. The results showed that the level of

incorporation into neomycin was higher with this precursor (14%) than with glucose (4%). D-ribose was only very slightly labeled.

The four sub-units of neomycin have themselves been added as labeled compounds into the growth medium. D-ribose was least incorporated (6%) but its activity was found in all four fragments. Deoxystreptamine was most incorporated, but essentially only into the deoxystreptamine sub-unit. Neosamines B and C showed intermediate incorporation (24 and 39%, respectively) and their activity appeared in all three diamine fragments.

Majumdar and Majumdar (1969,1970) isolated from the fermentation broth of S. fradiae 3535 (Waksman and Lechevalier, 1949) three phosphorylated amino-sugar compounds which were characterized as neomycin B pyrophosphate, neomycin C pyrophosphate and neomycin C dipyrophosphate. They considered these compounds as intermediates of neomycin biosynthesis, while several amino acids, particularly glycine and L-serine favored the accumulation of these compounds.

A mutant was isolated from S. fradiae 3535 which was incapable of synthesizing neomycin in the absence of added deoxystreptamine. When the sub-units streptamine and 2-epistreptamine were introduced into the medium instead of deoxystreptamine these were incorporated into new antibiotics (Shier et al., 1969).

The kanamycins, which are produced by Streptomyces kanamyceticus, are aminoglucoside antibiotics closely related to neomycin. Kanamycin A, the major compound, contains deoxystreptamine and 3-amino-3-deoxy-D-glucose as well as 6-amino-3-deoxy-D-glucose. Mutants derived from S. kanamyceticus were shown by Murase et al. (1970) to produce new compounds, whose chemical structure and biological properties differed from kanamycins A, B and C. In the medium were found, e.g., 6-amino-6-deoxy-D-glucosyldeoxystreptamine and 3-amino-3-deoxy-D-glucosyldeoxystreptamine; one contained neamine and 3-amino-3-deoxy-D-glucose and the other paromamine and an unknown sugar as well as neamine and glucose. All these compounds showed biological activity against certain bacteria.

In this paper, the isolation of mutants from S. fradiae 3535 which synthesize parts of the neomycin molecule will be reported and, in addition, the capability of one mutant to form new compounds from sub-units of neomycin and other related amino-glucosidic antibiotics.

II. MATERIALS AND METHODS

A. Analytical

Bioassays: These were performed using two layer diffusion method. The assay medium contained: Difco peptone, 0.6%; Difco yeast extract, 0.3%; Difco beef extract, 0.15%; NaCl, 0.2%; and Difco agar, 1.5%. Penassay base agar (Baltimore Biological Laboratories, Cockeyville, Maryland) supplemented with 0.2% NaCl was used also. The medium was adjusted to pH 7.9 and autoclaved at 121°C for 15 min. The test organism was Staphylococcus aureus FDA 209. 12.7 mm paper dics (Schleicher & Schuell, Inc., Keene, N.H.) were used in this agar diffusion assay. After application of the paper discs the agar plates were incubated at 37°C for about 16 hr.

Chromatography: The fermentation broth was purified after harvest as follows: concentrated HCl was added to pH 2.5; shaken for 20 min; the mycelium centrifuged off; pH raised with NaOH 6 N to 7.9, and the precipitent centrifuged off. The purified broth was passed through a column (12 x 550 mm) containing 40 ml CG 50 (NH_4^+-form). Eluation was carried out with water and NH_4OH.

Fractions from the chromatographic separation were analyzed by thin layer chromatography using silica gel (Eastman Chromatogram Sheet, cat. no. 6061) and $CHCl_3$:CH_3OH:conc.NH_4OH (1:3:2) as developing solvent.

Neomycin and related compounds were detected on chromatograms by spraying with ninhydrin (Endo and Perlman, 1972). Another solvent system was n-propanol:pyridine:acetic acid:water (15:10:3:12) (Perlman - personal communication) and the compounds were detected with ninhydrin and aniline phthalate (Macek, 1963).

In addition, descending paper chromatography was used. The developing solvent was 2-butanone:isopropanol: 6.5-N NH_4OH (8:2:3) used with Whatman no. 1 paper. The neomycin-like compounds were detected with ninhydrin (Deshmukh, et al., 1969).

Paper Ionophoresis: The ionophoresis was carried out by the method of Maeda et al. (1969) using Whatman no. 1 paper and a pH 1.8 buffer containing acetic acid:formic acid:water (20:2:78). The voltage was 50 volt/cm and operation time was 40 min.

B. Microbiological

Production of Mutants by Nitrosoguanidine and Ultraviolet Light: The method described by Delic et al. (1970) was mainly followed in producing mutants by nitrosoguanidine.

In this method the spores from a well sporulated slant of S. fradiae 3535 were suspended in sterile water. The suspension was filtered through sterile cotton and centrifuged for 15 min at 1000 x g. The spore pellet was resuspended in 1 ml of sterile water. The suspension was

transferred to a nitrosoguanidine solution, which was prepared by dissolving 90 mg of nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) (Aldrich Chemical Company, Milwaukee, Wis.) in pH 9.0, 0.05 M Tris-maleate buffer. The spore suspension after mixing in nitrosoguanidine solution was incubated at 30°C in a water bath with gentle shaking. 3.0 ml samples were drawn after the intervals of 15 and 40 min and centrifuged in the cold (4°C) as described. The spore pellet was diluted for plating (Delic et al., 1970). The medium for isolation of mutants was V-8 agar 10 ml/plate.

A modification of the described method was also used. Samples drawn from the spore-nitrosoguanidine suspension were not centrifuged but were diluted directly for plating (Shier et al., 1969).

Production of Mutants by Ultraviolet Light: The preparation of spore suspensions was similar to that described above. After centrifugation the spore pellet was suspended in 25 ml of sterile water in a Petri plate and the suspension was agitated by a magnetic stirrer. A germicidal lamp G30T8 (General Electric) placed 37 cm above the plate provided the source of ultraviolet light. Samples were removed every 30 sec and diluted before plating on the agar.

Fermentation Conditions: All fermentation experiments were carried out on a rotary shaker (Controlled Environment Incubator Shaker; New Brunswick Scientific Co., Inc., New Brunswick, N.J.). An incubation temperature of 28-30°C was used and the speed of the shaker was 300 rpm. Inocula for production consisted of 10 ml of cultures incubated in 1" x 6" test tubes for three days. For production of neomycin and related metabolites the incubation time was 5 days unless otherwise noted. Fermentation was carried out in 250 ml Erlenmeyer flasks with 100 ml of medium.

Composition of Media: "V-8" agar was used as a stock culture medium and the medium for isolation of mutants. It contained V-8 juice (Campbell Soup Company, Camden, N.Y.) 500 ml, moist baker's yeast 10 g, agar 20 g, and distilled water 500 ml. The medium was adjusted to pH 7.0 with KOH and autoclaved at 121°C for 20 min.

The medium for neomycin production contained soy bean meal 30 g, D-glucose (Matheson Coleman & Bell) 30 g, $CaCO_3$ (powder) 10 g, in 1000 ml of distilled water. The pH was adjusted to 7.2 after boiling and cooling. The medium was autoclaved at 121°C for 20 min.

Evaluation of Antibiotic Potential of the Mutant's Metabolites: After the chromatograms were developed and neomycin-like compounds detected with ninhydrin their activity was determined on the agar plates as described

above using S. aureus FDA 209 as a test organism.

C. Chemicals Used

2-deoxystreptaminehydrochloride (Schering Laboratories)

D-glucosaminehydrochloride (Eastman Organic Chemicals)

Neomycin B sulfate (S. B. Penick Company)

Neamine and neobiosamine prepared by Dr. T. Endo from Neomycin B.

Paromamine base (Parke, Davis & Company)

Tris-(hydroxymethyl)-aminomethane and maleic acid (Aldrich Chemical Co., Inc.)

III. RESULTS AND DISCUSSION

A. Isolation of Mutants

1. Natural variants

In our search for mutants with unusual characteristics we used as criteria the decrease in biosynthesis of antibiotic. The activity was measured directly from the medium after purification. If a notable change in activity had occurred, the fermentation sample was analyzed by thin layer chromatography to see whether the usual mixture of neomycins B and C are present. For detection of activity of the spots bioautography was run.

100 natural variants obtained from the original culture of Streptomyces fradiae 3535 were isolated to find which produced only a portion of the neomycin molecule. While the neomycin production assay varied between 500-2000 µg/ml, the components were shown by TLC to be neomycin B and C. Isolate no. 83 was selected for further investigation as representing the wild type (original) strain.

2. Mutations by ultraviolet light

Over two hundred colonies were tested from every level of radiation (see Figure 1) but no mutants of the type looked for were discovered. Generally the production of antibiotic activity remained high after ultraviolet treatment.

3. Mutations by nitrosoguanidine

Nitrosoguanidine turned out to be more effective than ultraviolet light. All mutants for further investigation were isolated after NTG treatment. The method of Delic et al. (1970) gave the killing curve of spores as presented in Figure 2 whereas modification of the method as presented by Shier et al. (1969) gave the curve in Figure 3.

Four mutants were selected for further study on the basis of decreased antibiotic production. Of these strains 136a and 136II produced no activity and strains 152a and 174IV produced small amounts of neomycin-like activity.

B. Metabolites from Selected Strains

The fermented broth from each strain was purified further after incubation and acid-base treatment in a GC 50 column (12 x 500 mm) by eluating with water and NH_4OH. The eluants were analyzed by thin layer and paper chromatography as well as by ionophoresis. The activity of the various compounds on the chromatography sheet was detected by bioautography.

The results obtained by thin layer and paper chromatography and ionophoresis are given in Tables 1, 2 and 3, respectively. The compounds showing antibacterial activity have been underlined. An asterisk indicates that the compound was detected only by the bioautography.

The R_g-values (the migration distance of other compounds was compared to that of glucosamine) of known reference compounds are given in the first columns.

The strains 136a and 136II were very much alike. Both contained neobiosamine, R_g-value 1.10 and glucosamine, as well as an unknown compound with R_g-value 0.95. The compound with the R_g-value of 0.80-0.85 was the main compound produced by both the strains, but it was present also in the fermented media from other strains, even though in minute amount. This compound had R_g-values of 0.40 by paper chromatography and 1.55 by ionophoresis. It was biologically inactive and gave an intensive purple color with ninhydrin. It was clearly separated from deoxystreptamine by paper chromatography and ionophoresis.

Both strains 136a and 136II produced a compound with R_g-value of 0.65-0.70 lacking activity, while all other strains produced it as well, but it seemed to be anti-bacterially active.

Strain 136a also produced neomycin (R_g-value of 0.50) as identified by thin layer chromatography. On paper chromatography spots for both strains were in the position of neomycin with the R_g-value of 0.15. Each one was active.

Strain 83, a "natural" isolate from the parent culture, seemed to produce neobiosamine, glucosamine and the unknown compound with R_g-value of 0.85 in trace amounts. These were noted only by thin layer chromatography. The

main compound was neomycin B (R_g-value 0.50) while neomycin C (R_g-value of 0.65) was produced as well. Both these compounds were antibacterially active. The other three compounds were detected by bioautography. The compound with the R_g-value of 0.75 was obviously neamine. The two other R_g-values of 0.30 and 0.25 belong perhaps to mono-N-actyl (in the neamine fragments) derivatives of neomycin B and C (Rinehart, 1964). According to paper chromatographic analysis samples of fermentation broths for strain 83 contained two active compounds, neamine (R_g = 0.20) and neomycin (R_g = 0.10). Ionophoresis showed three active compounds, neomycin (R_g = 1.55) and two additional ones with R_g-values 1.30 and 1.15, which were detected only by bioautography.

The metabolites from strains 152a and 174IV were very similar to those of strain 83. These strains produced neobiosamine and glucosamine as well as an unknown compound with R_g-value of 0.90-0.95. Another unknown compound with R_g-value of 0.80 was present in a greater amount in these strains than in strain 83. The anti-bacterially active compounds were the same as those produced by strain 83 according to thin layer and paper chromatography, but ionophoresis showed higher R_g-values for the two active compounds of strain 152a and 174IV than for that of strain 83.

The unknown compound with R_g-value of 0.85 produced by strain 136a was hydrolyzed in HCl 6-N at 110°C for 6 hr. The hydrolysate was evaporated to dryness and analyzed by thin layer chromatography. Comparison of these results with those obtained from the hydrolysate of neomycin B showed that the unknown compound contained deoxystreptamine and an unidentified compound, which gave intensive purple color with ninhydrin. It was neither neosamine B nor C. No ribose was detected as in the hydrolysate of neomycin B.

The capability of strain 136a to synthesize active compounds when sub-units of neomycin or related compounds were added to the medium was studied.

Paromamine as well as paromamine and neobiosamine were added to the culture medium at a concentration of 500 µg/ml medium after three days incubation in test tubes. Incubation was continued for 6 more days. No antibacterial activity was detected in samples taken during the incubation period.

When neamine alone and neamine with neobiosamine were added in the concentration of 500 µg/ml medium after three days incubation, the antibacterial activity decreased in both cases during the ensuing incubation of 6 days (Figure 4). The explanation for the phenomenon may be that the streptomycete metabolizes the active neamine.

Neamine was clearly poisonous for strain 136a in the concentration of 500 µg/ml medium, because the amount of

living hypha decreased drastically in its presence.

The same experiment was repeated as such, but the additions of neamine and of a neamine-neobiosamine mixture were done after 1 day's incubation. The changes in antibacterial activity are presented in Figure 5. The antibacterial activity in both cases decreased sharply at first, then slowed further but continued in neamine containing medium until the fifth day, whereas activity increased sharply after a 4-day incubation in the neamine and neobiosamine containing medium. The effect of medium composition on production of antibacterial activity was also investigated, when neamine as well as neamine and neobiosamine were added to the medium in Erlenmeyer flasks after 1 day's incubation. The medium after incubation was treated as above and analyzed by thin layer and paper chromatography as well as ionophoresis. The results are presented in Tables 1, 2 and 3. Thin layer chromatography showed that neobiosamine was present in the medium only, when neamine and neobiosamine were added. The same thing was shown by paper chromatography. Glucosamine was present in both media according to thin layer chromatography and ionophoresis. The unknown compound with R_g-value of 0.80 by TLC, 0.40 by paper chromatography and 1.55 by ionophoresis was the main compound in both media. Neamine was present in both media, but when only neamine was added the amount of this compound was greater.

The other active compound with R_g-value of 0.60 was also present and it was thought to be neomycin C. The amount of this compound seemed to be greater when both neamine and neobiosamine were added in the medium. The compound with R_g-value of 0.45 was likely neomycin B. Its amount was so small that it was detected only by bioautography.

In accordance with paper chromatography both active compounds were neomycin, but ionophoresis gave an R_g-value of 1.40, which was very close to that of the compound found in the medium of strains 152a and 174IV.

According to these results, it seems that the lack of neobiosamine in the medium, when only neamine was added, prevented the biosynthesis of antibacterially active compounds. Neamine was converted into the compound (R_g-value 0.80) characteristic of strain 136a. When both neamine and neobiosamine were present, neamine was converted in greater amounts into the compound with an R_g-value of 0.60 (obviously neomycin C) and perhaps into neomycin B.

C. Summary

Nine aminoglucosidic compounds were isolated and detected from the growth medium of Streptomyces fradiae 3535. Five of the compounds had antibacterial properties as measured by inhibition of S. aureus, that is, three unidentified ones in addition to neamine and neomycin.

A mutant strain was isolated from S. fradiae by treatment with nitrosoguanidine which produced a new compound. It was shown to contain deoxystreptamine and an unidentified substance giving intensive purple color with ninhydrin. When neamine was added to the fermentation medium of the mutant after 1 day's incubation, the antibacterial activity due to neamine decreased during the course of incubation. Addition of a mixture of neamine and neobiosamine resulted in an increase of antibacterial activity.

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Table 1

Thin layer chromatographic analysis of cationic metabolites from S. fradiae mutants and strain 83

Reference compounds	R_g [1] of the compounds produced by the strains							
	Standards	83	136a	136II	152V	174IV	136a[2]	136a[3]
Neobiosamine	1.10	1.15	1.10	1.10	1.15	1.10	-	1.05
Glucosamine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		-	0.95	0.95	0.90	0.95	0.90	0.90
		0.85	0.85	0.85	0.80	0.80	0.80	0.80
Deoxystreptoamine	0.80	-	-	-	-	-	-	-
Neamine	0.75	0.75+	-	-	0.75+	0.75+	0.70	0.70
		0.65	0.70	0.65	0.65	0.65	0.60	0.60
Neomycin B	0.55	0.50	0.50+	-	0.50	0.50	0.45+	0.45+
		0.30+	-	-	0.35+	0.35+	-	-
		0.25+	-	-	0.25+	0.25+	-	-

Developing solvent: $CHCl_3$:CH_3OH:conc.NH_4OH (1:3:2).
Detection with ninhydrin.
Underlined = antibacterially active.
+ = detected only by bioautography.
1) R_g = motility with reference to glucosamine.
2) = neamine added to the fermentation medium.
3) = neamine and neobiosamine added to the fermentation medium.

Table 2

Paper chromatographic analysis of cationic metabolites from S. fradiae mutants and strain 83

Reference compounds	R_g [1] of the compounds produced by the strains							
	Standards	83	136a	136II	152a	174IV	136a [2]	136a [3]
Neobiosamine	1.95	-	-	-	-	-	-	2.05
Glucosamine	1.00	-	-	-	-	-	-	-
Deoxystreptamine		-	0.70	-	-	-	-	-
	0.50	-	-	-	-	-	-	-
		-	0.40	0.40	-	-	0.40	0.40
Neamine	0.20	0.20	-	-	0.20	0.20	0.20	0.20
Neomycin B	0.15	0.10	0.15	0.15	0.10	0.10	0.10	0.10

Developing solvent: 2-butanone:iso-propanol:6.5-N NH_4OH (8:2:3).
Detection with ninhydrin.
Underlined = antibacterially active.
1) R_g = motility with reference to glucosamine.
2) = neamine added to the fermentation medium.
3) = neamine and neobiosamine added to the fermentation medium.

Table 3

Paper ionophoresis analysis of cationic metabolites from S. fradiae mutants and strain 83

Reference compounds	R_g [1] of the compounds produced by the strains							
	Standards	83	136a	136II	152a	174IV	136a [2]	136a [3]
Deoxystreptamine		-	-	-	-	-	1.95	1.95
	1.80	-	-	-	-	-	-	-
Neamine	1.70	-	-	-	-	-	1.70	1.70
		-	1.55	1.55	-	-	1.55	1.55
Neomycin B	1.55	1.55	-	-	1.55	1.55	-	-
		-	-	-	1.45+	1.45+	1.40	1.40
Neobiosamine	1.45	-	1.45	1.45	-	-	-	-
		1.30+	-	-	1.40+	1.40+	-	-
		1.15+	-	-	-	-	-	-
Glucosamine	1.00	1.00	1.00	1.10	1.05	1.00	1.05	1.05

Buffer: pH 1.8; acetic acid:formic acid:water (20:2:78); voltage 50 volt/cm.
Detection with ninhydrin.
Underlined = antibacterially active.
\+ = detected only by bioautography.
1) R_g = motility with reference to glucosamine.
2) = neamine added to growing culture.
3) = neamine and neobiosamine added to growing culture.

Figure 1. Survivors of S. fradiae spores after UV treatment.

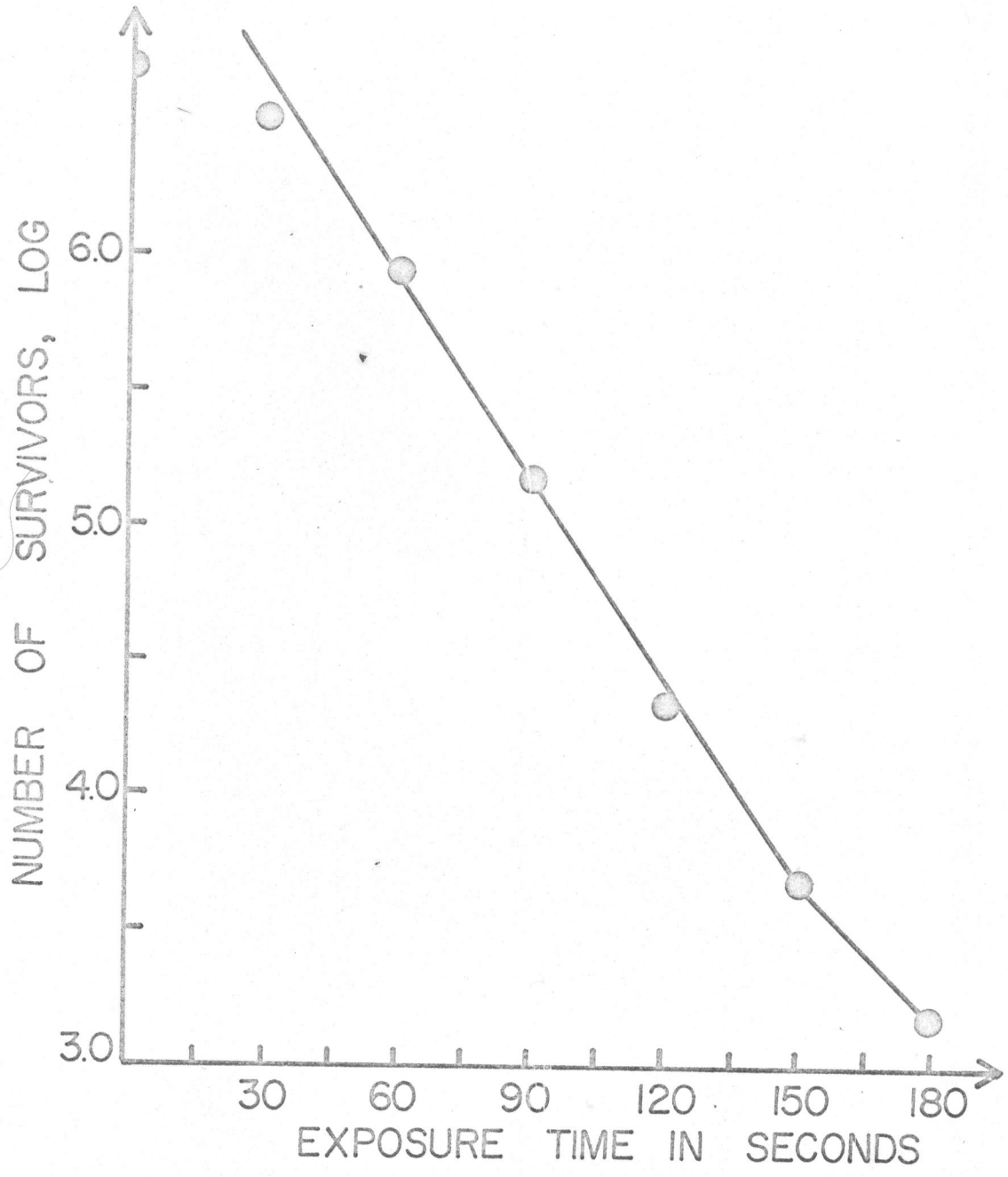

Figure 2. Survivors of S. fradiae spores after NTG treatment.

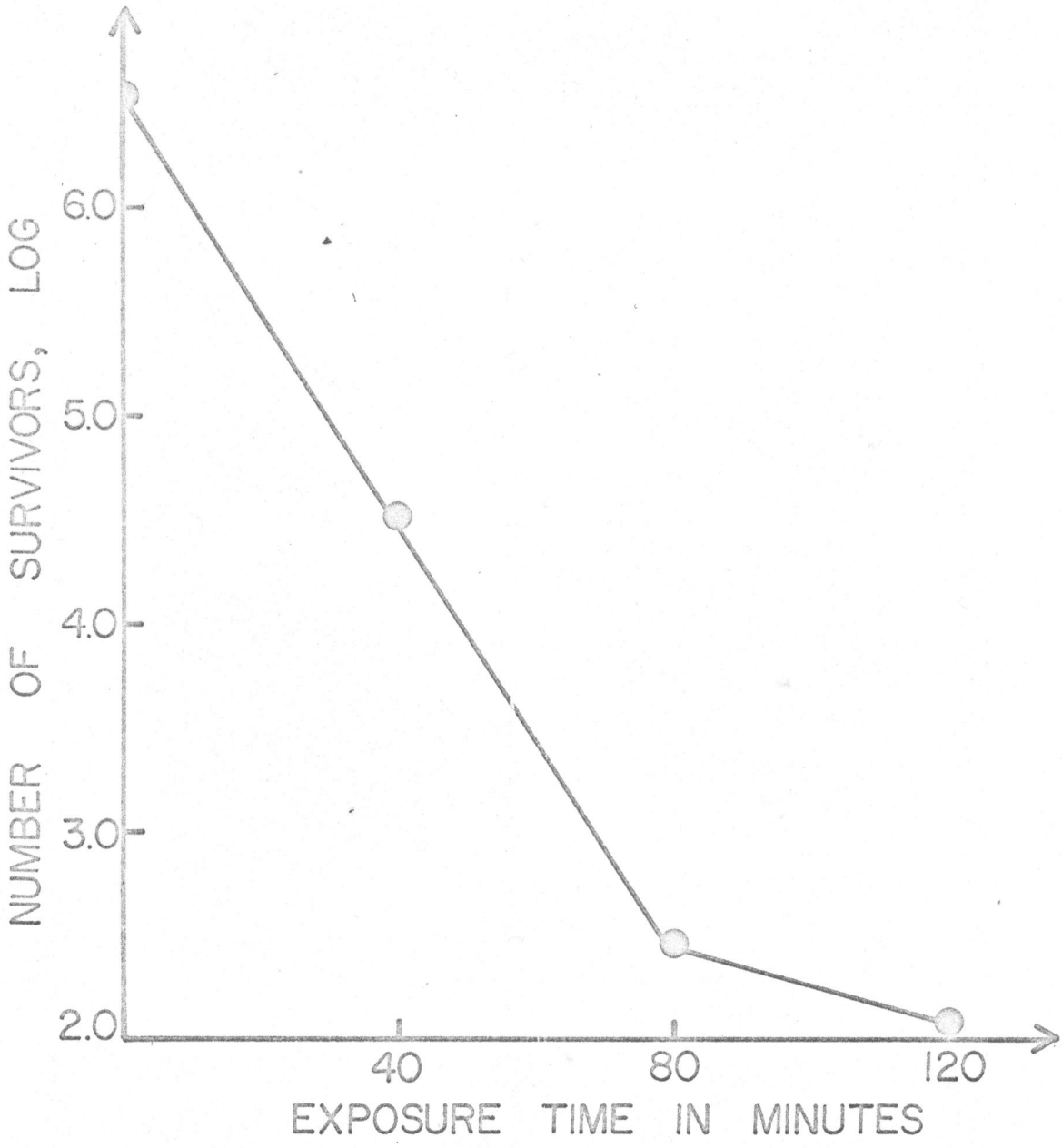

Figure 3. Survivors of S. fradiae spores after NTG treatment.

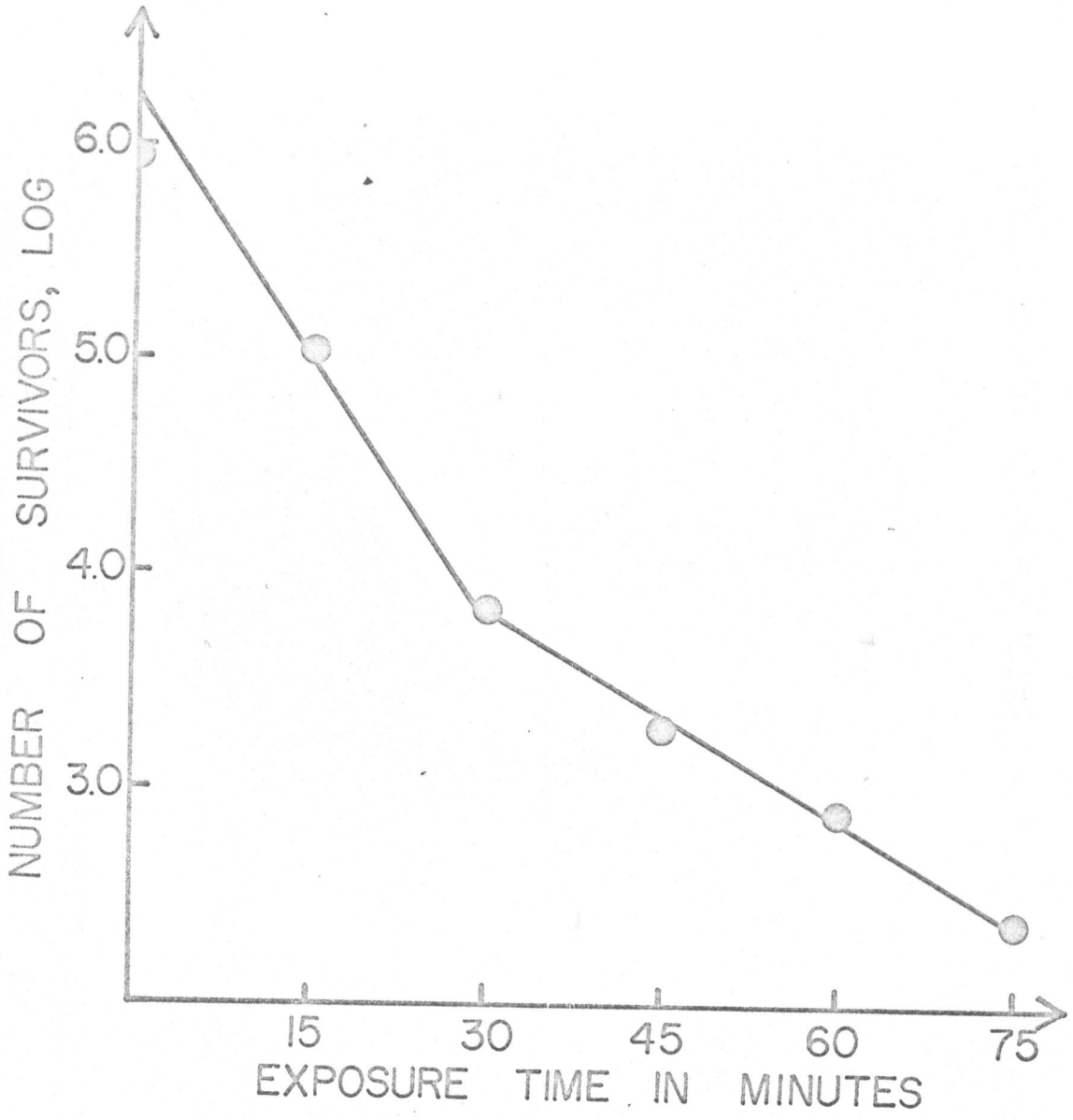

Figure 4. Changes of activity during incubation, neamine and a mixture of neamine and neobiosamine added after three days incubation.

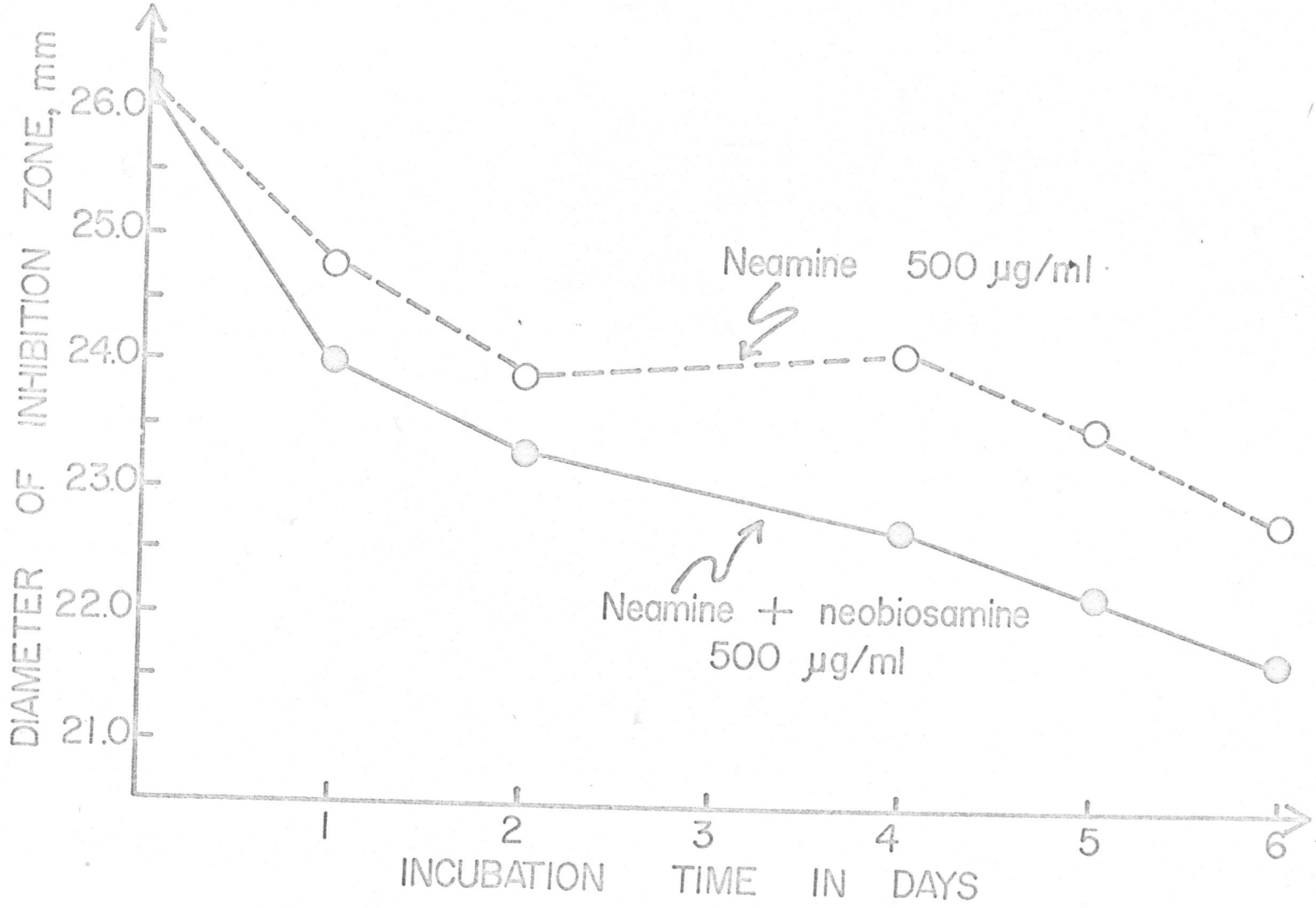

Figure 5. Changes of activity during incubation, neamine and a mixture of neamine and neobiosamine added after one day's incubation.