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).

KALAMAZOO, MICHIGAN RECEIVED DECEMBER 21, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

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).

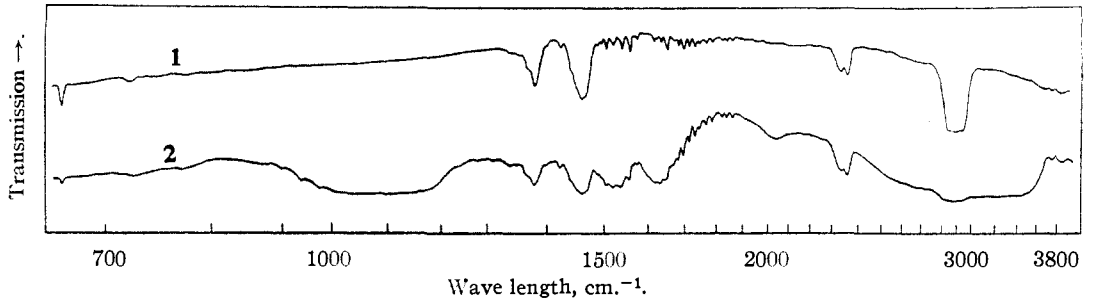

Fig. 1.—Infrared absorption spectrum: 1, liquid petrolatum, thickness 0.05 mm.; 2, neomycin sulfate suspended in liquid petrolatum.

subtilis assay organism. The residual activity remaining after acid hydrolysis has been isolated as a crystalline base which differs from neomycin both chemically and bacteriologically. We have proposed the name "neamine" for this degradation product which will be described in a separate paper.[10] This is the first instance to our knowledge where an antibiotic has been degraded to give a new substance with marked antibacterial properties.

Neomycin does not show any absorption in the ultraviolet between 212 and 360 mμ. The infrared spectrum (Fig. 1) shows typical polyamide type absorption, and some absorption due to bending vibrations of free amino groups. There is no evidence of aromatic rings. Neomycin forms crystalline salts with *p*-hydroxyazobenzene-*p'*-sulfonic acid and with ammonium reineckate. The hydrochloride and sulfate are amorphous. Neomycin and its acid hydrolysates give a typical color with ninhydrin, an atypical color with Pauly reagent, negative Sakaguchi and maltol tests. The purified neomycin described in this paper has been chromatographed over carbon. The chromatography is unique in that water alone serves as solvent and eluting agent. We have been unable to increase the potency of these preparations by conversion to the crystalline *p*-hydroxyazobenzene-*p'*-sulfonate, repeated recrystallization and regeneration to either the hydrochloride or sulfate. This indicates that the neomycin used in these studies approaches ultimate purity.

Neomycin has been distributed counter-currently between *n*-butanol and water using 2-ethylbutyric acid buffered to *p*H 6.9 as the carrying agent. By application of the single-withdrawal technique[11] neomycin was distributed as an unsymmetrical curve as shown in Fig. 2. These curves were obtained by reading the optical rotation of alternate fractions. The curve is skewed as would be expected with a distribution coefficient of 4.10 using the single-withdrawal technique. The distribution curves for neamine and for a mixture of neomycin and neamine are also shown in Fig. 2. These studies indicate that neomycin is a single entity.

Antibacterial studies on neomycin indicate that it is very active against a variety of Gram-positive and Gram-negative organisms, including the tuberculosis organism. The results of these studies are given in Table I.

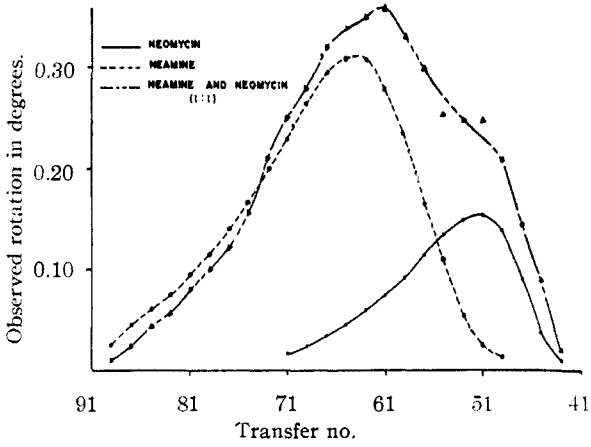

Fig. 2.—Distribution patterns of neomycin and neamine.

TABLE I

ANTIBACTERIAL SPECTRUM STUDIES ON NEOMYCIN

Organism	Mcg. per ml. required to inhibit
Staph. albus	0.01
Staph. aureus	.01
Strep. fecalis	.11
B. anthracis	.02
A. aerogenes	.14
E. typhosa	.03
E. coli	.11
Ps. aeruginosa	.33
Br. abortus	.05
K. pneumoniae	.10
Proteus vulgaris	.13
Salmonella schott.	.11
M. tuberculosis (607)	.29

Acute toxicity tests in mice indicate an LD_{50} of approximately 80 mg. per kg. intravenously and 400 mg. per kg. subcutaneously. Thus, on a weight basis, neomycin is more toxic than streptomycin.

Acknowledgments.—The authors are indebted to Dr. G. F. Cartland for active direction and continued interest in this problem. We wish to acknowledge the assistance of various members of the Antibiotics Research Department in certain phases of this research. We are also grateful to Dr. J. L. Johnson for the infrared spectrum and Mr. W. A. Struck for the microanalytical data.

(10) B. E. Leach and Charlotte M. Teeters, THIS JOURNAL, **73**, 2794 (1951).

(11) L. C. Craig and D. Craig in A. Weissberger, "Technique of Organic Chemistry," Vol. III, Interscience Publishers, Inc., New York, N. Y., 1950, pp. 285–292.

Experimental

Assay and Standards.—The method described by Loo, *et al.*,[12] for the assay of streptomycin was used for the determination of neomycin activity. In order to make the assay more sensitive the plates were prepared with only the agar seed layer of *B. subtilis*. The neomycin primary standard was a dried preparation with an assigned potency of 150 units per mg. supplied to us by Dr. Waksman. The most active preparations of neomycin sulfate prepared in our laboratories have assayed approximately 200 Waksman units per mg. For convenience, one of these preparations (Research No. 9291) was adopted as a working standard and assay results are reported in terms of mcg. of neomycin sulfate. The primary standard supplied by Dr. Waksman would thus assay 750 mcg. per mg. against this working standard.

Preparation of Crude Neomycin Sulfate.—Submerged fermentations of neomycin-producing culture, *Streptomyces* (No. 3535), were harvested at a potency of 750 to 2500 mcg. per ml.[13] The fermentations were carried out in iron tanks coated with Heresite.[14] The medium had the following composition: glucose 25 g., brewers yeast 2.5 g., ammonium sulfate 5 g., calcium carbonate 8 g., potassium chloride 4 g., potassium acid phosphate 0.4 g., soybean meal 25 g. and tap water to make one liter. The culture medium was seeded with a 5% volume of a 48-hour inoculum.

The fermentation broth harvested at 90 hr. had a volume of 6260 l. and assayed 750 mcg. per ml. representing 4700 g. of neomycin sulfate. This was adjusted from *p*H 7.9 to *p*H 2.7 with 57 l. of technical grade sulfuric acid. The mycelial cake was removed by filtration through a plate and frame filter press using 180 kg. of filter-aid.[15] The filtrate, 6180 l., was adjusted to *p*H 8.0 with 50% sodium hydroxide solution and stirred with 180 kg. of activated carbon[16] for one hour. The carbon was collected on a plate and frame filter press using 68 kg. of filter-aid, and washed once with 1200 l. of water. Considerable activity is lost by this washing operation, and its omission has since been found preferable. The neomycin was recovered from the carbon by four elutions with 10% acetone at *p*H 2.0. This required 5 l. of concentrated sulfuric acid. The eluates were pooled giving a volume of 970 l. which assayed 1040 mcg. per ml. representing 1010 g. of neomycin sulfate. The crude antibiotic was precipitated by the addition of 2800 l. of acetone to the pooled eluates. The active precipitate was collected and dissolved in 59 l. of water. The aqueous pool after adjustment to *p*H 6.2 with sodium hydroxide contained 1695 g. of solids assaying 425 mcg. per mg. The over-all yield was 15%.

Picrate Process.—Thirty-four liters of the aqueous pool described above, containing 977 g. of solids assaying 425 mcg. per mg. was treated with 1000 g. of picric acid in 4 l. of acetone. After refrigeration overnight, the milky precipitate was collected on a filter using Super-Cel[15] as the filter-aid. The gummy product was dissolved in 10 l. of acetone, filtered and treated with 75 ml. of concentrated sulfuric acid. The precipitated neomycin sulfate was collected, washed thoroughly with acetone and dissolved in 4800 ml. of water. The solution was adjusted to *p*H 4.7 with sodium hydroxide and freeze-dried to yield 210 g. of material assaying 570 mcg. per mg. In subsequent runs the picrate process has been omitted and the pooled aqueous concentrates have been chromatographed directly over carbon.

Carbon Chromatography of Crude Neomycin Sulfate.—A 4-inch Pyrex column was packed with an aqueous slurry containing 1500 g. of Darco G-60 and 1500 g. of Celite #545; the liquid holdup was 7 l. The starting material, 208 g. of crude neomycin sulfate, was dissolved in 400 ml. of water, introduced into the top of the column and developed with water. The effluent from the column was collected in fractions and assayed as shown in Table II.

(12) Y. H. Loo, P. S. Skell, H. H. Thornberry, J. Ehrlich, J. M. McGuire, G. M. Savage and J. C. Sylvester, *J. Bact.*, **70**, 701 (1945).

(13) H. A. Nelson, K. M. Calhoun and D. R. Colingsworth, Abstracts of 118th Meeting, Am. Chem. Soc., p. 16A (Sept., 1950).

(14) A phenolic resin coating applied by Heresite and Chemical Company, Manitowoc, Wisconsin.

(15) Celite 545 and Super-Cel are diatomaceous earths supplied by Johns-Manville.

(16) Darco G-60, supplied by the Darco Corporation.

TABLE II

CARBON CHROMATOGRAPHY OF NEOMYCIN SULFATE

Fraction	Ml.	Neomycin mcg.[a] × 10^6	%	Total solids, g.	Approx purity, mcg./mg.
Starting material	400	119	100	208	570
1	500			2.65	
2	500			16.10	
3	500			9.85	
4	500			4.80	
5	500	0.21	0.18	3.90	50
6	500	2.56	2.26	5.65	450
7	500	5.30	4.47	8.30	640
8	500	6.63	5.60	8.25	800
9	500	7.78	6.66	8.15	950
10	500	7.08	5.98	7.15	990
11	500	6.04	5.10	5.55	1080
12	500	4.70	3.97	4.20	1120
13	500	4.04	3.41	3.40	1190
14	500	3.16	2.67	3.05	1040
15	500	3.29	2.78	2.70	1220
16	500	2.88	2.43	2.55	1120
17	500	2.56	2.16	2.35	1090
18	5500			17.22	

[a] Bio-assay *vs.* neomycin sulfate, Research No. 9291.

Column fractions 7–18 were combined and freeze-dried to yield 68 g. of neomycin sulfate assaying 1000 mcg. per mg. The yield over the carbon column was 57%. The optical rotation was $[\alpha]^{26}D$ +47.8° (*c*, 1% in water).

Anal. Found: C, 27.86; H, 5.70; N, 5.20; S, 10.89; ash, 4.37.

Crystalline Neomycin *p*-Hydroxyazobenzene-*p'*-sulfonate.—One-half gram of neomycin sulfate was dissolved in 25 ml. of 15% methylcellosolve and treated with 0.5 g. of *p*-hydroxyazobenzene-*p'*-sulfonic acid. The precipitate was redissolved by heating on the steam-bath; a crystalline product separated upon slow cooling. Recrystallization from a 15% solution of methylcellosolve yielded 337 mg. of crystals assaying 480 mcg. per mg. The crystals had no definite melting point. The optical rotation was $[\alpha]^{25}D$ +29.5° (*c*, 0.5% in methanol).

Anal. Found: C, 47.01; H, 5.23; N, 10.55; S, 8.44.

One hundred and forty milligrams of thrice recrystallized neomycin *p*-hydroxyazobenzene-*p'*-sulfonate was suspended in 25 ml. of acetone and treated with 4 drops of concentrated hydrochloric acid. The white precipitate was collected, washed with acetone and dried to yield 40 mg. of amorphous neomycin hydrochloride assaying 1015 mcg. per mg.

Crystalline Neomycin Reineckate.—Thirty milligrams of neomycin sulfate was dissolved in 2 ml. of water and treated with 100 mg. of ammonium reineckate dissolved in 4 ml. of water. The precipitate was redissolved by warming and allowed to crystallize slowly. These crystals were collected and recrystallized from 6 ml. of warm water yielding 24.7 mg. of product assaying 445 mcg. per mg.

Paper Chromatography of Neomycin.—Descending paper chromatograms were run using water-saturated *n*-butanol containing 2% *p*-toluenesulfonic acid monohydrate as described by Peterson and Reineke.[17] The papergrams were developed for 18 hr. in a 12 in. by 24 in. battery jar closed with a sheet of plate glass. The optimum amount of neomycin under these conditions is 2.0 to 8.0 mcg. Considerable streaking is observed if larger amounts are used. The paper strips are thoroughly air-dried, placed for 10 minutes on a large glass tray containing a layer of agar seeded with *B. subtilis*, and removed. These trays are then incubated at 37° for 18 hr., and the zones of inhibition are accurately measured or photographed by the technique described by Drake.[18] Neomycin has an R_f value of 0.3–0.45 depending upon how well the chamber is saturated with

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

(18) N. A. Drake, *ibid.*, **72**, 3803 (1950).

solvent vapor. Typical results given in Fig. 3 indicate only one antibacterial component in the purified neomycin sulfate preparations. Reproducibility requires use of the same number of paper strips per chamber and equal amounts of the same fresh solvents for each run. As a routine procedure two 5 × 22 in. strips are used per chamber with 150 ml. of solvent in the bottom of the battery jar and 150 ml. in the developing tray.

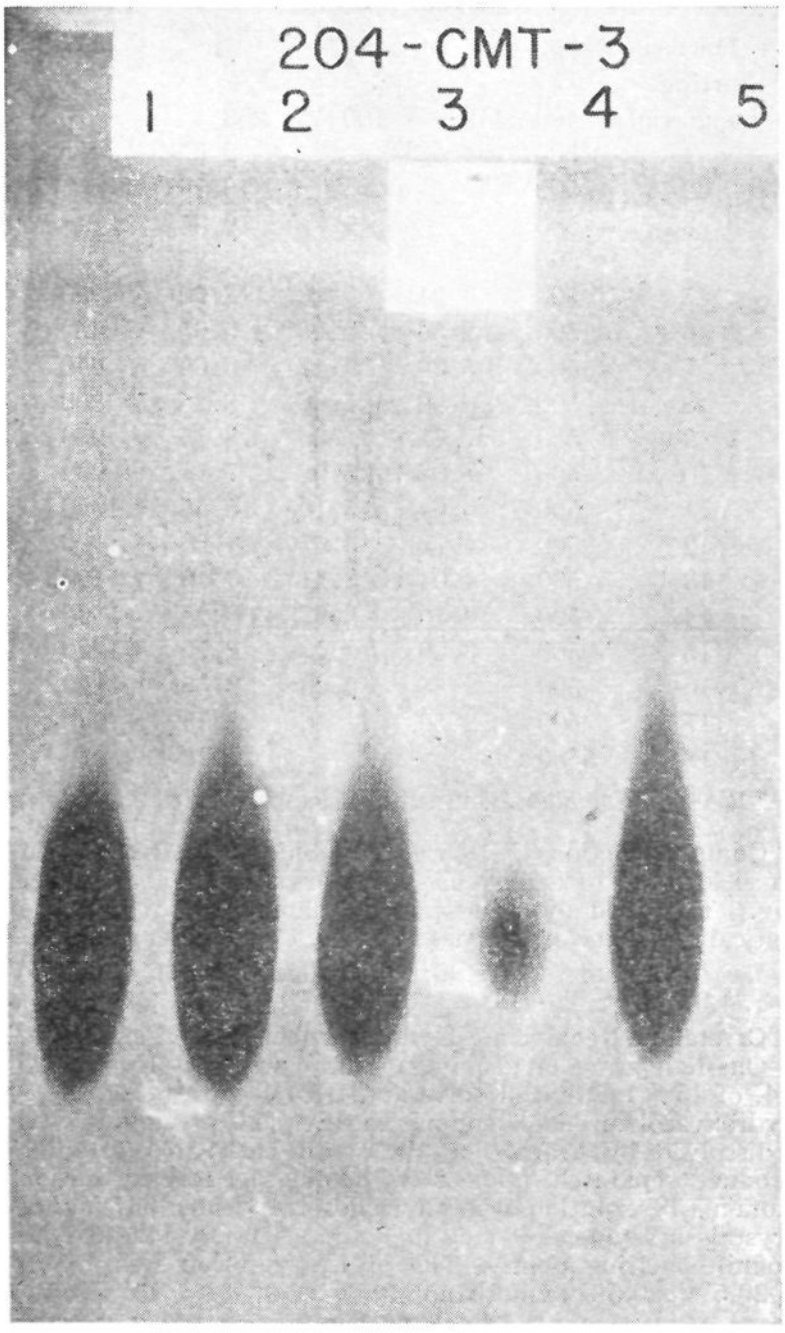

Fig. 3.—Descending paper chromatography of purified neomycin sulfate preparations using 2% *p*-toluenesulfonic acid monohydrate in water-saturated *n*-butanol as the developing solvent. The development period was 18 hours at room temperature. Zones 1, 2, 3 and 5 represent 8 mcg. of neomycin and zone 4 represents 2 mcg. of neomycin. A one inch square of paper is shown on the chromatogram.

Alkaline Hydrolysis of Neomycin Sulfate.—Ten grams of neomycin sulfate was dissolved in 30 ml. of water and treated with 500 ml. of 5% barium hydroxide octahydrate. The barium sulfate was removed by filtration, and the clear filtrate was heated under reflux for 16 hr. The solution was cooled, adjusted to *p*H 4.5 with sulfuric acid and filtered. The filtrate and washes were freeze-dried to yield 8.3 g. of material assaying 1220 mcg. per mg. The specific rotation and biological activity were unchanged after this treatment.

Acid Hydrolysis of Neomycin.—Neomycin hydrochloride (33 mg.) was dissolved in 2 ml. of 6 *N* HCl and heated in a sealed tube at 120° for 7 hr. Considerable charring occurred during the hydrolysis. The hydrolysate was dried repeatedly to remove the excess hydrochloric acid. The centrifuged solution was assayed repeatedly with variation from 30 to 85 mcg. per mg. with an average of 60 mcg. per mg. or 6% of the *B. subtilis* activity of the starting material. The hydrolysate gave positive tests with Brady reagent, Fehling solution and Tollens reagent indicating formation of an aldehyde by the hydrolysis. Paper chromatography of the hydrolysate using 50% *n*-butanol, 25% acetic acid and 25% water by volume as the developing solvent gave 4 colored zones with ninhydrin. The R_f values were 0.06, 0.22, 0.46 and 0.66, respectively. None of the known amino acids gave these R_f values when mixed with the hydrolysate. The ninhydrin-positive spot with R_f 0.06 was the bioactive component. Under these same conditions neomycin does not move from the point of application, therefore the acid degrades neomycin to another substance possessing antibacterial properties. We have proposed the *name neamine for this product.*

Counter-current Distribution of Neomycin.—One gram of neomycin sulfate was distributed in a 41-tube train[19] using a system containing *n*-butanol, water and 5% 2-ethylbutyric acid. The solvent mixture was adjusted to *p*H 6.9 with sodium hydroxide. Figure 2 shows the distribution curve for neomycin obtained by reading the optical rotation of alternate fractions after a total of 90 transfers. The biological activity, using *B. subtilis* as the test organism, followed the optical rotation. All the neomycin was transferred out of the train by the upper phase. The neomycin gave a peak in transfer number 51; the distribution coefficient is 4.10 using the formula developed by Craig[11] for an apparatus operated by the single-withdrawal technique. There is no evidence from these experiments that neomycin contains more than one component. Neamine, the acid-degradation product of neomycin, has a distribution coefficient of 1.78. Also given in Fig. 2 are the distribution curves of neamine and a mixture of neamine and neomycin.

KALAMAZOO, MICH. RECEIVED JANUARY 2, 1951

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