JCI The Journal of Clinical Investigation

NEOMYCIN—PRODUCTION AND ANTIBIOTIC PROPERTIES

Selman A. Waksman, Hubert A. Lechevalier, Dale A. Harris

J Clin Invest. 1949;28(5):934-939. https://doi.org/10.1172/JCI102182.

Research Article

NEOMYCIN—PRODUCTION AND ANTIBIOTIC PROPERTIES [1, 2, 3]

By SELMAN A. WAKSMAN, HUBERT A. LECHEVALIER, AND DALE A. HARRIS

(From the Department of Microbiology, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N. J.)

ANTIBIOTIC SURVEYS

During the last 10 years, a large number of antibiotics which are active against Gram-negative and Gram-positive bacteria, mycobacteria, rickettsiae, and certain of the larger viruses were isolated (1) from various species and strains of the genus *Streptomyces*. This served to focus attention on the actinomycetes as potential producers of antimicrobial agents that might possess promising chemotherapeutic properties. The fact that nearly 20 to 50% of these organisms possess antimicrobial activities served to heighten this interest. Numerous surveys have been conducted. Particular attention has been paid to the formation and isolation of antibiotics that would possess the following characteristics: (a) High activity against Gram-negative bacteria and mycobacteria; (b) antibiotic action against streptomycin-resistant bacteria; (c) low toxicity to animals; (d) other desirable properties, such as activity against rickettsiae, viruses, tumors and phages.

In our own laboratories, large numbers of actinomycetes were isolated from various natural substrates and tested for their antimicrobial activities. The agar-cross-streak method, frequently supplemented by other procedures, was commonly used for screening purposes. Those cultures that proved to be most active were selected and grown in liquid media. Only a small number of these were found capable of giving rise to active antibiotics. The most promising were selected for further studies. For example, of some 300 freshly isolated cultures tested by the above method, only 10 exhibited activities that justified further study. All these cultures proved to be highly active against mycobacteria.

Only few of the cultures, however, that gave good activity by the agar-streak method yielded filtrates which possessed corresponding potency. This may be due to a variety of factors, such as the formation by a single organism of more than one antibiotic or the production of different antibiotics under different conditions of culture. One of these cultures proved to be highly promising and was selected for more detailed investigations. This culture was entered into the Collection as No. 3535. The nature of its antimicrobial spectrum, as compared to corresponding spectra of known antibiotic-producing organisms and measured by the agar-cross-streak method, is shown in Table I. The spectrum of 3535 was found to be quite distinct from those of the streptomycin-producing *Streptomyces griseus* and streptothricin-producing *Streptomyces lavendulae*. The streptomycin-resistant strain of *Escherichia coli* and the Bodenheimer organism were found to be sensitive to the antibiotic activity of 3535; further, the antibiotic

TABLE I

Antibiotic spectrum of culture No. 3535 as compared to spectra of streptothricin-producing (S. lavendulae) *and streptomycin-producing* (S. griseus) *organisms*

Agar-cross-streak method. Zone of inhibition, in mm.

Test organisms*	Culture No. 3535	*S. griseus*	*S. lavendulae*
		3463	3516
Escherichia coli SS	18	Active	20
E. coli RS	25	None	Active
E. coli DS	NG	GG	NG
Bacillus subtilis	21	Active	22
B. mycoides	20	Active	3
B. cereus	18	Active	5
Staphylococcus aureus	19	Active	19
Bodenheimer's culture	19	None	Active
Pseudomonas aeruginosa	3	LA	LA
Proteus vulgaris	22	Active	Active
Mycobacterium 607	24	25	25
M. avium	20	20	23
M. ranae	15	25	23
M. phlei	28	27	26

* SS = streptomycin-sensitive; RS = streptomycin-resistant; DS = streptomycin-dependent; NG = no growth; GG = good growth; LA = limited activity.

[1] Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11–12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

[2] Paper in the Journal Series, New Jersey Experiment Station, Rutgers University—The State University of New Jersey, Department of Microbiology.

[3] These investigations were supported by a grant from the Rutgers Research and Endowment Foundation.

TABLE II

Effect of $CaCO_3$ upon the production of antibiotic 3535

Incubation, days	No $CaCO_3$		$CaCO_3$, 1%	
	pH	*u./ml.*	*pH*	*u./ml.*
2	5.6	25	6.8	35
3	5.8	47	6.7	88
5	6.1	68	7.8	172
6	7.7	156	8.0	200

substance produced by 3535 cannot replace streptomycin in making possible the growth of the streptomycin-dependent strain of *E. coli*. The activity of 3535 upon *Bacillus mycoides* and *Bacillus cereus* serves to differentiate this antibiotic from streptothricin. The various mycobacteria were found to be sensitive alike to the antibiotic produced by 3535 and to the other two antibiotics.

METHODS

Formation and isolation of antibiotic 3535

The next steps in the study of the new antibiotic comprised the development of suitable media for its maximum production. Fortunately, media previously found to be best for the production of streptomycin proved suitable. A medium containing 5 gm. peptone, 5 gm. meat extract, 5 gm. glucose, 5 gm. NaCl, 1,000 ml. tap water gave good results. Various modifications were later introduced. The reaction of the medium tended to become acid during the early stage of growth; this favored early lysis of the culture. This effect was overcome by addition of $CaCO_3$, as brought out in Table II. This could also be accomplished by increasing the peptone content of the medium to 1 or even 2%, particularly when vegetable peptones, such as soya peptone, were used, or by reducing the sugar content. If sufficient peptone were used, the glucose content could also be increased, with a delaying but favorable effect on the production of the antibiotic. The results of a typical experiment are given in Table III. The addition of a small amount of zinc (1 mg. to 10 mg. $ZnSO_4 \cdot 7H_2O$ per liter) was later found to exert a favorable effect, yielding culture filtrates with an activity of 500 to 1,000 u./ml., as shown in Table IV. Larger amounts of zinc proved to be injurious, however. Meat extract could be replaced by yeast extract. Glucose could be replaced by starch in shaken cultures. When distilled water was used, no activity was obtained unless zinc was added.

Aeration proved to be an important factor in the production of the antibiotic, the amount of air required being apparently less than for the production of streptomycin and streptothricin. The reduced aeration could be accomplished by increasing the volume of the medium in the

TABLE III

Influence of composition of medium on the production of antibiotic 3535

Constituents of medium, gm./liter	N	N_1	N_2	N_3	No. 3
Meat extract	5		5	5	5
Soya peptone	10	10	20	10	
Bacto peptone					5
NaCl	5	5	5	5	5
Glucose	10	10	10	20	10
Tap water	1000	1000	1000	1000	1000

Medium	Incubation, days					
	3		4		6	
	*pH**	*u./ml.*†	*pH*	*u./ml.*	*pH*	*u./ml.*
N	8.3	100	8.4	240	8.7	240
N_1	8.2	<10	8.2	10	8.4	75
N_2	8.6	150	8.6	240	9.0	150
N_3	7.4	15	7.8	240	7.9	100
No. 3	8.4	<10	8.5	10	8.8	10

* Initial pH of medium 6.5–6.8.

† u./ml. = *E. coli* dilution units, as measured by agar streak dilution method.

TABLE IV

Influence of composition of improved media upon the production of antibiotic 3535

Dilution units per 1 ml. of medium; cup readings made against a neomycin standard

Nature of medium	pH	*E. coli*	*B. mycoides*	*S. aureus*	*B. subtilis*	Cup readings *u./ml.*
three days incubation						
N_2*	7.8	50	240	150	750	35
N_4†	7.6	50	300	300	>1,000	24
N_8	7.5	25	100	75	500	28
N_9	7.4	150	>1,000	>1,000	>1,000	154
N_{7a}	6.5	<30	<30	<30	240	<5
four days incubation						
N_2	8.3	50	300	240	1,000	56
N_4	7.8	150	300	300	2,400	85
N_8	8.3	75	240	300	>1,000	68
N_9	8.0	500	2,400	1,500	>10,000	316
N_{7a}	6.0	<10	30	10	>100	<5
six days incubation						
N_2	8.7	100	300	240	1,000	56
N_4	8.7	200	1,000	900	>3,000	128
N_8	8.7	90	240	240	1,000	74
N_9	8.7	900	7,500	3,000	>10,000	407
N_{7a}	8.4	30	150	100	>300	24

* N_2 = Soya peptone—2%, glucose—1%, NaCl—0.5%, meat extract—0.5%, tap H_2O—1000 ml.

† N_4 = N_2 minus meat extract; N_8 = N_2 minus NaCl; N_9 = N_2 + 10 mg./liter $ZnSO_4 \cdot 7H_2O$; N_{7a} = N_2 + 2% glucose.

shaken flasks or by adding a small amount of agar to the medium. A higher temperature (35° C.) was found to be more favorable for rapid formation of neomycin than a lower temperature (26–28° C.); however, a higher level of activity was reached upon prolonged incubation at 28° C.

In measuring the potency or concentration of the new antibiotic, the agar plate dilution method was first used, since it not only allowed determination of the total activity but also tended to establish the antibacterial spectrum of the culture. The agar-diffusion or cup method, which has proved so successful in measuring the potency of other antibiotics, required certain modifications and a well defined standard. This is brought out in the following summary of the effect of different concentrations of antibiotic 3535 upon its antibacterial activity, as measured in terms of streptomycin units:

Concentration of antibiotic 3535	Activity, as measured in terms of streptomycin
mg./ml.	*u./mg.*
1.00	10
0.10	18
0.01	94

When measured by the agar-streak dilution method, the same preparations gave 30 *E. coli* units per 1 mg.

The antibiotic was removed from the broth by procedures similar to those previously found to be effective in the isolation of streptothricin (2) and streptomycin (3).

RESULTS

Antimicrobial properties of antibiotic 3535

The first crude preparations gave an antibacterial spectrum which was quite characteristic of this antibiotic. This is brought out in Table V. One would expect from the results of the agar-cross-streak tests that the new antibiotic would be active alike upon the streptomycin-sensitive and the streptomycin-resistant organisms, as well as upon the streptothricin-sensitive and -resistant organisms. This was actually found to be the case. The high activity of the new antibiotic upon the various mycobacteria proved to be especially interesting. When the sensitivity of the human pathogenic culture of *Mycobacterium tuberculosis* H37Rv and of the streptomycin-resistant strain H37RvR was determined by turbidimetric procedures in the Dubos Tween medium, both cultures were found to be equally sensitive. The new antibiotic was similar to streptomycin in its lack of activity upon fungi.

The differences in the antimicrobial spectra of streptomycin and the new antibiotic, combined with certain chemical differences between the two antibiotics, emphasized the fact that we were dealing with a new type of antibiotic substance. It was, therefore, designated as *Neomycin.*

TABLE V

Comparative antibacterial spectra of neomycin and streptomycin

Amounts required to inhibit growth of organism in 1 ml. of culture

Organism	Neomycin*	Streptomycin†
	u./ml.	*μg./ml.*
Aerobacter aerogenes	0.625	0.5–2.5
Bacillus anthracis	0.156	0.375
B. mycoides	0.1–0.5	0.1–3.8
B. subtilis	0.02–0.1	0.12–1.0
Brucella abortus	1.25–5.0	0.5–3.75
B. melitensis	0.625–2.5	0.5
B. suis	0.312–2.5	0.5
Clostridium perfringens	>10.0	>104
Corynebacterium diphtheriae	0.156	0.375–3.75
Escherichia coli	1.25–2.5	0.3–3.75
E. coli R‡	1.5–5.0	>1,000
Hemophilus influenzae	1.25–2.5	1.56–5.0
H. pertussis	2.5	1.25–3.0
Klebsiella pneumoniae	0.312–0.625	0.625–8.0
K. pneumoniae R	0.312	>1,000
Malleomyces mallei	>10.0	10–>10.0
Mycobacterium avium	0.1–0.3	10
M. phlei	0.05–0.078	0.12
M. tuberculosis	<0.5	1.0–5.0
M. tuberculosis R	<0.5	>100
Neisseria intracellularis	1.25–2.5	5.0
Pasteurella pestis	0.625	0.75–1.5
P. tularensis	0.156	0.15–0.3
Phytomonas pruni	0.1	0.25
Proteus vulgaris	1.25–2.5	0.4–3.0
Pseudomonas aeruginosa	12.5–25.0	2.5–25.0
Salmonella typhosa	0.1–0.625	1.0–37.5
S. schottmülleri	0.4–0.7	2.0
Sarcina lutea	2.5	0.25
Serratia marcescens	1.25	1.0
Shigella paradysenteriae	0.25–0.5	0.25–3.75
Staphylococcus aureus	0.156–0.625	0.5–>16.0
Streptococcus faecalis	5.0	50.0
Vibrio comma	2.5	6.0–37.5
Various fungi	>10.0	>10.0

* Based on results obtained by F. Heilman at Mayo clinic; O. Graessle at Merck Institute; and in our own laboratories.

† Results reported by Waksman, S. A., and Schatz, A., Am. Pharm. Assoc., 1945, **34**, 273.

‡ Streptomycin-resistant.

Neomycin was found to be less favorable to the development of resistant strains of bacteria on contact with it than is usually found to be the case with streptomycin. A 20-hour-old agar culture of *E. coli* was suspended in water and plated out in nutrient agar containing varying amounts of neomycin. Of 22 billion cells added to each plate, only very few colonies developed. When pieces of agar were removed from the plates and added to sterile media, only the 5 u./ml. plate gave any bacterial growth; the 10 u./ml. and 25 u./ml. agar

plates gave no growth, thus pointing to the high bactericidal properties of neomycin. Figure 1 and Table VI illustrate the difference in the development of bacterial resistance to neomycin and streptomycin.

Plates containing varying amounts of neomycin were streaked with streptomycin-sensitive, -resistant and -dependent strains of *E. coli* (4); the first two strains were found to be sensitive alike to neomycin, and the last made no growth. This established further the marked difference in antibacterial behavior of neomycin and streptomycin. A comparison of the sensitivity of many strains of the same organism to neomycin revealed considerable variations. Most strains of *E. coli,* for example, were sensitive to 2.5 u./ml.; one

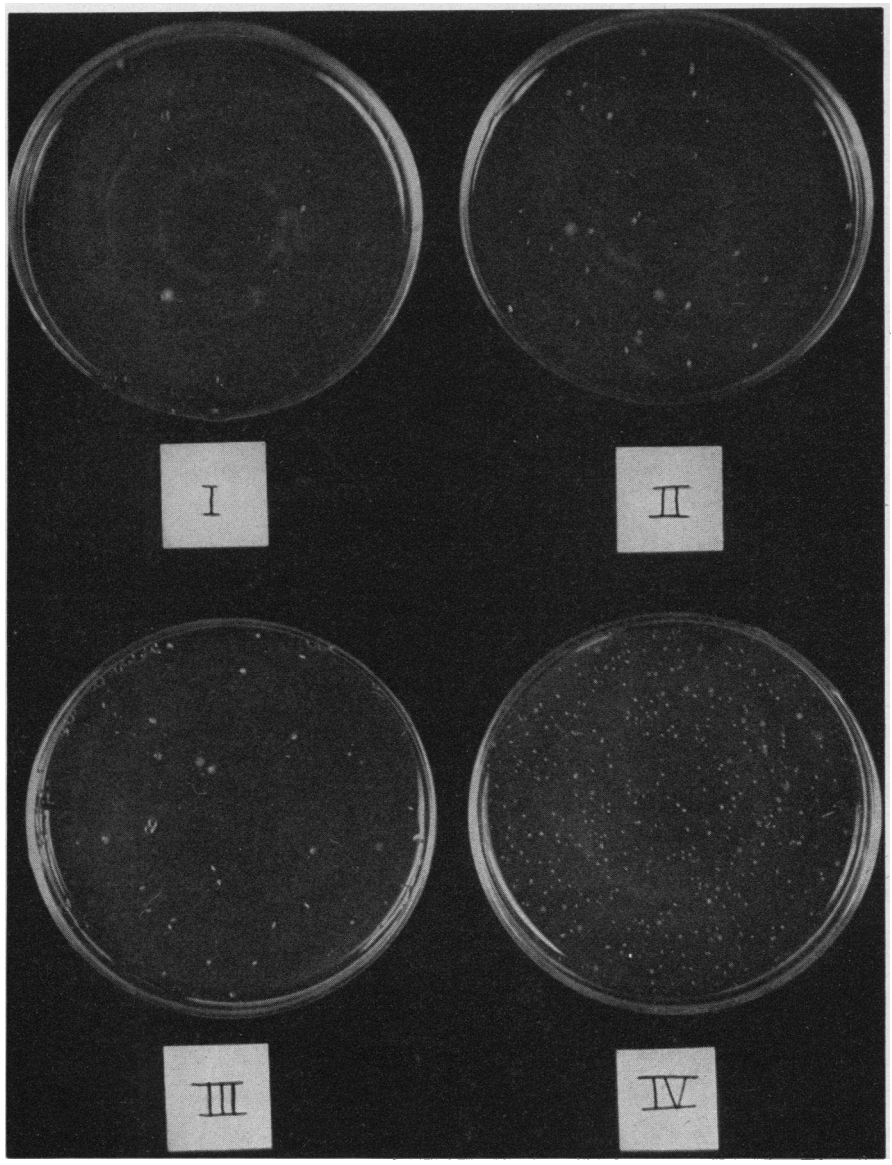

FIG. 1. RELATIVE SURVIVAL OF *E. coli* CELLS IN AGAR MEDIA CONTAINING NEOMYCIN AND STREPTOMYCIN

Number of cells added per plate I and II 66.7×10^6. Number of cells added to plates III and IV 66.7×10^7. Plates I and III contain 4 u. neomycin/ml.; plates II and IV contain 4 u. streptomycin/ml.

TABLE VI

Survival of E. coli *in plates containing varying concentrations of neomycin and streptomycin*

Incubation of plates	Neomycin (*u./ml.*)				Streptomycin (*μg./ml.*)			
	2	4	6	8	2	4	6	8
	Colonies developing on plate from 1 ml. of bacterial suspension*							
hrs.								
24	700	8	0	0	21,000	270	20	0
48	1,500	65	1	<1	4,600,000	560	70	26
72	1,700	108	2	<1	6,700,000	640	130	70
120	1,700	108	2	<1	7,400,000	640	130	70

* 1 ml. of 24-hour-old bacterial suspension contained 246 million cells.

strain, however (ATCC 6880), was resistant to 5 u./ml.

When broth or agar cultures of various bacteria containing sufficient neomycin to inhibit growth were incubated for longer periods, no further development of the bacteria occurred, thus pointing to the stability of this antibiotic, as contrasted to aureomycin, for example. Similar results were obtained with saprophytic mycobacteria and the pathogenic *M. tuberculosis*.

Identity of neomycin-producing organism 3535

The neomycin-producing culture was found to be quite distinct in its cultural and other properties from the other known antibiotic-producing species of *Streptomyces*. Its growth on synthetic and organic media was yellowish to brownish, but no soluble pigment was formed, thus placing it among the non-chromogenic forms. Aerial mycelium was readily produced on synthetic media; it was pigmented at first white, turning to rose-pink or flesh-pink or seashell pink (light-russett–vinaceous, according to Ridgway, XXXIX b–9″). On certain media, the mycelium tended to be patchy, gradually covering the surface of the vegetative growth. The sporulating hyphae were usually straight, either forming no spirals or only an occasional loose spiral. Among the type cultures already described in the literature, the above description was found to fit best with the organisms described by Waksman and Curtis as *Actinomyces* (*Streptomyces*) *fradiae* (5).

No. 3535 produced excellent growth in shaken cultures; it frequently tended to undergo lysis, especially in poorly buffered media. It has not been established as yet whether the lysis of the culture is due to infection with phage or to the action of an autolytic enzyme. In contrast to streptomycin, once neomycin was produced, however, it remained in the medium and was not readily destroyed.

Physical and chemical properties of neomycin

A detailed survey of the physical and chemical properties of neomycin will be presented elsewhere. It is sufficient to summarize some of these properties here.

Neomycin is highly resistant to the action of micro-organisms. It is heat-stable and is resistant to the action of acid (pH 2.0) at the temperature of boiling water.

Neomycin is favored in its antibacterial activity by an alkaline reaction of the medium. The best results were obtained with a pH of 7.0–8.0 of the agar and a pH of 7.0 of the buffer. The presence of glucose in the test medium reduces the potency of the antibiotic by favoring either acid production or growth of test organism.

Cysteine has apparently no marked effect upon the activity of neomycin. When 1 to 10 mg. of cysteine are added to 50 u. of neomycin in a phosphate buffer at pH 7.0 and solutions allowed to stand at room temperature for three to 20 hours, only a slight loss in activity occurs.

SUMMARY

The formation of a new antibiotic, designated as neomycin, by a culture of *Streptomyces* (No. 3535) closely related to *S. fradiae* is reported.

Neomycin is produced, under shaken or submerged conditions, in media similar to those used for the production of streptomycin by *S. griseus*. The culture tends at first to form acid and undergo lysis. This can be prevented by addition of $CaCO_3$ to sugar-rich media, or by reducing the sugar content of the medium, or by increasing the peptone content. Addition of a small amount of zinc has a favorable effect.

Neomycin belongs to the basic group of antibiotics, which includes a number of substances already described in the literature.

Neomycin is heat-stable. It is also stable to the action of micro-organisms. It is favored in its activity by an alkaline reaction of the medium.

It is not favored by the presence of glucose in the medium.

Neomycin is active against a large variety of bacteria, including Gram-positive and Gram-negative, as well as acid-fast, forms. It is active alike against streptomycin-sensitive and streptomycin-resistant strains of bacteria, including those of *M. tuberculosis* var. *hominis*. It is not active against fungi.

Neomycin is not only bacteriostatic but also strongly bactericidal. It does not readily allow development of resistant strains of bacteria among the sensitive forms.

ACKNOWLEDGMENT

The authors are indebted to Mr. W. P. Iverson of this laboratory for making the determinations of the relative developments of bacterial resistance to neomycin and to streptomycin; to Dr. A. Swart and Miss D. Hutchison for preparing and isolating some of the neomycin used in these studies.

BIBLIOGRAPHY

1. Waksman, S. A., Antibiotics. Biol. Rev., 1948, **23**, 452.
2a. Waksman, S. A., and Woodruff, H. B., Streptothricin, a new selective bacteriostatic and bactericidal agent, particularly active against gram-negative bacteria. Proc. Soc. Exper. Biol. & Med., 1942, **49**, 207.
b. Hutchison, D., Swart, E. A., and Waksman, S. A., Production, isolation, and antimicrobial, notably antituberculosis, properties of streptomycin. VI. Arch. Biochem., 1949, **22**, 16.
3. Schatz, A., Bugie, E., and Waksman, S. A., Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc. Soc. Exper. Biol. & Med., 1944, **55**, 66.
4. Iverson, W. P., and Waksman, S. A., Effect of nutrients upon growth of streptomycin-sensitive, -resistant and -dependent strains of *Escherichia coli*. Proc. Soc. Exper. Biol. & Med., 1948, **69**, 586.
5. Waksman, S. A., and Curtis, R. E., The actinomycetes of the soil. Soil Sci., 1916, **1**, 99.