Potassium (D-α-Phenoxyethyl)-penicillin.—To a stirred, cooled solution of 16.6 g. (0.1 mole) of *d*-α-phenoxypropionic acid in 100 ml. of tetrahydrofuran there was added 10.1 g. (0.1 mole) of triethylamine in one portion. The solution was cooled to −15° and 13.6 g. (0.1 mole) of isobutyl chloroformate was added dropwise at a rate which gave a final reaction temperature of −4°. Immediately there was added in one portion a chilled solution of 21.6 g. (0.1 mole) of 6-APA in 35 ml. of water and 15 ml. of triethylamine. The cooling bath was replaced at once by a water-bath at 55° and the reaction temperature rose to 25° within 5 minutes. The solution was stirred for 25 minutes at 25–27°, diluted with 250 ml. of chilled water and extracted twice with ether. It was then layered with ether, chilled, acidified to *p*H 2 with cold 3 *M* sulfuric acid, extracted twice with ether and the combined ether extracts washed with water and dried over anhydrous sodium sulfate for 10 minutes. To the dried ether solution was added 36.4 g. (0.1 mole) of a 50% solution of potassium 2-ethylhexanoate in butanol. The crystalline product was collected and dried to yield 31.7 g. (79%) of material which decomposed at 214–215°. Successive recrystallizations to constant optical rotation from water–butanol and water–acetone gave 17 g. (42%) of material which decomposed at 234.5–235°. This product had a purity of 99.2% by a phase-solubility assay; $[\alpha]^{20}D$ +252° (*c* 1 in water).

Anal. Calcd. for $C_{17}H_{19}KN_2O_5S$: C, 50.75; H, 4.78; N, 6.98. Found: C, 50.88; H, 4.82; N, 6.94.

Potassium (L-α-Phenoxyethyl)-penicillin.—A solution was prepared by mixing 8.3 g. (0.05 mole) of *l*-α-phenoxypropionic acid, 40 ml. of dry *p*-dioxane, 20 ml. of dry acetone and 8 ml. of triethylamine. To this stirred and cooled solution (*ca.* 0°) was added dropwise, during 10–15 minutes, 6.8 g. (0.05 mole) of isobutyl chloroformate in 10 ml. of *p*-dioxane while the temperature was maintained below 10°. After the addition was completed the mixture was stirred and cooled during 10 minutes, after which time a solution of 10.8 g. (0.05 mole) of 6-APA in 50 ml. of water and 8 ml. of triethylamine was added rapidly. The resulting solution was stirred 15 minutes at *ca.* 10° and then 2 hours at room temperature. After dilution with an equal volume of water the reaction mixture was extracted twice with 100-ml. portions of ether, the ethereal extracts being discarded. The clear aqueous solution was covered with 150 ml. of ether, cooled to 10°, and acidified to *p*H 2 with a cold 5 *M* sulfuric acid solution. The ethereal solution was separated, washed with cold water and dried for 10 minutes over anhydrous sodium sulfate. After filtration 25 ml. of a 50% solution of potassium 2-ethylhexanoate in butanol was added. The white crystalline material which separated was collected by filtration and recrystallized once from 10% aqueous butanol and once again from 10% aqueous acetone. This procedure afforded 9.5 g. (47%) of pure potassium (L-α-phenoxyethyl)-penicillin which decomposed at 238–239°, $[\alpha]^{24}D$ +218° (*c* 1 in water).

Anal. Calcd. for $C_{17}H_{19}KN_2O_5S$: C, 50.75; H, 4.78; N, 6.98. Found: C, 50.92; H, 4.97; N, 6.93.

The same material could be obtained by extensive recrystallizations of potassium (DL-α-phenoxyethyl)-penicillin from a butanol–water mixture. The product obtained in each case had a purity of 98.6–99.8% as determined by the phase-solubility method.[14]

SYRACUSE 1, N. Y.

[CONTRIBUTION FROM THE WILLIAM ALBERT NOYES LABORATORY OF CHEMISTRY, UNIVERSITY OF ILLINOIS, AND THE INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY]

Chemistry of the Neomycins. V. Differentiation of the Neomycin Complex. Identity of Framycetin and Neomycin B. Compounds Obtained from Methyl Neobiosaminide B

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Neomycin preparations as their N-acetyl derivatives could be resolved qualitatively and quantitatively by paper and column chromatographic techniques. Framycetin thus has been shown to be neomycin B admixed with small quantities of neomycin C. This conclusion was further supported on comparing the products obtained from framycetin and neomycin B by selective chemical degradations of their methyl neobiosaminide moieties. These degradation reactions are outlined in detail.

Soon after the discovery of neomycin by Waksman and Lechevalier,[1] in 1949, it became apparent that the antibacterial activity of the *Streptomyces fradiae* fermentation broth was not due to a single antibiotic but to a number of active substances entitled[2] the "neomycin complex." At the present time there is general agreement concerning the production by *S. fradiae* of two isomeric compounds called neomycin B and neomycin C.[3,4] A third substance, originally called neomycin A[5] but now termed neamine,[6,7,8] is known to arise as a hydrolytic cleavage product of neomycins B and C. The antibiotics catenulin,[9] kanamycin[10,11,12] and paromomycin[13] may also be considered as antibiotics somewhat related to but distinctly different from neomycins B and C.

Aside from these different antibiotics, several other antibiotic mixtures, apparently belonging to the neomycin BC group, also have been reported. Streptothricins BI and BII[14] were shown[15] to be identical with neomycins B and C, respectively.

(1) S. A. Waksman and H. A. Lechevalier, *Science*, **109**, 309 (1949).
(2) E. A. Swart, D. Hutchison and S. A. Waksman, *Arch. Biochem.*, **24**, 92 (1949).
(3) J. D. Dutcher, N. Hosansky, M. N. Donin and O. Wintersteiner, THIS JOURNAL, **73**, 1384 (1951).
(4) J. H. Ford, M. E. Bergy, A. A. Brooks, E. R. Garrett, J. Alberti, J. R. Dyer and H. E. Carter, *ibid.*, **77**, 5311 (1955).
(5) R. L. Peck, C. E. Hoffhine, Jr., P. Gale and K. Folkers, *ibid.*, **71**, 2590 (1949).
(6) B. E. Leach and C. M. Teeters, *ibid.*, **73**, 2794 (1951).
(7) B. E. Leach and C. M. Teeters, *ibid.*, **74**, 3187 (1952).
(8) J. D. Dutcher and M. N. Donin, *ibid.*, **74**, 3420 (1952).
(9) J. W. Davisson, I. A. Solomons and T. M. Lees, *Antibiotics & Chemotherapy*, **2**, 460 (1952).
(10) T. Takeuchi, T. Hikiji, K. Nitta, S. Yamazuki, S. Abe, H. Takayama and H. Umezawa, *J. Antibiotics (Japan)*, **10A**, 107 (1957).
(11) H. Umezawa, M. Ueda, K. Maeda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta and T. Takeuchi, *ibid.*, **10A**, 181 (1957).
(12) M. J. Cron, D. L. Evans, F. M. Palermiti, D. F. Whitehead, I. R. Hooper, P. Chu and R. U. Lemieux, THIS JOURNAL, **80**, 4741 (1958).
(13) T. H. Haskell, J. C. French and Q. R. Bartz, *ibid.*, **81**, 3480 (1959).
(14) H. Umezawa, T. Takeuchi and S. Yamagiwa, *J. Antibiotics (Japan)*, **3**, 833 (1950).
(15) K. Maeda, *ibid.*, **5**, 343 (1952).

Dextromycin[16] recently was shown[17] to consist of neomycin B admixed with small amounts of neomycin C. Other antibiotics, such as framycetin,[18,19] fradiomycin[20] and flavomycin,[21] have long been suspected of being closely related, if not identical, to neomycin B and C mixtures.

Much of the confusion that reigns here is due to the fact that the neomycins are frequently produced as mixtures of isomeric components of varying proportions. As such, they produce mixed crystalline derivatives with a variety of organic acids; the free bases or their inorganic salts produced as amorphous solids do not readily lend themselves to precise physical and chemical characterization. It thus was quite apparent that if proper characterization of neomycin preparations was to be made, individual components would have to be isolated and compared physically and chemically.

In the past a great variety of techniques have been employed with different degrees of success in the attempt to separate and differentiate various neomycin preparations.[2,3,4,22] Paper chromatographic techniques generally have been unsuccessful in resolving mixtures of the free neomycins or their salts, whereas some differentiation can be achieved with pure components.[23] With mixtures of neomycins B and C the problems of streaking and overlapping hinder the separation and resolution of these isomeric components on paper. Degradation products of the neomycins, such as neamine, are more easily differentiated from the neomycins[9] by paper chromatography.

The technique of countercurrent distribution has also been applied to this problem of neomycin differentiation. Swart[2,24] reported separation of the "neomycin complex" employing solvent systems developed by Plaut and McCormack[25] in their studies with the streptomycins. The solvent systems were composed of pentasols, fatty acid carriers, and borate or bicarbonate buffers. Later countercurrent distribution studies[26] indicated that no differentiation of neomycins B and C could be obtained with these solvent systems, since the partition coefficients were quite similar and varied greatly with solute concentration.

Both alumina and carbon chromatographic techniques also have been used extensively to separate mixtures of the isomeric neomycins.[3,4] Application of the Hagdahl composite column technique[27,28] to carbon chromatography has produced good separations of neomycins B and C in our work.

More recently, Pan and Dutcher[22] reported the paper chromatographic separation and semi-quantitative analysis of the N-acetyl derivatives of neomycins B and C. Since much of the difficulty encountered in paper chromatography of the neomycins conceivably is due to their great basicity, acetylation neutralized this effect and permitted their resolution. In the present studies, modification of this excellent technique to cellulose powder column chromatography has successfully been used to obtain pure N-acetylneomycin components. By applying this technique to the study of commercial neomycin and framycetin preparations, and by comparative degradative reactions with the major components, we have established the identity of framycetin and neomycin B.

Paper Chromatography of Neomycin Antibiotics.—The individual neomycin and neomycin-like antibiotics neamine, neomycin B, neomycin C, catenulin, kanamycin and framycetin were effectively compared by paper chromatography, employing sulfate salts and their respective N-acetyl derivatives. The sulfates were chromatographed in a *n*-propyl alcohol–glacial acetic acid–water (PAW 9:1:10) descending solvent system, whereas the N-acetyl derivatives were chromatographed according to the procedure of Pan and Dutcher.[22] The results (*cf.* Table I) indicate that the detection of minor isomeric neomycin components was possible only with the chromatography of the N-acetyl derivatives and not of the sulfate salts. The chromatography of the free bases or salts of neomycin mixtures generally led to confusion because the development proceeded with streaking and overlapping of components. In a descending solvent system, methyl alcohol–3% aqueous sodium chloride (2:1), employing buffered paper (1.0 *M* sodium sulfate, *p*H 2.4), apparent differences observed[23] earlier in the migration of framycetin and neomycin B were due to the presence of small amounts of neamine in the framycetin preparation. With this solvent system, however, pure neomycin B, neomycin C, neamine and catenulin can be differentiated readily.

TABLE I

PAPER CHROMATOGRAPHY OF NEOMYCIN ANTIBIOTICS

Antibiotic	R_f sulfate salt[a]	R_{ef} N-acetyl-neomycin B[b]
Neomycin B	0.26	1.00 (0.76)[c]
Neomycin C	.30	0.76 (1.00)[c]
Framycetin	.26	1.00 (0.76)[c]
Neamine	.43	0.96
Catenulin	.52	.85
Kanamycin	.57	.75

[a] PAW 9:1:10 solvent system. [b] BPyrW 6:4:3 solvent system; R_f, N-acetyl derivative/R_f, N-acetylneomycin B. [c] Minor component.

It became apparent from these paper chromatographic studies that neomycin B and framycetin preparations were very similar if not identical; therefore, it was felt desirable to make comparative chemical studies to establish their identity. Since mixtures of neomycins B and C as N-acetyl derivatives could be completely resolved on paper,

(16) K. Ogata, *ibid.*, **3**, 440 (1950).
(17) S. Tatsuoka, A. Miyake and H. Nawa, *ibid.*, **11A**, 193 (1958).
(18) E. C. Tremblay, P. Distouches and N. Karatchengeff, *Presse med.*, **61**, 292 (1953).
(19) M. M. Janot, H. Pénau, D. Van Stolk, G. Hagemann and L. Pénasse, *Bull. soc. chim. France*, 1458 (1954).
(20) Japanese dextrorotatory antibiotic produced by *Streptomyces fradiae* (commercial).
(21) K. Aiso, K. Miyaki, F. Yanagisawa, T. Arai and M. Hayashi, *J. Antibiotics (Japan)*, **3**, 87 (1950).
(22) S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **28**, 836 (1956).
(23) A. Saito and C. P. Schaffner, Abstr. IIIrd Intern. Congr. Biochem., Brussels, 1955, p. 98.
(24) E. A. Swart, H. A. Lechevalier and S. A. Waksman, THIS JOURNAL, **73**, 3255 (1951).
(25) G. W. E. Plaut and R. B. McCormack, *ibid.*, **71**, 2264 (1949).
(26) C. P. Schaffner, "Antibiotics Annual 1954–1955," Medical Encyclopedia, Inc., New York, N. Y., 1954, p. 153.
(27) L. Hagdahl, *Acta Chem. Scand.*, **2**, 574 (1948).
(28) L. Hagdahl, *Sci. Tools*, **1**, 21 (1954).

preparative methods employing this technique were investigated.

Cellulose Powder Column Chromatography of N-Acetylneomycins.—With a modification of the Pan and Dutcher solvent system,[22] solvent partition procedures involving countercurrent distribution or cellulose powder column partition chromatography were found to be effective in the separation of N-acetylneomycin mixtures on a preparative scale. Illustrated in Fig. 1 is a typical separation

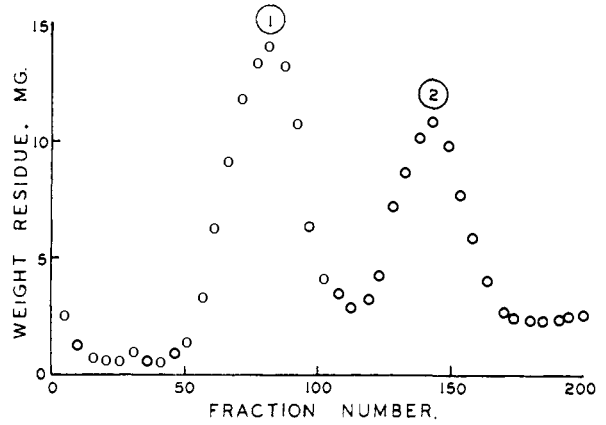

Fig. 1.—Cellulose powder partition chromatography of N-acetylneomycins: 1, N-acetylneomycin B; 2, N-acetylneomycin C.

obtained with a one-gram mixture composed of 60% N-acetylneomycin B and 40% N-acetylneomycin C as applied to a cellulose powder partition column. In direct correlation with results obtained on paper chromatograms with this solvent system, the N-acetylneomycin B was eluted from the column before N-acetylneomycin C. Recovery of the N-acetylneomycins was practically the theoretical, and the respective components were obtained in crystalline form. In Fig. 2 the chromatographic separation of a one-gram sample of N-acetylframycetin is illustrated. Typical of a commercial neomycin preparation, the presence of a major amount of N-acetylneomycin B material, admixed with a minor amount of N-acetylneomycin C, is detected. Comparisons (*cf.* Table II) of the optical rotation data from the N-acetyl components derived from neomycin and framycetin preparations have been made. From these studies it is clear that framycetin is primarily neomycin B admixed with small amounts (3–5%) of neomycin C.

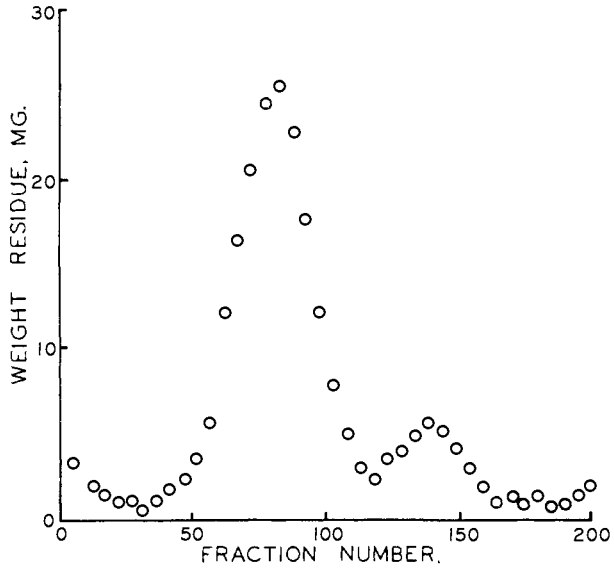

Fig. 2.—Cellulose powder partition chromatography of N-acetylframycetin.

TABLE II

SPECIFIC ROTATION OF N-ACETYLNEOMYCINS[a]

Source	N-Acetylneomycin B	N-Acetylneomycin C
Neomycin[b]	+47.8°	(+94.5°)[d]
Framycetin[c]	+47.6°	(+93.9°)[d]

[a] Specific rotations are measured at 28° in 4 mg./ml. aqueous solutions and are corrected for moisture. [b] N-Acetylneomycin components derived from neomycin sulfate, Upjohn 4362-RCA-5A,U-4567. [c] N-Acetylneomycin components derived from framycetin sulfate, Roussel 3055-4. [d] Minor component.

Degradation Products from Neomycin B and Framycetin.—The conclusion derived from paper chromatographic studies of the intact neomycin B and framycetin molecules, that the antibiotics are identical, was substantiated by parallel degradative studies on the two antibiotics.

Neomycin B was shown in earlier studies[3,4,6,29] to consist of two smaller fragments of approximately equal size, neamine ($C_{12}H_{26}N_4O_6$) and neobiosamine B ($C_{11}H_{22}N_2O_8$).[30] On methanolysis of neomycin B, neamine and the methyl glycoside of neobiosamine, methyl neobiosaminide B, were obtained.[3,4,31] While the complete structural formula for neamine has not yet been assigned, it has been shown to contain deoxystreptamine (I)[32] and a $C_6H_{14}N_2O_4$ moiety of unknown structure.[31] Neamine is purified readily *via* its crystalline hydrochloride. However, the compound is quite stable to hydrolysis so that those conditions which are sufficiently vigorous to cleave the bond between deoxystreptamine and the second C_6N_2 fragment give rise to extensive degradation of the latter.

Similarly, methanolysis of framycetin was shown by Janot, *et al.*,[19] also to give neamine, together with a second methanolysis product, referred to by these workers as "fraction B" or "methyl-oxide." The striking similarity of its properties to those of methyl neobiosaminide B has been noted earlier.[33]

Methyl neobiosaminide B has been shown in preliminary reports from one of these laboratories to be a disaccharide,[3] composed of a diaminohexose, neosamine B,[33] and a pentose, D-ribose.[34] Neosamine B was obtained as the product of

(29) K. L. Rinehart, Jr., P. W. K. Woo, A. D. Argoudelis and A. M. Giesbrecht, THIS JOURNAL, **79**, 4567 (1957).

(30) The formula originally proposed for neobiosamine B, $C_{11}H_{22}N_2O_7$,[3] has been shown to be incorrect in a preliminary communication[29] of portions of the present work.

(31) (a) J. R. Dyer, Ph.D. Dissertation, University of Illinois, June, 1954; (b) P. D. Shaw, Ph.D. Dissertation, University of Illinois, August, 1957.

(32) F. A. Kuehl, M. N. Bishop and K. Folkers, THIS JOURNAL, **73**, 882 (1951).

(33) K. L. Rinehart, Jr., P. W. K. Woo and A. D. Argoudelis, *ibid.*, **80**, 6461 (1958).

(34) K. L. Rinehart, Jr., P. W. K. Woo and A. D. Argoudelis, *ibid.*, **79**, 4568 (1957).

vigorous hydrolysis of methyl neobiosaminide B, whereas D-ribose was isolated from the mild hydrolysis of benzoylated methyl neobiosaminide B. Similarly, vigorous hydrolysis of "fraction B," the "methyl-oside" from framycetin, gave "fraction C," considered to be an anhydrodiaminohexose,[19] whose reported properties were quite similar to those of neosamine B. Mild hydrolysis of framycetin by warming with a cation exchange resin gave "fraction D," believed to be a pentose but said to differ from all common pentoses, methylpentoses and deoxypentoses.[19]

At the beginning of the present studies, then, the similarities between the two antibiotics were apparent: (a) neomycin B and framycetin had similar rotations; (b) on methanolysis both gave neamine plus a methyl glycoside of similar properties; (c) the methyl glycoside could be degraded to a smaller fragment, a diaminohexose of similar properties; and (d) both antibiotics contained a pentose (certainly in the methyl neobiosaminide B and presumably in the "fraction B" or "methyl-oside" portion). However, the disparity in experimental conditions employed by the two groups and the lack of complete descriptions of conditions and products[19] rendered it desirable to undertake a single study employing identical, standardized conditions for the degradation of the two antibiotics. This has now been done and the study confirms the identity of the two.

Methanolysis of neomycin B in 0.38 *N* methanolic hydrogen chloride gave neamine and methyl neobiosaminide B; similar methanolysis of framycetin gave the same two products. The two are formed in nearly identical weight ratios from methanolyses of the two antibiotics (*cf.* Table III). The identity of neamine from methanolyses of the two antibiotics has been confirmed in the present study by means of paper chromatography.

TABLE III

METHANOLYSIS PRODUCTS FROM NEOMYCIN B AND FRAMYCETIN

	Neomycin B			Framycetin	
Study[a]	I	II	III	I	IV
Neamine					
Amount[b]	0.40 to 0.42	0.48	...	0.43	0.50
Methyl neobiosaminide B					
Amount[b]	0.47 to 0.48	0.34	...	0.54	0.50
$[\alpha]_D$	+24° to +25°	..	+22°	+30°	+23°

[a] I, present study; II, ref. 4; III, ref. 3; IV, ref. 19. [b] Relative to starting neomycin B or framycetin.

The methyl glycoside, methyl neobiosaminide B, from the two antibiotics also is the same. Rotations of the crude materials isolated from the reaction mixtures are very similar and compare well with those reported from both neomycin B and framycetin by earlier workers (*cf.* Table III). This material is, however, a mixture, and physical properties of the material isolated directly from the methanolysis are not particularly meaningful. Paper chromatograms of the crude product (from both framycetin and neomycin B) showed the presence of neamine and unreacted neomycin, as well as of methyl neobiosaminide. The nature of the mixture was confirmed by charcoal chromatography (*cf.* Fig. 3); the first eluates (fraction a) from the column were shown to contain sizable

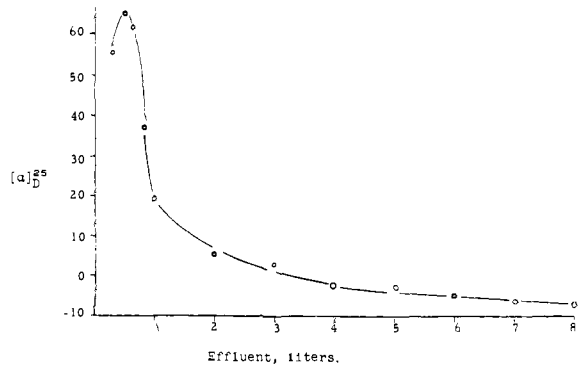

Fig. 3.—Chromatography of crude methyl neobiosaminide B; specific rotation of eluates.

amounts of neamine and neomycin, whereas later fractions (b and c) contained smaller quantities of these impurities. These early fractions (and d as well) undoubtedly also contained some methyl neobiosaminide C, since the starting neomycin B contained a small amount of neomycin C.[4] Although methyl neobiosaminide C is not distinguished from B on papergrams, it is known to emerge from a charcoal column before B.[35] Identities of the various components were determined by specific rotations, by papergrams and by carbon–nitrogen ratios; the latter values were useful criteria since the C/N ratios for neamine, neomycin and methyl neobiosaminide B differ greatly.

In later fractions (e through g), however, the effluent contained only methyl neobiosaminide B. Although the specific rotation steadily decreased, as noted in Fig. 3, this presumably was due to the prior emergence from the column of methyl α-neobiosaminide B, followed by the β-glycoside. This conclusion, that mixtures of anomeric forms were the cause of the rotation drop,[35b] was shown

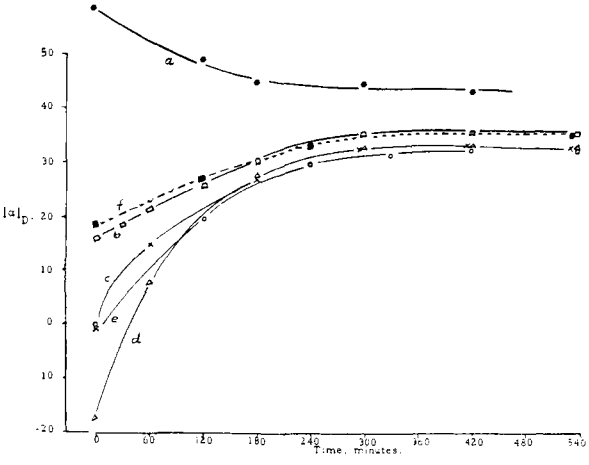

Fig. 4.—Specific rotations of methyl neobiosaminide B hydrolysates: a, methyl neobiosaminide B from fraction c; b, from fraction e; c, from fraction f; d, from fraction g; e, from a second chromatographic run; f, from framycetin.

to be correct by hydrolysis under mild conditions of the methyl neobiosaminide B fractions to neobiosamine B of constant mutarotation value, $[\alpha]^{25}D$ + 34° (*cf.* Fig. 4).

(35) (a) P. W. K. Woo, Ph.D. Thesis, University of Illinois, August, 1958; (b) A. D. Argoudelis, Ph.D. Thesis, University of Illinois, August, 1959.

The quantities of methyl neobiosaminide B obtained from methanolysis of the small available sample of framycetin did not allow its purification by charcoal chromatography. Instead, it was purified by preparative paper chromatography, which separated it from neamine and neomycin, but not from methyl neobiosaminide C. The specific rotation of the sample was lowered by this procedure from $[\alpha]^{25}D + 30°$ to $[\alpha]^{25}D + 19°$. Mild hydrolysis of this sample gave essentially the same mutarotation value, $[\alpha]^{25}D + 36°$, for neobiosamine B as did those of methyl neobiosaminide B samples from neomycin (*cf.* Fig. 4). The slightly higher value probably reflects the presence of a small amount of neobiosamine C, expected since framycetin has been shown to contain a small amount of neomycin C in addition to the predominant B isomer (*cf.* above). From the mutarotation values of pure neobiosamines B and C, +34° and +104°, respectively,[29] the proportion of neomycin C in framycetin is estimated to be about 3%, in good agreement (*cf.* Fig. 2) with the value obtained from chromatography of the intact N-acetylframycetin.

The identity of methyl neobiosaminide B from the two antibiotics has been substantiated further by their hydrolyses to neosamine B and to ribose. Neosamine B was obtained by hydrolysis of methyl neobiosaminide B (from both neomycin B and framycetin) in refluxing 6 *N* hydrochloric acid. The compound obtained from the two sources was shown to be identical by papergram behavior and by rotation (*cf.* Table IV).

TABLE IV

DEGRADATION PRODUCTS OF METHYL NEOBIOSAMINIDE B

Compound From	Neomycin B[a]	Framycetin[a]
Neobiosamine B		
R_f (BAW 2:2:1)[a,b]	0.190[b]	0.187[b]
R_f (PAW 10:1:9)	0.540	0.541
$[\alpha]^{25}D$	+34°	+36°
Neosamine B		
R_f (BAW 2:2:1)	0.252	0.250
R_f (PAW 10:1:9)	0.610	0.604
$[\alpha]^{25}D$	+17.5°	+15.6°
D-Ribose		
R_f (BAW 4:1:5)	0.287	0.289 (0.290)[c]
R_f (BAW 2:2:1)	.508	.509 (.510)[c]
R_f (PAW 10:1:9)	.640	.642 (.644)[c]
$[\alpha]^{28}D$	−16°	... (−23°)

[a] All R_f values for the same compound were determined from a single chromatogram containing the various samples. [b] Solvent systems are described in footnote 39 (*cf.* text). [c] Values in parentheses refer to an authentic sample of D-ribose.

The pentose obtained, both from framycetin and from neomycin B, was shown to be ribose. Direct hydrolysis of the glycosidic bond in neobiosamine B is difficult, due to inhibition by the positively charged ammonium group of protonation required for cleavage of the glycosidic bond. Thus, under conditions severe enough to cause glycosidic cleavage, the pentose is degraded to furfural.[4,32a] In the present study this difficulty has been circumvented by removal of the basic amino group. In one sequence this has been accomplished by conversion of the amino group to the neutral benzamido group. Hydrolysis of this N-benzoyl derivative gave D-ribose, identified by papergrams and rotation. A similar procedure was employed for the isolation of D-ribose from methyl N,N′-dibenzoylneobiosaminide C.[34] In a second reaction sequence the amino groups of methyl neobiosaminide B (from neomycin) were removed by treatment with silver nitrite; hydrolysis of the deamination product gave ribose, identified from color reactions and R_f-values. The deamination procedure also was employed for the identification of ribose from framycetin methyl neobiosaminide B (*cf.* Table IV).

Molecular Formula of Neobiosamine B.—Neosamine is a diaminohexose, $C_6H_{14}N_2O_4$. This is shown clearly by microanalytical values for its dihydrochloride and its dipicrate, and by those for the N,N′-bis-dinitrophenyl derivative of its reduction product, neosaminol B, as well as by its positive reactions toward Fehling and Elson–Morgan reagents and its periodate uptake.[36,37] The former investigators of framycetin[19] assigned the formula $C_6H_{12}N_2O_3$ to their "fraction C," and considered this compound to be an anhydrodiaminohexose, arising from the dehydration of a diaminohexose. In the present studies no compound corresponding to an anhydrodiaminohexose has been obtained. Whether this is due to the somewhat less strenuous hydrolysis procedure employed (6 *N* acid *vs.* 7 *N* acid employed in the earlier study), or whether neosamine B and "fraction C" are the same compound is uncertain. The rotation of "fraction C" is very similar to that obtained in the present study for neosamine B, and the reported melting point of the picrate of "fraction C," 126°, is the same as that found in the present study for neosamine B picrate, 125–126.5°. It would, perhaps, be surprising if the anhydro derivative of neosamine B had the same rotation as the parent diaminohexose, since 1,6-anhydromonoaminohexoses have generally quite different rotations from the parent monoaminohexoses.[38] In this respect it may be noted that "fraction C" consumed five moles of periodate,[19] as expected for a diaminohexose, rather than the lower number expected for an anhydrodiaminohexose. It seems likely, then, that "fraction C" may have been slightly impure neosamine B.

The investigators of framycetin stated that the pentose obtained by them differed from known compounds.[19] In the present study, however, it is clearly shown to be ribose. Whether degradation of the pentose occurred in the earlier hydrolysis procedure has not been established.

From the molecular formulas for neosamine B and ribose, $C_6H_{14}N_2O_4$ and $C_5H_{10}O_5$, the molecular formula of neobiosamine B must be $C_{11}H_{22}N_2O_8$ and that of its methyl glycoside $C_{12}H_{24}N_2O_8$. Chromatographically purified methyl neobiosaminide B, its perchlorate and its N,N′-bis-dinitrophenyl derivative, neobiosamine B, its N,N′-bis-dinitrophenyl derivative, and its borohydride re-

(36) K. L. Rinehart, Jr., and A. D. Argoudelis, paper in preparation.

(37) All nitrogen in the parent neomycin occurs in the form of primary amino groups, hence neosamine B must contain two primary amino groups.

(38) R. Kuhn, W. Bister and H. Fischer, *Ann.*, **617**, 109 (1958).

duction product neobiosaminol B, all gave microanalyses in agreement with the molecular formulas $C_{11}H_{22}N_2O_8$ for neobiosamine B and $C_{12}H_{24}N_2O_8$ for its methyl glycoside. Although earlier workers suggested the molecular formula $C_{12}H_{24}N_2O_7$ for methyl neobiosaminide B and postulated it to be the methyl glycoside of a diaminodeoxyhexose-pentose disaccharide,[3] the present data exclude this possibility.

In summary, then, the identity of framycetin and neomycin B rests on their chromatographic behavior, their nearly identical optical rotations, and their degradation products. Methanolysis of either antibiotic gives the same two degradation products, neamine and methyl neobiosaminide B. Samples of the latter compound from the two antibiotics have been shown to be identical by their physical properties (R_f-values and rotation) and by their further degradation to the same products, neosamine B and D-ribose. Identity of neosamine B samples from the two antibiotics has been established by rotation and papergram behavior, identity of ribose by color tests and R_f-values.

Experimental[39]

Preparation of N-Acetylneomycins.—Since the neomycin free base was acetylated more easily than the sulfate salts, the neomycin sulfate salts were converted to the free base by use of the anion exchange resin, Amberlite IRA400[40] (hydroxyl phase). Five and one-half grams of neomycin sulfate[41] was dissolved in 250 ml. of distilled water; the resultant solution was passed over 100 ml. of Amberlite IRA400 anion exchange resin in the hydroxyl phase. The eluates and aqueous washes were concentrated to dryness *in vacuo*. The neomycin free base was dissolved in 300 ml. of absolute methyl alcohol; 100 ml. of acetic anhydride was added, and the resultant solution was shaken for 24 hours at room temperature or until the solution produced a negative ninhydrin reaction. The N-acetylneomycin was precipitated from solution by the addition of 1 liter of anhydrous ether. The precipitate was washed several times with small aliquots of ether and finally dried *in vacuo*. The entire procedure was repeated with framycetin sulfate.[41]

Cellulose Powder Column Partition Chromatography.—The chromatographic columns employed in the separation of N-acetylneomycins were prepared by packing glass columns, 25 × 1100 mm., with small aliquots (2.0 g.) of Whatman powdered cellulose, ashless grade for chromatography. A cellulose column, 25 × 1000 mm., was thus prepared by mechanical packing. The N-acetylneomycin sample (1.0 g.) was mixed with an equal weight of the cellulose powder and placed on top of the column. A few aliquots of cellulose powder were packed on top of the band of the N-acetylneomycin and cellulose mixture.

The one-phase solvent system consisting of *n*-butyl alcohol–pyridine–water (3:2:2) was prepared immediately prior to use and applied directly to the column. The column was allowed to flow without pressure, and the eluate was collected in 5.0-ml. aliquots. Column fractions were analyzed by the paper chromatographic procedure of Pan and Dutcher[22] and by weight residue analyses (*cf.* Fig. 1). Column fractions containing individual N-acetylneomycins were pooled, extracted with petroleum ether, and concentrated to dryness *in vacuo*. The residues were crystallized from methyl alcohol–ethyl ether solvent mixtures under anhydrous conditions. The weights of N-acetylneomycin B and N-acetylneomycin C isolated were, respectively, 0.58 and 0.39 mg. (97 and 98% of theoretical).

Preparation of Methyl Neobiosaminide B.—Methyl neobiosaminide B was prepared by methanolysis of neomycin B according to a previous procedure.[4,32a] In a typical run 30.0 g. of dried neomycin B sulfate, $[\alpha]^{25}D$ + 52.5°,[4] was heated for 3 hours under reflux with 3.6 liters of 0.38 *N* hydrogen chloride in scrupulously dried methanol. The clear solution was cooled in ice and diluted with 1.2 liters of anhydrous ether to precipitate neamine hydrochloride, which was filtered. The filtrate was concentrated to 0.3 liter and treated with 3.6 liters of anhydrous ether to precipitate crude methyl neobiosaminide B hyrochloride. In six runs the weight of neamine hydrochloride fraction isolated varied from 12.0 to 12.5 g. (78–81% of theoretical), whereas that of crude methyl neobiosaminide B hydrochloride fraction $[\alpha]^{25}D$ + 23.6 to + 24.6° (*c* 2.1 to 2.9, water), varied from 14.0 to 14.5 g. (107–110% of theoretical).

Chromatography of Crude Methyl Neobiosaminide B.—Chromatographic separation was effected by a method previously employed for the purification of neomycins B and C.[4] Chromatographic columns were prepared from slurries composed of two parts of Darco G-60, one part of Celite 545 and nine parts of de-ionized water; the mixture was adjusted to *p*H 2 with sulfuric acid and stirred vigorously for 2 hours, then packed under nitrogen pressure and washed with water.

In a typical run, crude methyl neobiosaminide B hydrochloride from the above methanolyses was converted by passage over Dowex-2[42] (hydroxyl phase) to the free base. The base was dissolved in a minimum of water and added to a small amount of slurried Darco–Celite (*p*H 2, sulfuric acid) similar to that of the column. The column, 5 cm. × 120 cm., containing 390 g. of Darco–Celite, was eluted with water; the effluent was collected initially in 20-ml. fractions, subsequently in very dilute 200-ml. fractions. The specific rotation behavior of 8.5 liters of effluent is shown in Fig. 3, while specific rotations, observed rotations and concentrations of the initial 150 20-ml. fractions are shown in Fig. 5. Larger fractions (a to g), obtained by combining several of these small eluate fractions, are discussed below. Each of these was treated in the same manner. Papergrams were determined on the eluate fractions before combination. After combination these were passed over Dowex-2[42] and lyophilized to the free base.

Fraction a (from eluates 1 to 20, 0 to 0.4 liter, *cf.* Fig. 5) was estimated by graphical integration to contain 5.16 g. of material. This was shown by paper chromatography to be a mixture of neomycin B, R_f (BAW 2:2:1)[39] 0.00; and neamine, R_f (BAW 2:2:1) 0.20 to 0.50.

Anal. Calcd. for $C_{23}H_{46}N_6O_{13}$ (neomycin): C/N, 3.83. Calcd. for $C_{12}H_{26}N_4O_6$ (neamine): C/N, 3.00. Calcd. for $C_{12}H_{24}N_2O_8$ (methyl neobiosaminide): C/N, 6.00. Found: C/N, 3.24.

Fraction b free base (from eluates 20 to 30, 0.4 to 0.6 liter) had $[\alpha]^{25}D$ +74.4° (*c* 0.6, water). This fraction was shown by paper chromatography to be a mixture of neomycin B, R_f (BAW 2:2:1) 0.00 to 0.02; neamine, R_f (BAW 2:2:1) 0.20 to 0.50; and methyl neobiosaminide B, R_f (BAW 2:2:1) 0.80.

Anal. Found: C/N, 4.99.

Fraction c free base (from eluates 31 to 35, 0.6 to 0.7 liter), $[\alpha]^{25}D$ +62.8° (*c* 1.6, water), consisted largely of methyl neobiosaminide B, R_f (BAW 2:2:1) 0.81, but contained some neamine, R_f (BAW 2:2:1) 0.25 to 0.48.

Anal. Found: C/N, 5.3.

Fraction d free base (from eluates 36 to 40, 0.7 to 0.8 liters), $[\alpha]^{25}D$ +45° (*c* 2.2, water), consisted almost entirely

(39) Melting points were determined on a Kofler micro hot-stage and are corrected. Solvent systems and spray reagents employed for paper chromatography are abbreviated, and their abbreviations are described in the text as: PAW 9:1:10, a one-phase system, consisting of *n*-propyl alcohol, glacial acetic acid and water (9:1:10, by volume); BAW 2:2:1, a one-phase system, consisting of *t*-butyl alcohol, glacial acetic acid and water (2:2:1, by volume); PAW 10:1:9, a one-phase system, consisting of *n*-propyl alcohol, glacial acetic acid and water (10:1:9, by volume); BEW 4:1:5, an equilibrated two-phase system, from *n*-butyl alcohol, ethyl alcohol and water (4:1:5, by volume), consisting of an organic mobile phase and an aqueous stationary phase; BAW 4:1:5, a system identical to BEW 4:1:5, except that ethyl alcohol is replaced by acetic acid; NIN, a solution of 0.25 g. of ninhydrin in 50 ml. of pyridine and 50 ml. of 95% ethyl alcohol; AHP, a solution of 1.66 g. of phthalic acid and 0.93 g. of aniline in 100 ml. of water-saturated *n*-butyl alcohol; orcinol, a solution of 0.5 g. of orcinol and 15 g. of trichloroacetic acid in 100 ml. of water-saturated *n*-butyl alcohol.

(40) A quaternary base anion exchange resin sold by the Rohm and Haas Co.

(41) Neomycin sulfate: Upjohn Co. Lot No. 4362-RCA-5A. Micronized U-4567. Framycetin sulfate: Roussel Lot No. 3055-4.

(42) A strongly basic anion-exchange resin obtained from the Dow Chemical Co.

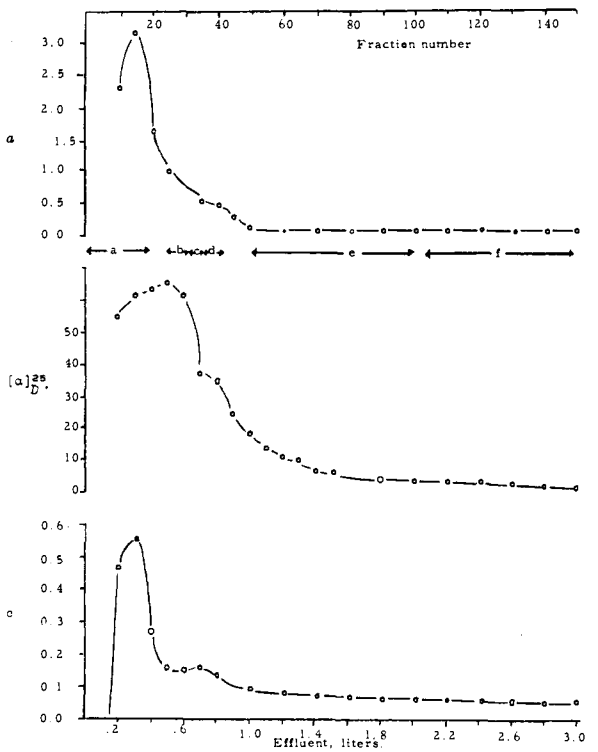

Fig. 5.—Properties of eluates from chromatography of crude methyl neobiosaminide B: α, observed rotation (°); $[\alpha]_D$, specific rotation (°); c, concentration (g./200 ml.).

of methyl neobiosaminide B, R_f (BAW 2:2:1) 0.83, 0.44[43]; with a trace of neamine, R_f (BAW 2:2:1) 0.30.

Anal. Found: C/N, 5.82.

Fraction e free base (eluates 50 to 100, 1.0 to 2.0 liters), $[\alpha]^{25}D$ +12.5° (c 1.3, water), contained only methyl neobiosaminide B, R_f (BAW 2:2:1) 0.82, 0.47.[43]

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot H_2O$: C, 42.14; H, 7.66; N, 8.19; C/N, 6.00. Found: C, 42.64; H, 7.61; N, 8.44; C/N, 5.88.

Fraction f free base (eluates 100 to 150, 2.0 to 3.0 liters), $[\alpha]^{25}D$ −3.3° (c 1.5, water), consisted of methyl neobiosaminide B, R_f (BAW 2:2:1) 0.82, 0.44.[43]

Anal. Found: C, 41.82; H, 7.42; N, 7.95; C/N, 6.05.

Fraction g free base (7.5 to 8.5 liters), $[\alpha]^{25}D$ −17° (c 1.2, water), contained only methyl neobiosaminide B, R_f (BAW 2:2:1) 0.82, 0.43.

Anal. Found: C, 42.01; H, 7.40; N, 8.21; C/N, 5.98.

The sample was dissolved in methanol, re-precipitated with ether and dried for 18 hours at 56° (0.1 mm.); it then sintered at 110°, darkened at 168–175° and melted at 192–200°.

Anal. Calcd. for $C_{12}H_{24}N_2O_8$: C, 44.48; H, 7.47; N, 8.65; C/N, 6.00. Found: C, 43.93; H, 7.32; N, 8.77; C/N, 5.84.

Methyl neobiosaminide B diperchlorate was prepared by passing an aqueous solution of 1.5 g. of the free base, $[\alpha]^{25}D$ −7.5° (c 0.6, water), through an anion exchange column containing Dowex-2[42] resin in the perchlorate phase. The eluate was lyophilized to give 1.2 g. (50%) of the perchlorate, $[\alpha]^{25}D$ −4.8° (c 1.0 water), m.p. 145–148° dec.

Anal. Calcd. for $C_{12}H_{24}N_2O_8 \cdot 2HClO_4$: C, 27.45; H, 4.99; N, 5.34. Found: C, 27.32; H, 5.29; N, 5.53.

(43) The multiple R_f-values frequently observed for methyl neobiosaminide B probably are due to differing ionic species of the compound. The same phenomenon has been observed in chromatograms of pure methyl neobiosaminide C,[35] as well as in chromatograms of amino acids [*cf.*, *e.g.*, H. K. Berry, H. E. Sutton, L. Cain and J. S. Berry, in "Biochemical Institute Studies IV," University of Texas Publication No. 5109, May 1, 1951, pp. 43, 47; and S. Aronoff, *Science*, **110**, 590 (1949)].

Methyl N,N′-Bis-(2,4-dinitrophenyl)-neobiosaminide B.—A mixture of 0.350 g. of methyl neobiosaminide B, 2.2 g. of sodium bicarbonate and 2.15 g. of 2,4-dinitrofluorobenzene in 60 ml. of 80% ethanol was shaken for 4 hours and allowed to stand for 12 hours at room temperature, then dried under reduced pressure. The residue was dissolved into methanol and purified by partition chromatography over a silicic acid–Celite column, employing an ethanol–chloroform–water (1:9:1) solvent system. After a forerun of 2,4-dinitrofluorobenzene and 2,4-dinitrophenol, methyl N,N′-bis-(2,4-dinitrophenyl)-neobiosaminide B was eluted as the major band. Solvent was removed under reduced pressure to give the bright yellow derivative, R_f (BEW 4:1:5[39]) 0.905, m.p. 164–166°.

Anal. Calcd. for $C_{22}H_{28}N_6O_{16}$: C, 43.94; H, 4.29; N, 12.83. Found: C, 43.88; H, 4.05; N, 12.79.

Neobiosamine B. I. Preparation.—A solution of 1.5 g. of methyl neobiosaminide B, $[\alpha]^{26}D$ −3° (c 1.2, water) in 40 ml. of 1 N aqueous hydrochloric acid was heated for 8 hours at 83°, then decolorized with charcoal and lyophilized. The residue was purified by repeated solution in methanol and precipitation with ether. The white precipitate of **neobiosamine B dihydrochloride** (0.95 g., 58%) was filtered and dried *in vacuo;* the material sintered 150° and darkened 198–200°. Papergrams of the material showed one spot, purple to NIN[39] and AHP,[39] R_f (BAW 2:2:1) 0.25.

Anal. Calcd. for $C_{11}H_{22}N_2O_8 \cdot 2HCl$: C, 34.47; H, 6.31; N, 7.31. Found: C, 34.19; H, 6.62; N, 6.75.

II. Mutarotation Value from Hydrolysis of Methyl Neobiosaminide B.—Each of a number of solutions of methyl neobiosaminide B in 1 N aqueous hydrochloric acid was divided into sealed tubes. The tubes were immersed in a constant temperature bath at 89° and removed at intervals for rotation determinations. Material for runs I to IV was obtained from the typical chromatographic run described above, while that for run V was from a second chromatographic run. Rotations of the individual samples are given in Table V and summarized in Fig. 4. Paper chromatograms of hydrolysates gave in every case except run I only one spot, positive to both ninhydrin and AHP. R_f values (BAW 2:2:1) were: run I (420 min.), 0.25; run II (600 min.), 0.25; run III (420 min.), 0.25; run IV (540 min.), 0.25. A papergram of run I (420 min.) showed in addition the presence of neamine.

N,N′-Bis-(2,4-dinitrophenyl)-neobiosamine B was prepared by the procedure described above for methyl N,N′-bis-(2,4-dinitrophenyl)-neobiosaminide B, from 0.269 g. of neobiosamine B dihydrochloride, 2 g. of 2,4-dinitrofluorobenzene and 2 g. of sodium bicarbonate in 60 ml. of 80% ethanol. The bright yellow compound obtained weighed 0.450 g. (40%), and had m.p. 165–169°, R_f (BEW 4:1:5) 0.895, $[\alpha]^{26}D$ +30° (c 0.39, methanol).

Anal. Calcd. for $C_{28}H_{26}N_6O_{16}$: C, 43.03; H, 4.08; N, 13.08. Found: C, 42.89; H, 3.71; N, 13.46.

Neobiosaminol B.—A solution of 0.540 g. of neobiosamine B dihydrochloride and 0.42 g. of sodium borohydride in 20 ml. of water was allowed to stand for 30 minutes in an ice-bath, then for 2 hours at room temperature. After it had been acidified to pH 2 with hydrochloric acid, the solution, which gave a negative Fehling test, was evaporated to dryness *in vacuo*. The white hygroscopic powder was freed of borate (negative flame test) by repeated addition and evaporation of methanolic hydrogen chloride, of other inorganic salts by solution in a minimum of methanol. Addition of ether to the methanolic solution precipitated the crude hydrochloride. One-half of this was purified by recrystallization from ethanol–ether; pure **neobiosaminol B dihydrochloride** (93 mg., 38%) decomposed 186–190°, had R_f (BAW 2:2:1) 0.22.

Anal. Calcd. for $C_{11}H_{24}N_2O_8 \cdot 2HCl$: C, 34.28; H, 6.80; N, 7.27. Found: C, 34.51; H, 6.83; N, 6.96.

D-Ribose. I. Identification. A. Via Deamination of Methyl Neobiosaminide B.—A mixture of 60 mg. of methyl neobiosaminide B, 250 mg. of silver nitrite and 4 ml. of 20% acetic acid was allowed to stand for 1 hour at 0° and for 7 hours at room temperature. Silver chloride was precipitated by the addition of 0.5 ml. of concentrated hydrochloric acid and removed by filtration. The filtrate was lyophilized to give a hygroscopic white powder, which gave a negative AHP test and a slightly positive NIN test.

TABLE V

MUTAROTATION VALUES OF METHYL NEOBIOSAMINIDE B HYDROLYSES

Run	Methyl neobiosaminide $[\alpha]^{25}D$	Concn., g./100 ml.	$[\alpha]^{25}D$ at t min. 0	30	60	120	180	240	300	330	420
I	+62.8°	0.487	+59.5°			49.2°	45.1°		45.1°		3.14°
II	+12.5	1.055	+16.5	18.5°	22°	26.3	30.2		36.1		36.0
III	− 3.3	1.329	0		+15		27.1		33.1		33.1
IV	−17	1.217	−17		+ 7.4		27.9		32.0		32.8
V	− 1.5	1.666	0			+20.8		30.3°		31.2°	

A solution of 4.2 mg. of the white powder in 1 ml. of 1 *N* aqueous hydrochloric acid was kept for one hour in a sealed tube at 80°. Possible NIN-positive material was removed by addition of a small amount of Dowex-50[44] (hydrogen phase). The filtrate was lyophilized to give a pale yellow hygroscopic solid, negative to NIN and strongly positive to AHP. Paper chromatograms showed this material to contain ribose; R_f-values[39] of sample: 0.287 (BAW 4:1:5), 0.508 (BAW 2:2:1), 0.640 (PAW 10:1:9); R_f - values of authentic ribose: 0.290 (BAW 4:1:5), 0.510 (BAW 2:2:1), 0.644 (PAW 10:1:9).

B. *Via* **Methyl N,N′,O-Tribenzoylneobiosaminide B.**—To a mixture of 0.600 g. of methyl neobiosaminide B, $[\alpha]^{25}D$ −11.8° (*c* 0.8, water), 15.7 ml. of 1 *N* aqueous sodium hydroxide and 6.6 ml. of benzoyl chloride was added, in portions, 13.2 ml. of 25% aqueous sodium hydroxide. The temperature was maintained at 5–10° and the mixture was shaken vigorously after each addition of alkali, then shaken for 6 hours at room temperature, finally allowed to stand for 24 hours at room temperature. The supernatant was decanted and the gummy, polybenzoylated organic residue was heated for 4 hours under reflux with 83 ml. of 0.25 *N* sodium hydroxide in 80% methanol. The solution was cooled, adjusted to *p*H 7 with hydrochloric acid and evaporated to dryness *in vacuo*. The residue was crystallized from methanol to give 0.550 g. (56%) of white needles, sintering 203°, melting 215–220°, negative to NIN. The infrared spectrum (Nujol) showed both aromatic ester (1718 cm.$^{-1}$) and, somewhat stronger, amide (1645 cm.$^{-1}$) carbonyl absorption, while analytical values agreed with its formulation as a tribenzoyl derivative.

Anal. Calcd. for $C_{12}H_{21}N_2O_8(C_6H_5CO)_3 \cdot H_2O$: C, 58.82; H, 6.21; N, 4.41. Found: C, 59.00; H, 6.61; N, 4.31.

A solution of 43.8 mg. of the tribenzoyl derivative in 2 ml. of 1 *N* aqueous hydrochloric acid was held for 2 hours at 99° (sealed tube), cooled and extracted with ether to remove benzoic acid. The aqueous phase was lyophilized to a hygroscopic pale yellow powder. Papergrams of this material showed it to contain ribose. In Table VI, R_f-values are compared to those of known pentoses. Each R_f-value is the average of three values.

TABLE VI

PAPER CHROMATOGRAPHY OF PENTOSE SUGARS

Compound	R_f value + average deviation BAW 2:2:1[a]	BAW 4:1:5[a]	Spot color AHP[a]	Orcinol[a]
Ribose	0.596 + 0.006	0.308 + 0.005	Red	..[b]
Lyxose	.547 + .004	.248 + .006	Red	..
Xylose	.542 + .005	.254 + .006	Red	..
Arabinose	.536 + .006	.257 + .007	Red	..
Xylulose	.671 + .009	.334 + .007	Brown	Green
Ribulose	.692 + .007	.330 + .006	Brown	Green
Hydrolysate	.597 + .006	.308 + .006	Red	..

[a] *cf.* footnote 39. [b] Negative test.

In a preliminary hydrolytic experiment it had been shown that the maximum amount of ribose was formed after about 1 hour. A solution of 60 mg. of the tribenzoyl derivative in 3 ml. of 1 *N* aqueous hydrochloric acid was divided into three sealed tubes, which were removed at 1-, 3- and 5-hour intervals and treated as described above. The red AHP-positive (NIN-negative) ribose spot was most intense after 1 hour, weak after 3 or 5 hours; R_f-values of the sample were 0.585 (BAW 2:2:1), 0.308 (BAW 4:1:5); those of ribose were 0.583 (BAW 2:2:1), 0.308 (BAW 4:1:5). A second spot, R_f 0.250 (BAW 2:2:1), 0.240 (BAW 4:1:5), positive to both AHP and NIN, increased with time and was attributed to neosamine B, perhaps mixed with neobiosamine B. Two other AHP-positive spots also were observed; these had higher R_f-values than the two above and reached maximum intensity after 3 hours, then decreased.

II. **Isolation.**—A solution of 0.400 g. of crude methyl N,N′,O-tribenzoylneobiosaminide B in 20 ml. of 1 *N* aqueous hydrochloric acid was heated for 1 hour under reflux. Precipitated benzoic acid was filtered from the cooled solution and the filtrate was neutralized to *p*H 6.8 by the addition of Dowex 2[42] (hydroxyl phase). The resin was filtered and the filtrate, which gave strong NIN and AHP tests, was concentrated. Neosamine and neobiosamine were removed by repeated passage of the filtrate over a Dowex 50[44] column (hydrogen phase). The effluent, neutralized to *p*H 6.5 by Dowex-2 (hydroxyl phase), gave a negative NIN test but strongly positive AHP test. Lyophilization gave 60 mg. of white, highly hygroscopic, powder, of which 20 mg. was purified by a descending preparative paper chromatogram. The chromatogram was developed for 24 hours with BAW 2:2:1 and air-dried for 12 hours. A zone, R_f 0.635, located by means of narrow strips from each edge and the center of the paper, was cut from the paper and extracted with water, then lyophilized to give 4.5 mg. of white powder. The powder was shown to be D-ribose by its papergram behavior, R_f 0.654 (BAW 2:2:1), 0.351 (BAW 4:1:5), and rotation, $[\alpha]^{28}D$ −16 ± 5° (*c* 0.25, water), the average of 10 readings. An authentic sample of D-ribose, determined simultaneously, had R_f 0.652 (BAW 2:2:1), 0.350 (BAW 4:1:5), $[\alpha]^{25}D$ −23.2° (*c* 1, water).

Neosamine B.—A solution of 2.000 g. of methyl neobiosaminide B free base (fraction g above) in 40 ml. of 6 *N* aqueous hydrochloric acid was heated for 90 minutes under reflux. The cooled black solution was diluted, decolorized with Norit, and evaporated to dryness under reduced pressure. The yellow resinous solid was crystallized repeatedly from methanol–ether, filtered and dried *in vacuo* to give 0.909 g. (60%) of white **neosamine B dihydrochloride**, $[\alpha]^{25}D$ +17.5° (*c* 0.9, water), d. 135–150°. A papergram gave two purple spots with NIN, R_f (BAW 2:2:1) 0.23 (strong), 0.49 (weak), and a single yellow-brown spot with AHP, R_f (BAW 2:2:1) 0.23.

Anal. Calcd. for $C_6H_{14}N_2O_4 \cdot 2HCl$: C, 28.69; H, 6.41; N, 11.16. Found: C, 28.41; H, 6.61; N, 10.76.

N,N′-Bis-(2,4-dinitrophenyl)-neosamine B was prepared by the procedure described above for the preparation of methyl N,N′-bis-(2,4-dinitrophenyl)-neobiosaminide B, except that the solvent employed for chromatography was the organic phase of the mixture chloroform–methanol (75:25, volume) saturated with water as the mobile phase. From 0.301 g. of neosamine B was obtained 0.310 g. (40%) of the bright yellow DNP derivative, R_f 0.914 (BEW), m.p. 155–158°, $[\alpha]^{26}D$ −44° (*c* 0.31, methanol).

Anal. Calcd. for $C_{18}H_{18}N_6O_{12}$: C, 42.39; H, 3.56; N, 16.48. Found: C, 42.51; H, 3.31; N, 16.34.

Neosamine B Picrate.[45]—A concentrated aqueous solution of neosamine B dihydrochloride was added to a nearly saturated aqueous solution of picric acid. On concentration of the solution, picric acid was obtained first, and then the crude picrate. Repeated recrystallization from water gave the pure picrate, m.p. 125.0–126.5°, $[\alpha]D$ +13° (*c* 0.94, water).

Neosaminol B was prepared by borohydride reduction, as described above for the preparation of neobiosaminol B, from 0.502 g. of neosamine B dihydrochloride, $[\alpha]^{25}D$ +17.5° (*c* 0.9, water), and 0.434 g. of sodium borohydride in 20 ml. of water. The crude hydrochloride of the re-

(44) A strongly acidic cation-exchange resin obtained from the Dow Chemical Co.

(45) Prepared by Dr. T. P. Culbertson.

duction product gave a single spot on paper chromatography, positive to NIN and periodate–permanganate, negative to AHP, R_f 0.157 (BAW 2:2:1), while a simultaneous papergram of the starting diaminohexose showed one spot, positive to both NIN and AHP, R_f 0.201 (BAW 2:2:1).

The crude reduction product was converted to its dinitrophenyl derivative by the procedure described above for the preparation of N,N′-bis-(2,4-dinitrophenyl)-neosamine B. The deep yellow **N,N′-bis-(2,4-dinitrophenyl)-neosaminol** B isolated weighed 0.350 g. (30% from neosamine B dihydrochloride), had m.p. 118–122°, $[\alpha]^{26}D$ +30.5° (*c* 0.39, methanol), R_f (BEW 4:1:5) 0.912.

Anal. Calcd. for $C_{18}H_{20}N_6O_{12}$: C, 42.22; H, 3.95; N, 16.42. Found: C, 41.89; H, 3.86; N, 15.85.

Degradation Products of Framycetin.—Papergrams of framycetin sulfate[41] $[\alpha]^{26}D$ +47.4° (*c* 0.4, water), showed a single NIN-positive spot, R_f (BAW 2:2:1) 0.000, (PAW 10:1:9) 0.184; a simultaneous papergram of neomycin B sulfate had R_f (BAW 2:2:1) 0.000, (PAW 10:1:9) 0.184.

I. **Methyl neobiosaminide** B was obtained by methanolysis of 0.744 g. of framycetin under conditions identical to those employed above for methanolysis of neomycin B. Neamine hydrochloride obtained weighed 0.322 g. and had R_f (BAW 2:2:1) 0.131, (PAW 10:1:9) 0.485; on the same papergram a sample from methanolysis of neomycin B had R_f (BAW 2:2:1) 0.130, (PAW 10:1:9) 0.487. Methyl neobiosaminide B hydrochloride obtained weighed 0.404 g. and had $[\alpha]^{25}D$ +30° (*c* 1.3, water), R_f (BAW 2:2:1) 0.801, (PAW 10:1:9) 0.698. On the same papergram an authentic sample of methyl neobiosaminide B dihydrochloride from methanolysis of neomycin B had R_f (BAW 2:2:1) 0.804, (PAW 10:1:9) 0.699.

A sample of methyl neobiosaminide B hydrochloride from the framycetin methanolysis was purified by preparative descending paper chromatography, employing as solvent system BAW 2:2:1. The purified hydrochloride, $[\alpha]D$ +19.4° (*c* 0.35, 1 *N* aqueous hydrochloric acid), (21.7 mg., corresponding to 17.8 mg. of free base) was dissolved in 5 ml. of 1 *N* aqueous hydrochloric acid and heated in sealed tubes for varying lengths of time at 89°. Optical rotation data are summarized in the following table (*cf.* also Table V) and in Fig. 4. A paper chromatogram of the 360-minute hydrolysate showed one spot, positive to NIN and AHP, R_f (BAW 2:2:1) 0.187, (PAW 10:1:9) 0.541; on the same papergram neobiosamine B dihydrochloride, from neomycin B, had R_f (BAW 2:2:1) 0.190, (PAW 10:1:9) 0.540.

Time, min.	0	120	240	360
$[\alpha]^{25}D$	+19.4°	25	33.3	36

II. **D-Ribose.**—A solution of 50 mg. of methyl neobiosaminide B hydrochloride, from framycetin methanolysis, was deaminated and the deamination product hydrolyzed, precisely as described above for the identification of ribose from neomycin B. The pale yellow hygroscopic solid isolated was negative to NIN, strongly positive to AHP, R_f (BAW 4:5:1) 0.289, (BAW 2:2:1) 0.509, (PAW 10:1:9) 0.642. On the same papergrams the deamination–hydrolysis product from authentic methyl neobiosaminide B (neomycin B starting material) had R_f (BAW 4:1:5) 0.287, (BAW 2:2:1) 0.508, (PAW 10:1:9) 0.640, while authentic ribose had R_f (BAW 4:1:5) 0.290, (BAW 2:2:1) 0.510, (PAW 10:1:9) 0.644.

III. **Neosamine** B was prepared by hydrolysis of 105 mg. of methyl neobiosaminide B hydrochloride (from framycetin), previously purified by preparative descending paper chromatography (BAW 2:2:1). The hydrolysis and isolation procedures were precisely those employed to obtain neosamine B from methyl neobiosaminide B dihydrochloride (neomycin starting material; *cf.* above). The yield of neosamine B hydrochloride, $[\alpha]^{25}D$ +15.6° (*c* 1.07, water), was 46 mg. (70%). A papergram of the sample gave R_f (BAW 2:2:1) 0.250, (PAW 10:1:9) 0.604; on the same papergram an authentic sample of neosamine B dihydrochloride obtained from neomycin B gave R_f (BAW 2:2:1) 0.252, (PAW 10:1:9) 0.610. Both samples gave strongly positive tests with NIN and AHP. The infrared spectra of purified neosamine B (from neomycin B or framycetin) do not contain carbonyl absorption.

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Chemistry of the Tetracycline Antibiotics. II. Bromination of Dedimethylaminotetracyclines

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The reaction products obtained by brominating several different dedimethylaminotetracyclines are described as well as some further reactions of these bromo derivatives. The conversion of 12a-deoxydedimethylaminotetracycline to a known degradation product of oxytetracycline, dedimethylaminoterrarubein, is described.

In the course of chemical studies dealing with the tetracycline antibiotics, bromination reactions were carried out on three key tetracycline derivatives lacking the dimethylamino substituent. The compounds investigated were dedimethylaminotetracycline,[1] dedimethylamino-7-chlorotetracycline[1,2] and 12a-deoxydedimethylaminotetracycline.

Bromination of a chloroform suspension of dedimethylaminotetracycline at room temperature with one equivalent of N-bromosuccinimide (NBS) led to a crystalline monobromosubstitution product. The same substance was also isolated in poorer yield from reaction of dedimethylaminotetracycline with bromine in acetic acid containing sodium acetate. Introduction of the bromine was observed to result in a 15 mμ hypsochromic shift in the ultraviolet maximum in 0.1 *N* hydrochloric acid to 350 mμ (log ε 3.494) and in the ap-

(1) J. H. Boothe, G. E. Bonvicino, C. W. Waller, J. P. Petisi, R. G. Wilkinson and R. B. Broschard, THIS JOURNAL, **80**, 1654 (1958).

(2) C. R. Stephens, L. H. Conover, R. Pasternack, F. A. Hochstein, W. T. Moreland, P. P. Regna, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **76**, 3568 (1954).