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Neamine, an Antibacterial Degradation Product of Neomycin

BY BYRON E. LEACH AND CHARLOTTE M. TEETERS

This work was undertaken in an attempt to determine the structure of an acid degradation product of neomycin and, ultimately, to elucidate the structure of this antibiotic. Neomycin has been degraded with acid to yield a crystalline antibacterial agent for which the name neamine is proposed. Analytical data indicate an empirical formula of $C_6H_{12-14}N_2O_3$ or possibly the dimer $C_{12}H_{26}N_4O_6$. A method is given for the preparation of neamine which consists of acid hydrolysis of neomycin, adsorption on Amberlite IRC-50 and elution, chromatography over carbon and, finally, crystallization from ammoniacal methanol. The preparation and properties of the crystalline N,N'-dibenzoyl-, pentaacetyl- and amorphous pentabenzoyl derivatives are described. The results of countercurrent distribution and paper chromatographic studies are also given. The antibacterial properties of neamine are compared with those of neomycin.

Introduction

Neomycin, an antibiotic substance described by Waksman and Lechevalier,[1] has been degraded with acid to yield a crystalline base for which we propose the name neamine. Neamine has been found to be active against a variety of Gram-positive and Gram-negative organisms, but not as active as the parent compound against most of the organisms tested. To our knowledge, this is the first instance in which degradation of an antibiotic molecule gave another substance with pronounced antibacterial properties.

The neomycin used in these studies was prepared from culture filtrates of *Streptomyces fradiae* (No. 3535) by a process which will be published as a separate paper.[2] The neomycin sulfate was degraded by heating under reflux with 6 *N* sulfuric acid. The sulfate ions were removed with barium hydroxide and the neamine was adsorbed on Amberlite IRC-50 in the sodium form. The active fraction was eluted with *N* hydrochloric acid and freeze-dried. Chromatography of the product over carbon gave relatively pure neamine hydrochloride. Crystalline neamine was obtained from ammoniacal methanol; m.p. (dec.) 256°, specific rotation $[\alpha]^{25}D + 123°$.

Elementary analyses and molecular weight determinations indicate an empirical formula of $C_6H_{12-14}N_2O_3$ for neamine. However, molecular weight determinations on the pentaacetyl derivative cryoscopically in water and in camphor does not exclude the possibility of the dimer $C_{12}H_{26}N_4O_6$. Potentiometric titration indicates that the nitrogens are basic. Since all of the nitrogen is liberated with nitrous acid, primary amino groups are indicated.

Neomycin after hydrolysis with acid yields at least one-third of its weight as neamine based on bioassays.

Neamine shows no absorption in the ultraviolet between 212 and 360 mμ. The infrared spectrum (see Fig. 1) indicates the presence of OH and/or NH and C–O and/or C–N groups. The absence of ketone, aldehyde, ester, carbonyl, lactone or free carboxyl groups is also indicated. Paper chromatography studies, using a *n*-butanol–acetic acid–water mixture show that neamine is different from neomycin (see Fig. 2).

Crystalline neamine reacted with benzoyl chloride to give a crystalline N-benzoyl derivative. Treatment of neamine with acetic anhydride and pyridine yielded a crystalline pentaacetyl derivative. An amorphous pentabenzoyl derivative was also prepared.

Neamine has been subjected to countercurrent distribution studies in the Craig all glass apparatus,[3] using *n*-butanol, water and 2-ethylbutyric acid buffered to *p*H 6.9 with sodium hydroxide. Neamine has a distribution coefficient of 1.78 in this system. The curve (see Fig. 3) was obtained by employing the single-withdrawal technique. The unsymmetrical curve is to be expected from the distribution coefficient and the small number of units in the train.

Periodic acid oxidation of neamine did not produce any acid or base in 24 hours. Neamine consumed 2.8 moles of periodic acid in 3 hours and approximately 4 moles in 24 hours. N,N'-Dibenzoylneamine required 2.6 moles of periodic acid in 3 hours and no acid was produced, even after 24 hours.

Antibacterial studies on neamine indicate that it is active against Gram-positive and Gram-negative organisms, including the tuberculosis organism. Results of these studies are given in Table I along with neomycin for comparison. The slopes of the bioassay curves (see Fig. 4) for neamine and neomycin, using *B. subtilis* as the test organism, differ markedly. The data indicate that the antibacterial activity of neamine is different from that of neomycin.

TABLE I

ANTIBACTERIAL SPECTRUM STUDIES ON NEAMINE AND NEOMYCIN

Organism	Neamine	Neomycin
	Expressed in micrograms per ml. required to inhibit	
Br. bronchiseptica	3.3	0.5
S. aureus	0.13	.017
E. coli	1.0	.1
B. subtilis	1.0	.01
S. schottmuelleri	1.0	.1
E. typhosa	0.17	.033
K. pneumoniae	0.5	.05
Proteus vulgaris	<0.33	.1
M. tuberculosis (607)	5.0	.33

In mice, neamine has an LD_{50} of 320 mg. per kg. intravenously and 1250 mg. per kg. subcutaneously. Mice survived a daily dose of 900 mg. per kg. subcutaneously for 14 days.

Acknowledgments.—The authors are indebted to Drs. G. F. Cartland and J. S. Evans for their

(1) S. A. Waksman and H. A. Lechevalier, *Science*, **109**, 305 (1949).

(2) B. E. Leach, W. H. DeVries, H. A. Nelson, W. G. Jackson and J. S. Evans, THIS JOURNAL, **73**, 2797 (1951).

(3) A. Weissberger, "Technique of Organic Chemistry," Vol. III, Interscience Publishers, Inc., New York, N. Y., 1950, pp. 285–292.

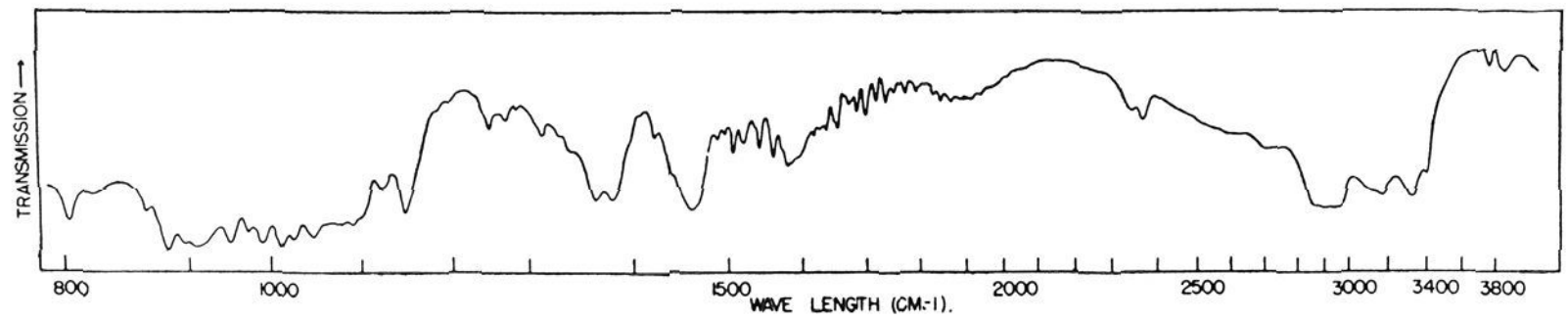

Fig. 1.—Infrared absorption spectrum of neamine measured in liquid petrolatum (Nujol) suspension, using a Perkin–Elmer Model 12C Spectrometer.

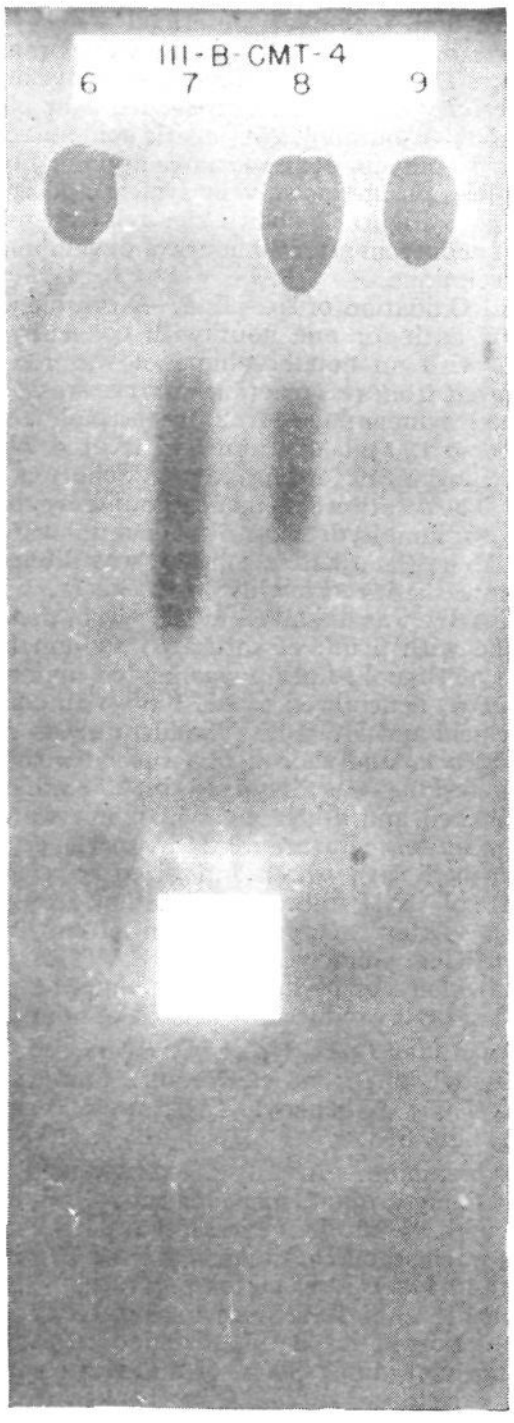

Fig. 2.—Paper chromatography of neamine and neomycin using 50% *n*-butanol, 25% acetic acid and 25% water as the developing solvent at room temperature; development period 60 hours; zones 6 and 9, 8 mcg. of neomycin; zone 7, 16 mcg. of neamine; zone 8, 8 mcg. of neamine and 8 mcg. of neomycin. A one-inch square of paper is shown on the chromatogram.

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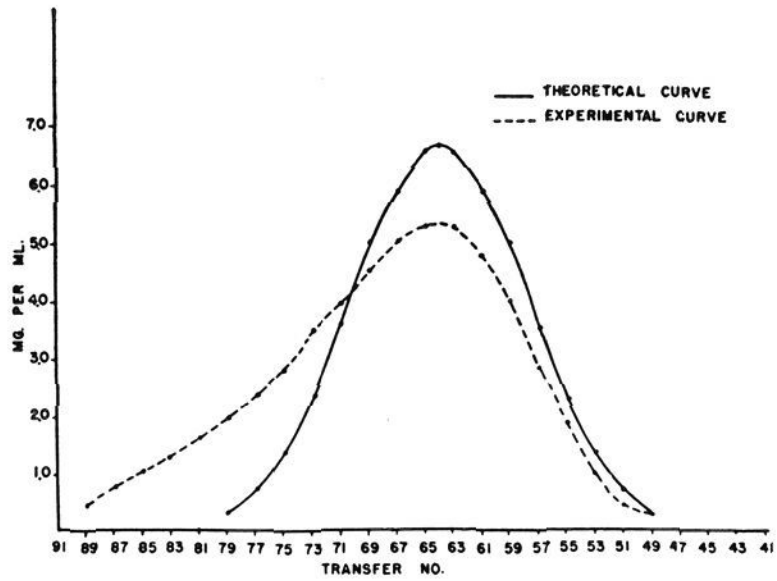

Fig. 3.—Distribution pattern of neamine.

Physics Department for the infrared spectrum, ultraviolet studies and microanalytical data.

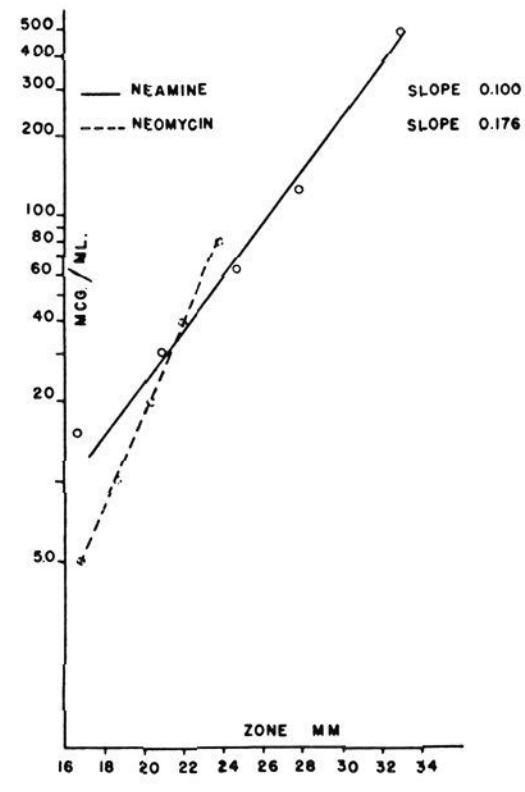

Fig. 4.—Slopes of the assay curves for neamine and neomycin using *B. subtilis* as the test organism. The log of the concentration in mcg. per ml. is plotted against the diameter of the zone of inhibition expressed in mm.

Experimental

The neomycin used in these studies was prepared from culture filtrates of *Streptomyces fradiae* (No. 3535). This culture was supplied to us by Dr. S. A. Waksman. The fermentation broth was harvested by a process which will be published as a separate paper.[2]

Hydrolysis of Neomycin Sulfate.—Neomycin sulfate (500 g.) was dissolved in 2400 ml. of 6 *N* sulfuric acid and heated under reflux for 7 hours. Considerable charring occurred during the hydrolysis. The reaction mixture was allowed to cool overnight and diluted to approximately 6 l. with water. The excess sulfuric acid was removed as barium sulfate. This required 2–3 kg. of barium hydroxide to raise the *p*H to 7.5. The barium sulfate was removed by filtration and the precipitate was washed thoroughly with water. The filtrate and washings were combined giving 11.6 l. of solution containing 273 g. of solids.

Amberlite IRC-50 Process.—The entire 11.6 l. of solution described above was passed over a 2 inch × 18 inch bed of Amberlite IRC-50 in the sodium form at a flow rate of 10 ml. per minute (0.5 ml. per sq. cm. per minute). After a wash with water, the activity was eluted with 1.2 *N* hydrochloric acid. The main band of activity contained 143 g. of solids in 1280 ml. of solution.

Carbon Chromatogram.—A 4-inch Pyrex column was packed with a slurry of 1430 g. of Darco G-60 and 1430 g. of Dicalite. The liquid hold-up was 6200 ml. The aqueous solution above containing 143 g. of solids in 1280 ml. was poured on top of the carbon column and developed with water. The flow rate was 25 ml. per minute using 12 lb. of air pressure. The eluates were collected and assayed as tabulated below:

TABLE II

CARBON CHROMATOGRAPHY OF NEAMINE

Fraction	Volume, ml.	*p*H	Total g. per fraction	α observed	$[\alpha]^{25}D$
0[a]	1280	6.7	143.0	....	
1	5525	...	1.08	0	
2	300	7.0	3.48	0	
3	300	6.9	6.21	0	
4	300	6.9	6.60	0	
5	300	6.6	7.05	0	
6	300	4.9	18.60	+3.90	+66.4
7	300	4.8	24.60	+6.70	+86.1
8	300	4.8	20.10	+5.48	+86.1
9	300	4.9	12.60	+3.50	+83.5
10	300	5.0	7.71	+2.14	+87.8
11	300	5.1	4.74	+1.32	+88.0
12	300	5.2	1.77	+0.51	+91.0
13	300	5.4	0.90	+0.25	+87.7
14	300	5.5	.66	+0.19	+91.0
15	300	5.5	.57	+0.14	+77.7
16	300	5.6	.48	+0.11	+72.5
17	4000	...	2.72	....	
Total g. recovered			119.87		

[a] Starting material.

Column fractions 6–17 were combined and freeze-dried to yield 89.5 g. of material.

Crystallization of Neamine.—A 43.8-g. aliquot of the above dried product was dissolved in 50 ml. of concentrated aqueous ammonia and diluted to 4 l. with commercial methanol. Dry ammonia was bubbled into this solution until crystallization had begun. The mixture was then refrigerated overnight. The crystals were collected, washed thoroughly with anhydrous methanol and dried *in vacuo* to yield 27.3 g. The yield was 10% by weight from neomycin. The compound decomposes in a capillary tube at 256°. The specific rotation is $[\alpha]^{25}D$ +123° (*c*, 0.5% in water). An analytical sample was prepared by recrystallization from an ethanol–water mixture.

Anal. Calcd. for $C_6H_{14}N_2O_3$: C, 44.43; H, 8.70; N, 17.28; amino N, 17.28; n.e., 81; m.w., 162.19. Found: C, 44.66, 44.92; H, 8.13, 8.06; N, 17.35, 16.95; amino N, 17.45; n.e. (potentiometric titration), 81 ± 1; m.w. (cryoscopic in β-naphthol), 165, 177.

There are no OCH_3, $C\text{–}CH_3$, or $N\text{–}CH_3$ groups in neamine as determined by the Zeisel, Pregl and Kuhn–Roth methods, respectively. The molecule consumes 2.8 moles of periodic acid in 3 hours and approximately 4 moles in 24 hours.

Quantitative Estimation of the Neamine Content of Neomycin.—One gram of neomycin sulfate was heated under reflux for 4 hours with 50 ml. of *N* sulfuric acid. The excess acid was neutralized to *p*H 8.0 with 25% sodium hydroxide solution. This solution was assayed on the *B. subtilis* plate using crystalline neamine as the standard. The results indicated that 0.33 g. of neamine was produced.

Chemical and Physical Properties of Neamine

Infrared Spectrum.—Neamine shows (see Fig. 1) a characteristic infrared curve indicating OH and/or NH and C–O and/or C–N absorption. A single band at 1603 cm.$^{-1}$, and the absence of a band near 1510 cm.$^{-1}$ indicates that this compound is not a polyamide. No C=O absorption of any kind was detected between 1603 and 1800 cm.$^{-1}$, indicating the probable absence of carbonyl, ester, lactone and free carboxyl groups in this compound.

Paper Chromatography.—Papergrams run for 16 hours by the down-flow technique described by Peterson and Reineke[4] gave only one zone, with R_f values ranging from 0.05 to 0.10. The zone was ninhydrin-positive and showed antibiotic activity on a *B. subtilis* seeded agar plate. The solvent was 50% *n*-butanol, 25% acetic acid and 25% water by volume. Neomycin does not move appreciably from the point of application in this solvent system and is separated from neamine. Figure 2 shows the relative positions of neamine and neomycin after 60 hours of development by the down-flow technique.

Nitric Acid Oxidation of Neamine.—Neamine was heated on the steam-bath for one hour with concentrated nitric acid diluted with an equal volume of water. The only product isolated from the reaction mixture was oxalic acid.

N,N′-Dibenzoylneamine.—A 2.0-g. sample of neamine was dissolved in 100 ml. of water and treated with 17.4 g. of benzoyl chloride, according to the Schotten–Baumann procedure. The solution was made alkaline by the addition of 12 g. of sodium hydroxide dissolved in water. After stirring one hour, the alkaline solution was filtered and the residue was washed thoroughly with water. The polybenzoyl derivative was dissolved in 120 ml. of methanol and made alkaline with 6 ml. of saturated sodium hydroxide. The alkaline methanol solution was heated under reflux for 4 hours, filtered, neutralized to *p*H 6.85 with concentrated hydrochloric acid and filtered. The filtrate was evaporated to dryness *in vacuo* and thoroughly washed with ether and water. The residue was dissolved in 200 ml. of boiling methanol, filtered and added to 1 l. of hot water. After standing at room temperature for 24 hours the crystals were collected, washed with water and dried to yield 3.14 g. (69% yield) of N,N′-dibenzoylneamine, m.p. 323–326° (microblock). After recrystallization from methanol–water the melting point was 327–328°. This compound consumed 2.6 moles of periodate in 3 hours at room temperature and did not produce any acid in 24 hours.

Anal. Calcd. for $C_{20}H_{22}N_2O_5$: C, 64.86; H, 6.00; N, 7.58; N, benzoyl, 56.75; m.w., 370.40. Found: C, 64.80; H, 5.91; N, 7.50; N-benzoyl, 47.0; m.w. (cryoscopic in β-naphthol), 365, 393.

Pentaacetylneamine.—A 1.0-g. sample of crystalline neamine was finely ground, suspended in 75 ml. of pyridine and treated with 25 ml. of acetic anhydride. The reaction mixture was warmed on the steam-bath for 30 minutes and allowed to stand 4 days at room temperature. The pyridine and excess acetic anhydride were removed *in vacuo*. The residue was dissolved in 42 ml. of chloroform and passed over a column containing 30 g. of aluminum oxide. The column was developed with 2% methanol in chloroform. The main band obtained from this column was dried *in vacuo* and crystallized from ethyl acetate yielding 1.39 g. of crystals melting at 256°. Recrystallization of 300 mg. from ethyl acetate and methylcyclohexane (3:1) afforded 180 mg. of crystals melting at 261°.

Anal. Calcd. for $C_{16}H_{24}N_2O_8$: C, 51.61; H, 6.49; N, 7.52; O-acetyl, 34.7; total acetyl, 57.8; m.w., 372.37. Found: C, 52.08; H, 6.62; N, 7.68; O-acetyl 34.0; total acetyl, 48.1; m.w. (cryoscopic in β-naphthol), 361, 387; (cryoscopic in water), 592; (Rast camphor), 502, 657.

Pentabenzoylneamine.—A 0.5-g. sample of neamine was suspended in 50 ml. of dry pyridine and treated with 15.7 g. of benzoyl chloride. The resulting solution was stirred at room temperature for 24 hours. The pyridine was removed

(4) D. H. Peterson and L. M. Reineke, THIS JOURNAL, **72**, 3598 (1950).

in vacuo and the residue was dissolved in 100 ml. of methanol. The benzoyl derivative was precipitated by the addition of 300 ml. of water. The precipitate was collected and dissolved in 300 ml. of ether. The ethereal solution was washed four times with 100-ml. portions of 5% sodium bicarbonate, twice with 100-ml. portions of 5% hydrochloric acid, twice with water, and was dried over sodium sulfate. The ether was evaporated to dryness, the residue was dissolved in 50 ml. of ethyl acetate and the pentabenzoyl derivative was precipitated with heptane as an amorphous powder. The yield was 890 mg. of pentabenzoylneamine melting at 135–140°.

Anal. Calcd. for $C_{41}H_{34}N_2O_8$: C, 72.09; H, 5.02; N, 4.10; total benzoyl, 76.2. Found: C, 71.18, 71.38; H, 5.72, 5.48; N, 3.69, 3.93; total benzoyl, 67.2.

Countercurrent Distribution of Neamine.—A 1.0-g. sample of crystalline neamine was distributed in a 41-tube train[5] using a system containing *n*-butanol, water, 5% 2-ethylbutyric acid and the *p*H of the resulting mixture was adjusted to 6.9 with sodium hydroxide. The neamine emerged as a single band from the end of the train which was operated by the single-withdrawal technique. The peak appeared in tube 64; the distribution coefficient is thus calculated to be 1.78. A withdrawal curve is always slightly skewed and with a distribution coefficient of 1.78 the curve tends to be even more unsymmetrical. The theoretical curve (probability curve) does not fit perfectly, therefore, with the experimental values. Figure 2 shows the curve obtained by reading the optical rotation of alternate fractions. The biological activity followed the optical rotation. The data indicate that neamine is a single component when distributed countercurrently under the conditions described above.

Biological Assay of Neamine.—Neamine and neomycin are assayed by an adaptation of the paper-disc method described by Loo, *et al.*,[6] for the assay of streptomycin using *B. subtilis* as the test organism. The agar plates are prepared with only a seed layer of *B. subtilis* to make the assay more sensitive. The assay curves for neamine and neomycin are given in Fig. 4. The log of the concentration is plotted against the diameter of the zone of inhibition in millimeters. The results indicate that neamine diffuses faster in agar than neomycin, consequently, it is a different antibiotic substance.

(5) The all-glass Craig countercurrent distribution apparatus was made by the Physics Department, The Upjohn Company, Kalamazoo, Michigan.

(6) Y. H. Loo, P. S. Skell, H. H. Thornberry, J. Ehrlich, J. M. McGuire, G. M. Savage and J. C. Sylvester, *J. Bact.*, **50**, 701 (1945).

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The Isolation and Characterization of Neomycin

BY BYRON E. LEACH, WILLIAM H. DEVRIES, HARRISON A. NELSON, WILLIAM G. JACKSON AND JOHN S. EVANS

A method is described for the preparation of neomycin sulfate. This method gives a product approaching ultimate purity which is satisfactory for chemical, pharmacological and clinical studies.

Introduction

Neomycin has been described by Waksman and Lechevalier[1] as an antibiotic substance produced by culture 3535, a strain of *Streptomyces fradiae*. It is very active against a variety of Gram-positive and Gram-negative bacteria including streptomycin-resistant strains.[1–4] It is also noteworthy that microörganisms do not readily develop resistance to the action of this antibiotic. The term neomycin applies to the antibacterial fraction of the culture broth and should not be confused with the antifungal fraction described by Swart, *et al.*,[5] to which the name fradicin has been assigned. The purpose of this paper is to describe the isolation, purification and some of the chemical properties of the neomycin that has been produced in these laboratories.

Early studies on neomycin concentrates by Swart, *et al.*,[6] indicated the presence of several antibacterial components for which the term "neomycin complex" was introduced. Peck and co-workers[7] have reported the isolation of neomycin A. Regna and Murphy[8] described a preparation which they called neomycin B. These reports have raised the question of the identity of the various neomycin preparations that have been submitted for clinical study. In an effort to clarify this problem, Swart, *et al.*,[9] have analyzed commercial preparations by the counter-current distribution technique. They interpret their results as indicative of at least three very closely related substances which are grouped under their definition of neomycin.

The purified neomycin preparations described in this paper have been subjected to a countercurrent distribution system different from that employed by Waksman's group.[9] The results, and also those obtained by paper chromatography, indicate the presence of only one active component. This is the type of neomycin which has been distributed from this Laboratory for clinical tests.

Neomycin, meeting these criteria of homogeneity, is extremely stable toward alkali since it withstands 18 hours of heating under reflux with excess barium hydroxide. This indicates a greater stability than previously reported by Waksman, *et al.*[4] Neomycin is stable at *p*H 2.0 at room temperature for at least 24 hours, but heating with 1 *N* or 6 *N* mineral acid results in extensive degradation with charring, particularly when 6 *N* acid is used. This degradation mixture still retains some biological activity against the *B.*

(1) S. A. Waksman and H. A. Lechevalier, *Science*, **109**, 305 (1949).

(2) S. A. Waksman, J. Frankel and O. Graessle, *J. Bact.*, **58**, 229 (1949).

(3) S. A. Waksman, H. Lechevalier and D. A. Harris, *J. Clin. Investigation*, **28**, 934 (1949).

(4) S. A. Waksman, E. Katz and H. Lechevalier, *J. Lab. Clin. Med.*, **36**, 93 (1950).

(5) E. A. Swart, A. H. Romano and S. A. Waksman, *Proc. Soc. Exp. Biol. Med.*, **73**, 376 (1950).

(6) E. A. Swart, D. Hutchison and S. A. Waksman, *Arch. Biochem.*, **24**, 92 (1949).

(7) R. L. Peck, C. E. Hoffhine, Jr., P. Gale and K. Folkers, THIS JOURNAL, **71**, 2590 (1949).

(8) P. P. Regna and Frances X. Murphy, *ibid.*, **72**, 1045 (1950).

(9) E. A. Swart, H. A. Lechevalier and S. A. Waksman, Abstracts of 118th Meeting Am. Chem. Soc., p. 33C (Sept., 1950).