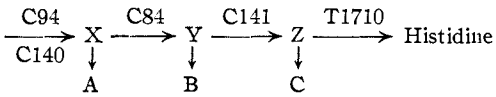
of the apparent structural relationships with the other compounds and its accumulation by both fungi.

The Biosynthesis of Histidine.—A previous publication[4] presented genetic and biochemical evidence that the histidine mutants of neurospora are related from the standpoint of biosynthesis of histidine according to the scheme



A, B and C refer to the compounds accumulated by the various mutants and they were not considered to be intermediates in the biosynthesis. These compounds have now been identified as: A = I, B = II and C = III. These compounds do not support the growth of any of the mutants and the discovery of the phosphate esters of I and II in the mycelium of strain C141 suggests the possibility that these esters correspond to the actual intermediates X and Y indicated in the above scheme. Since phosphate esters in general are not taken up by growing neurospora, it is necessary to use enzymatic methods to determine whether these esters are actually intermediates in histidine biosynthesis. Experiments of this kind are in progress. Since the first compound accumulated in the series contains a trihydroxypropyl side chain, it is a particularly attractive hypothesis that the carbon chain of histidine is derived in quite a direct fashion from pentose phosphate.

Compound III isolated from an *E. coli* histidine mutant by Vogel, *et al.*,[12] provided an important clue as to the nature of the biosynthetic pathway. It was found that his isolated L-histidinol was utilized slowly by another histidineless *E. coli* mutant. By selection from this second mutant, a strain was obtained which utilized L-histidinol 75% as well as histidine. If not histidinol, but its phosphate ester is the true intermediate, results such as this can be easily explained. Perhaps what was being selected for was an organism with a histidinol phosphorylating enzyme.

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PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Streptomyces Antibiotics. XXV. Isolation of Neomycin A

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Neomycin A has been isolated and characterized as the crystalline free base and as the crystalline picrate and *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate. Neomycin A sulfate and hydrochloride were obtained as amorphous white solids. The steps leading to the isolation of neomycin A comprised chromatography of "neomycin complex" on alumina, formation of picrate, conversion to hydrochloride, rechromatography on alumina, and formation and recrystallization of the *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate. An alternative procedure involved acid-treatment of "neomycin complex," formation and recrystallization of the picrate. Acetylated and benzoylated neomycin A as amorphous solids were prepared. Catalytic deacetylation yielded crystalline N-acetylneomycin A. Neomycin A may occur as a minor constituent of the "neomycin complex." Acid hydrolysis forms additional amounts of neomycin A, showing the presence of conjugated neomycin A.

Some of the chemical and biological properties of crystalline salts of neomycin A have been reported.[1] The methods used for separation of neomycin A from crude concentrates and for the preparation of pure salts of this antibiotic are described herein.

Early studies of neomycin[2,3] indicated that the biological activity of culture filtrates of *Streptomyces fradiae* was due to a single antibiotic entity. Very shortly, however, it was recognized that this activity was in reality due to a mixture of at least three antibiotic components, and the term "neomycin complex" was suggested to describe the crude concentrates obtained from culture filtrates.[4] Another antibiotic substance, active mainly against fungi, was also recognized as an elaboration product of *Streptomyces fradiae* and was later designated fradicin.[4,5] The latter substance is not considered as part of the neomycin complex, as it is separated at an early stage of purification. Purification studies on the neomycin complex have thus far resulted in the isolation of three antibiotic entities which have been designated neomycin A,[1] B[6,7] and C.[7]

Our early purification results led us to believe that crude concentrates of neomycin were yielding at least two antibiotically active substances. Chromatographic fractionation was followed by means of the cup assay employing *B. subtilis*. Since neomycin A appeared to diffuse more readily in agar than did other active components of the neomycin complex, this assay facilitated our work. The steps leading to pure neomycin A comprised the following: chromatography of crude concen-

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

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

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

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

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

(6) P. P. Regna and F. X. Murphy, THIS JOURNAL, **72**, 1045 (1950).

(7) J. D. Dutcher, N. Hosansky, M. N. Dorien and O. Wintersteiner, *ibid.*, **73**, 1384 (1951).

trates on alumina, separation of picrate, conversion to hydrochloride, rechromatography on alumina to give neomycin A-rich fractions, conversion to *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate of neomycin A, and finally conversion to neomycin A hydrochloride or sulfate.

It was found, as purification progressed, that neomycin A was a minor component of the neomycin complex. The isolation of this component without undue difficulties was probably facilitated by the guidance of the high biological activity of the substance. When tested by streak-dilution assays against *E. coli* or *M. tuberculosis*, neomycin A showed activity somewhat less than that of crude concentrates of neomycin complex.

The activity recovered from the picric acid step was greater than that present in the original concentrate used, in some cases. This led to the conclusion that conjugates of neomycin A present in the crude neomycin complex were being hydrolyzed during this procedure.

Since neomycin A had been isolated without hydrolytic procedures, the significantly increased yields of neomycin A obtained after hydrolytic steps established the presence of considerable amounts of this substance in combined or conjugated form in the crude complex. This indicated that a hydrolytic step could be included in the isolation scheme for neomycin A.

Neomycin A is rather stable to acid and remains active after heating at reflux temperature in 1 *N* hydrochloric acid for four hours. To liberate neomycin A in the crude concentrates, the latter were heated in aqueous acid. The "hydrolysates" were considerably more active than the original material when tested by the cup assay procedure. Neomycin A was isolated from such acid-treated concentrates as a crystalline picrate. Recrystallization of the picrate gave pure (by solubility analysis) neomycin A picrate without the necessity of chromatography. The picrate was readily converted to neomycin A hydrochloride or sulfate.

Neomycin A sulfate obtained from the pure picrate was a white, amorphous powder which showed an activity of about 1500 units/mg. based on the cup assay with a standard preparation of neomycin complex supplied by Dr. Waksman. Neomycin A hydrochloride, prepared from the picrate was likewise a white, amorphous powder. This salt showed an activity of about 1700 units/mg. Analytical data obtained on the hydrochloride and on the sulfate were in agreement with the values calculated for the corresponding salts of the base, $C_{12}H_{26}N_4O_6$.

Neomycin A free base was prepared from the sulfate by treatment with barium hydroxide. The base crystallized from water or aqueous ethanol and showed an activity of about 2200 units/mg.

Acetylation of neomycin A gave an amorphous acetyl derivative which was microbiologically inactive by cup assay. Deacetylation with methanolic ammonia gave a crystalline microbiologically inactive N-acetyl derivative. The analytical data on N-acetylneomycin were in agreement with the formula $C_{12}H_{22}N_4O_6(CH_3CO)_4$. The benzoyl derivative of neomycin A was prepared and found to be a microbiologically inactive, amorphous solid.

The activity of neomycin A hydrochloride when tested with a variety of microörganisms is shown in Table I. A pharmacological test showed neomycin A hydrochloride to be tolerated in single subcutaneous injection in mice at about 21 mg./20 g. mouse.

TABLE I

ACTIVITY OF NEOMYCIN A HYDROCHLORIDE TOWARD VARIOUS MICROÖRGANISMS

Organism	Concn. in γ/ml. required for complete inhibition
E. coli W	50
E. coli C	50
E. coli (neomycin-resistant)	50
K. pneumoniae	25–50
S. aureus	5–12
B. bodenheimer	25–50
B. mesentericus	1–2.5
C. pseudodiphthericum	1–5
B. cereus	12–50
M. lysodeikticus	10–100
B. subtilis	4–5

The properties of neamine, obtained as an acid degradation product of neomycin have been described.[8] Comparison of a sample of neamine free base[9] with neomycin A showed that the two substances are identical.

Experimental

The initial steps of obtaining crude neomycin concentrates were analogous to those already described.[2]

Chromatographic Purification of Neomycin Concentrates.—Various adsorbents, including silica gel and charcoal, were tried in chromatographic purification of crude neomycin concentrates,[2] but alumina was found to give the best results. A slurry of sulfuric acid-washed alumina in methanol was used to prepare the columns. Aqueous methanol was used as developing solvent. Data from typical chromatographic experiments are summarized in Table II.

TABLE II

CHROMATOGRAPHY OF NEOMYCIN CONCENTRATE ON ALUMINA

Material used	Eluate, ml.	Eluting methanol, %	Solids, g.	Activity, units/mg.	Units recovered
2 g., 150 u./mg. in	250	90	0.505	<10	
20 ml. of 88%	250	85	.125	<20	
MeOH on 100 g.	500	80	.232	250	58,000
of alumina	250	70	.126	700	88,000
	250	50	.196	400	78,000
	250	Water	.170	125	21,000
	Total recovered:		1.354 g.		245,000 units
12 g., 350 u./mg.	300	90	...	..	.....
in 100 ml. of	300	90	3.68	230	850,000
90% MeOH on	300	90	2.16	380	820,000
300 g. of alumina	500	85	1.73	448	770,000
	500	80	1.13	858	970,000
	500	70	0.97	344	330,000
	Total recovered:		9.67 g.		3,740,000 units

Neomycin A Picrate as Intermediate in Purification.—Ninety grams of crude neomycin complex (100 units/mg.) was dissolved in 540 ml. of water and the solution was heated to 70°. To this solution, was added a nearly boiling solution of 135 g. of picric acid in 2700 ml. of water. A gummy precipitate separated as the solution cooled. The supernatant was removed by decanting and the picrate was rinsed with water. The crude picrate was dissolved in 300 ml. of acetone and the solution was poured with stirring into 3 l. of acetone containing 50 ml. of concentrated hy-

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

(9) Sample kindly supplied by Dr. B. E. Leach of the Upjohn Company.

drochloric acid. An amorphous precipitate separated. The precipitate was collected on a filter, washed with acetone, and dried; yield 35.2 g., activity 328 units/mg.

Crystalline Neomycin A Picrate.—It was found that preparation of the crystalline picrate of neomycin A is prevented or made very difficult by the presence of small amounts of neomycin B. Accordingly, samples of neomycin A were treated with hot mineral acid before preparation of the picrate.

Forty grams of neomycin complex (about 270 units/mg.) was dissolved in 200 ml. of 2.5 *N* hydrochloric acid and the solution was maintained at the reflux temperature for one hour. The solution was cooled, neutralized to *p*H 6–7, and mixed with a hot solution of 60 g. of picric acid in 1200 ml. of water. This solution was treated with 9.2 g. of Darco G-60, heated to boiling and filtered. The filtrate was allowed to cool slowly, with stirring. The crystalline neomycin A picrate which separated was collected on a filter, washed with water and dried in air; yield 51 g. The product was recrystallized from 765 ml. of hot water (yield 30 g.), and twice from about 400 ml. of hot water to give 11.5 g. of product which melted at 262–265° (dec.), and which had an activity of about 750 units/mg.

Anal. Calcd. for $C_{36}H_{38}N_{16}O_{34}\cdot 3H_2O$: C, 33.44; H, 3.43; N, 17.34. Calcd. for $C_{36}H_{38}N_{16}O_{34}$: C, 34.90; H, 3.09; N, 8.09. Found (dried at 100° *in vacuo*): C, 35.19, 34.96; H, 3.24, 3.18; N, 18.84, 18.81.

Potentiometric titration of a sample of neomycin A picrate dried at 25° *in vacuo* showed an equivalent weight of 328; calcd. for $C_{36}H_{38}N_{16}O_{34}\cdot 3H_2O$: equiv. wt., 323.

Neomycin A Helianthate and *p*-(*p'*-Hydroxyphenylazo)-benzene Sulfonate.—A solution of 149 mg. of a neomycin A-rich fraction ($[\alpha]^{25}D$ +34° (*c* 1.11 in water); activity about 700 units/mg.) in 5 ml. of 40% methanol was warmed to 50°. To this solution, was added a hot solution of 245 mg. of methyl orange in 5 ml. of water. A precipitate formed immediately. After cooling the solution, the precipitate was collected on a filter, washed with water and dried; yield 230 mg. of dark red powder, m.p. 220–230° (dec.). The main portion of the neomycin A helianthate was suspended in 5 ml. of methanol containing 1 ml. of 2.5 *N* hydrochloric acid and the mixture was ground in a mortar. The mixture was then poured into a filter which had a small pad of Darco G-60. The colorless filtrate was poured into ten volumes of acetone and a white, amorphous neomycin A hydrochloride separated; yield 80 mg., $[\alpha]^{25}D$ +50° (*c* 1.0 in water), decomposition range 170–230°.

A solution of 60 mg. of the above-mentioned neomycin A hydrochloride in 2 ml. of 50% methanol was mixed with a solution of 118 mg. of sodium *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate in 4 ml. of hot water. Shortly after mixing, crystals began to separate. The crystalline product was recrystallized twice from about 6 ml. of 20% methanol to give 40 mg. of yellow crystals which melted with decomposition over the range, 204–214°. Samples taken after each crystallization melted with decomposition over the same range. The ultraviolet absorption spectrum of a solution of this product in phosphate buffer (*p*H 8, *M*/20) showed a prominent band at 3700 Å. ($E^{1\%}_{1\ cm.}$ 414).

Anal. Calcd. for $C_{12}H_{26}N_4O_6(C_{12}H_{10}N_2O_4S)_4$: C, 50.20; H, 4.63; N, 11.71. Found: C, 50.42; H, 5.01; N, 11.20; wt. loss on drying at 100° *in vacuo*, 6.9.

Amorphous Neomycin A Hydrochloride.—A 4-g. sample of three-times crystallized neomycin A *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate was dissolved in a mixture of 20 ml. of 1.25 *N* hydrochloric acid and 20 ml. of *n*-butanol. The phases were separated and the aqueous phase was extracted five times with 10-ml. portions of water-saturated butanol to remove the sulfonic acid. The colorless water solution was then poured into fifteen volumes of acetone to give 635 mg. of white amorphous neomycin A hydrochloride, $[\alpha]^{25}D$ +83° (*c* 1.0 in water). A second crop was obtained from the mother liquor by addition of more acetone. When heated on the microblock, the hydrochloride began to darken at about 220° and melted with decomposition at about 250–260°.

Anal. Calcd. for $C_{12}H_{26}N_4O_6\cdot 4HCl$: C, 30.78; H, 6.46; N, 11.97; Cl, 30.29. Found: C, 32.52; H, 6.86; N, 11.57; NH_2-N, 11.25; Cl, 28.65.

Potentiometric titration of a sample of neomycin A hydrochloride gave an equivalent weight of about 128 with midpoint *p*H value of 7.70.

A solution of 7.3 g. of recrystallized neomycin A picrate in 25 ml. of acetone was poured with stirring into 250 ml. of acetone containing 7.0 ml. of concentrated hydrochloric acid. The white precipitate which formed immediately was collected on a filter, washed with acetone and ether, and dried *in vacuo;* yield 3.3 g. of amorphous neomycin A hydrochloride. In order to remove traces of picric acid, this sample was dissolved in a mixture of 7.0 ml. of water containing some hydrochloric acid and 26.5 ml. of methanol, and the solution was poured into a filter which had a 6.6-g. pad of Darco G-60. The combined filtrate and wash was added to 400 ml. of acetone to give 2.6 g. of white, amorphous neomycin A hydrochloride, $[\alpha]^{25}D$ +81.5° (*c* 1.01 in water), activity about 1700 units/mg.

Anal. Calcd. for $C_{12}H_{26}N_4O_6\cdot 4HCl$: C, 30.78; H, 6.46; N, 11.97; Cl, 30.29. Found: C, 32.13, 32.17; H, 6.66, 6.48; N, 11.72, 11.98; Cl, 30.08, 30.09; S, nil; P, nil; C-methyl, nil; OCH_3, 0.52; NCH_3, nil.

Polarographic examination showed no oxidation–reduction groups. Potentiometric titration of a sample dried to constant weight *in vacuo* at 100° gave an equivalent weight of 115.

Neomycin A Sulfate.—Seven grams of crystalline neomycin A picrate was dissolved in 125 ml. of methanol. To this solution, 10 ml. of 50% sulfuric acid in 10 ml. of methanol was added with stirring. Neomycin A sulfate precipitated as a white, amorphous solid; it was collected on a filter, washed with methanol, and dissolved in 10 ml. of water. The aqueous solution was filtered through a 1-g. pad of Darco G-60 to remove traces of picric acid, and the filtrate was poured into 400 ml. of acetone. The white precipitate was dissolved in 10 ml. of water and reprecipitated in 700 ml. of 1:1 methanol–acetone to give 2.0 g. of white, granular neomycin A sulfate, $[\alpha]^{25}D$ +75.5° (*c* 1.0 in water). This salt showed an activity of about 1500 units/mg.

Anal. Calcd. for $C_{12}H_{26}N_4O_6\cdot 2H_2SO_4$: C, 27.79; H, 5.83; N, 10.81; SO_4, 37.05. Found: C, 27.96; H, 6.01; N, 9.74; SO_4, 38.82.

Neomycin A Free Base.—To a solution of 1.24 g. of neomycin A sulfate in 25 ml. of water at 60–70°, 53.67 ml. of 0.0468 *N* barium hydroxide solution was added. A stream of nitrogen was used to maintain a carbon dioxide-free atmosphere. After keeping the mixture at 60–70° for a few minutes, the solution was cooled and filtered to remove barium sulfate. The filtrate was concentrated to a volume of 5 ml., and crystals began to separate. Addition of ethanol and cooling brought about satisfactory crystallization. The crystals softened and darkened slightly at 230° and melted at 250–260° (dec.). The product was recrystallized twice from aqueous alcohol to give 259 mg. of neomycin A which decomposed over the range 225–260°, $[\alpha]^{25}D$ +112.8° (*c* 1.0 in water). It showed an activity of about 2200 units/ml.

Anal. Calcd. for $C_{12}H_{26}N_4O_6$: C, 44.71; H, 8.13; N, 17.38. Found: C, 44.62; H, 8.46; N, 16.98.

Potentiometric titration gave a value of 89 for the equivalent weight.

A solution of 4.5 g. of neomycin A hydrochloride in 6 ml. of concentrated ammonium hydroxide was diluted with 400 ml. of methanol. Liquid ammonia was added to this solution until crystallization began. After standing for about one hour in the coldroom, the crystalline product was collected on a filter, washed with methanol and ether, and dried *in vacuo*. The product was twice recrystallized from aqueous ethanol to give 1.65 g. of needles which sintered at about 235° and melted at about 250–260° (dec.) (microblock), $[\alpha]^{25}D$ +123.5° (*c* 1.0 in water).

Anal. Calcd. for $C_{12}H_{26}N_4O_6$: C, 44.71; H, 8.13; N, 17.38. Found: C, 45.11; H, 8.13; N, 17.16.

Acetylation of Neomycin A.—Two and one-half grams of neomycin A hydrochloride was mixed with 1.7 g. of fused sodium acetate and 100 ml. of acetic anhydride and the mixture was held at reflux temperature for 1.5 hours. Sodium chloride was removed by filtration of the hot mixture, and the filtrate was evaporated to dryness. The residue was completely soluble in chloroform. After evaporation, the residue was dissolved in 40 ml. of acetone and the solution was poured into 400 ml. of petroleum ether to give 3.45 g. of amorphous acetylated neomycin A, $[\alpha]^{25}D$ +74° (*c* 1.0 in methanol).

TABLE III

ACETYLATION PRODUCTS OF NEOMYCIN A

	Fraction	Volume, ml.	Wt. of solid, g.	$[\alpha]^{25}D$ (c 1.0 in methanol)	Analytical data[a] C, %	H, %	N, %	Acetyl, %	Mol. wt.[b]
	More solvent-soluble portion								
1	Benzene	50	1.3	+80°	50.48	6.22	7.14	50.76	740
2	Benzene	100	0.4	+83					
3	$CHCl_3$	50	2.5	+90	49.12	6.87	7.24	54.38	673
4	$CHCl_3$	200	1.1	+91					
5	Acetone	400	0.1						
	Less solvent-soluble portion								
1	$CHCl_3$	50	2.70	+71°	49.90	6.74	7.77	32.00	534
2	$CHCl_3$	100	1.41	+62	49.06	6.79	7.76	33.45	566
3	Acetone	350	0.15	+52					
4	Methanol	350	0.05						

[a] Calcd. for $C_{12}H_{19}N_4O_6(CH_3CO)_7$: C, 50.64; H, 6.54; N, 9.09; CH_3CO, 49.5; mol. wt., 616.6. [b] Ebullioscopic determination.

Another sample of acetylated neomycin A, prepared as described above from 3.0 g. of neomycin A, was dissolved in 100 ml. of chloroform and the solution was shaken with 100 ml. of water. The chloroform layer was dried, evaporated to a volume of 20 ml. and precipitated with petroleum ether to give 1.97 g. of amorphous acetylated neomycin A (more solvent soluble portion), $[\alpha]^{25}D$ +65° (*c* 1.0 in methanol). The water layer from extraction of chloroform was evaporated to dryness, dissolved in 20 ml. of 1:1 acetone–methanol, and poured into ten volumes of petroleum ether. Since the product precipitated as an oil, the whole mixture was evaporated to dryness, and the residue was redissolved in 25 ml. of chloroform. Addition of this solution to petroleum ether gave 1.97 g. of amorphous acetylated neomycin A (less solvent soluble portion).

A 6.1-gram sample of the more soluble form of acetylated neomycin A was dissolved in 25 ml. of benzene and chromatographed on a column of 60 g. of Darco G-60 and 20 g. of polycel fiber. The column was developed with benzene, chloroform and finally stripped with acetone. The results are summarized in Table III.

A 4.5-gram sample of the less soluble form of acetylated neomycin A was dissolved in 20 ml. of chloroform and chromatographed on a column of 45 g. of Darco G-60 and 15 g. of polycel fiber. Chloroform was used first as a developing solvent, followed by acetone, and the column was finally stripped with methanol. The results obtained are summarized in Table III.

N-Acetylneomycin A.—A solution of 2.45 g. of the more solvent soluble portion of acetylated neomycin A in 100 ml. of methanol was cooled in ice and saturated with dry gaseous ammonia. After standing overnight, the solution was evaporated to dryness. The residue readily redissolved in 30 ml. of methanol. On standing at 25° for a day, a considerable amount of granular material had deposited. This material was dissolved in 150 ml. of hot methanol, and a little Darco G-60 was added. The filtrate was evaporated to a volume of 25 ml. *in vacuo*. After a few minutes, crystallization occurred. The product was collected on a filter, washed with methanol and dried; yield 563 mg., m.p. 325–327°. A second crop, m.p. 325–328°, was obtained from the mother liquor by concentration to about 20 ml. The combined material was recrystallized from 35 ml. of methanol to give 720 mg. of product which melted at 332–334°. Further recrystallization of N-acetylneomycin A from methanol raised the melting point to a constant value of 334–336° (microblock), $[\alpha]^{25}D$ +87° (*c* 1.0 in water). On drying samples for analyses at 100° *in vacuo* for two hours, the weight loss was 5.1 and 5.6%.

Anal. Calcd. for $C_{12}H_{22}N_4O_6\cdot(CH_3CO)_4$: C, 48.97; H, 6.99; N, 11.42; CH_3CO, 35.10. Found: C, 49.03, 49.01; H, 6.83, 6.78; N, 11.45, 11.18; acetyl, 19.3 (alkaline hydrolysis), 36.6 (acid hydrolysis).

One hundred milligrams of N-acetylneomycin dissolved in 5 ml. of 2.5 *N* hydrochloric acid was held at reflux for two hours. The solution was cooled, neutralized and diluted to a volume of 10 ml. An aliquot of this solution showed an activity of 8540 units ml. by cup assay. Since N-acetylneomycin A shows no assayable activity in this assay, the activity observed in the hydrolysis solution provides evidence for substantial hydrolysis of N-acetylneomycin A to neomycin A. This was confirmed by isolation of crystalline neomycin A picrate from the main portion of the hydrolysate.

Benzoylation of Neomycin A.—A mixture of 2.5 g. of neomycin A hydrochloride, 15 ml. of pyridine and 6.5 ml. of benzoyl chloride was heated at reflux temperature for 15 minutes, cooled somewhat, and diluted with 50 ml. of chloroform. The chloroform solution was washed with water, dilute acid, dilute sodium bicarbonate solution, water, and dried over sodium sulfate. The solution was then concentrated to a volume of 30 ml. *in vacuo* and poured into 300 ml. of petroleum ether to give an amorphous precipitate of benzoylated neomycin A; yield 4.91 g., m.p. 165–170°, $[\alpha]^{25}D$ +97° (*c* 1.0 in methanol).

Anal. Calcd. for $C_{12}H_{19}N_4O_6(C_6H_5CO)_7$: C, 69.24; H, 5.81; N, 5.30. Found: C, 69.43; H, 5.05; N, 5.29.

A 4.65-g. sample of benzoylated neomycin A was dissolved in 20 ml. of benzene and chromatographed on a column of 45 g. of Darco G-60 and 15 g. of polycel fiber. The column was developed with benzene and stripped with acetone. The results are summarized in Table IV.

TABLE IV

BENZOYLATED NEOMYCIN A

Fraction	Volume, ml.	Wt. of solid, mg.	$[\alpha]^{25}D$ (c 1.0 in methanol)	Analytical data found[a] C, %	H, %	N, %	Mol. wt.[b]
1 Benzene	25	140[c]	+105°	69.43	5.63	4.99	895
2 Benzene	50	827	+104				
3 Benzene	100	874	+104	68.71	4.91	5.04	971
4 Benzene	300	632	+104				
5 Acetone	300	507	+ 97				

[a] Calcd. for $C_{12}H_{19}N_4O_6(C_6H_5CO)_7$: C, 69.24; H, 5.81; N, 5.30; mol. wt., 1058. [b] Ebullioscopic determination. [c] M.p. 175–180°.

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