stored in a vacuum desiccator over calcium chloride. The pyrone melted at 51° (reported 51.5°).

A weighed amount of the pyrone was placed in a 50-ml. flask equipped with a stirrer and a cork stopper. The flask was placed in a water-bath with a thermometer and stirrer. Weighed quantities of freshly distilled water were added from a weighing bottle, the water-bath heated above the upper consolute temperature, and the bath allowed to cool slowly, during which time the upper and lower solution temperatures were recorded; then more water was added and the procedure repeated. These temperatures were reproducible within ±0.5°. Readings were recorded at the temperature at which there was a definite separation into two phases. The data are plotted in Fig. 1.

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DEPARTMENT OF CHEMISTRY
UNIVERSITY OF LOUISVILLE
LOUISVILLE, KENTUCKY RECEIVED NOVEMBER 6, 1950

COMMUNICATIONS TO THE EDITOR

NEOMYCINS B AND C, AND SOME OF THEIR DEGRADATION PRODUCTS

Sir:

In the course of purification of the antibiotic complex termed neomycin by Waksman and Lechevalier,[1] we have obtained by chromatography of the hydrochloric acid salt[2] in 80% methanol over alumina two homogeneous biologically active fractions. One of these closely resembles the neomycin B described by Regna and Murphy,[3] while the properties of the other, named by us neomycin C, sharply differentiate it from neomycin A[4] (see Table I).

TABLE I

COMPARISON OF PROPERTIES OF NEOMYCINS

	Neomycin component B, amorphous hydrochloride	Neomycin component C, amorphous hydrochloride	Neomycin A[4] amorphous hydrochloride	Neomycin B[4] amorphous sulfate
Biopotency in[a] nutrient broth *vs. Klebsiella pn.*	265 units/mg.	180 units/mg.	20 units/mg.[b]	260 255 units/mg.
Biopotency on nutrient Agar *vs. B. subtilis*	86 units/mg.	121 units/mg.	1700 units/mg.[b] 710 units/mg.[b]	.....
$[\alpha]_D$	+54°	+80°	+83°	+58°

[a] The units are based on a comparison with a Waksman standard preparation. [b] These values determined with a sample of neomycin A hydrochloride kindly supplied by Dr. Karl Folkers.

Crystalline reineckates, picrolonates and *p*-(*p'*-hydroxyphenylazo)-benzenesulfonates have been prepared from both entities. In addition, antibacterially inactive, crystalline N-acetates have been obtained (*Neomycin B N-acetate*, needles from aqueous acetone; m.p. 200–205° with decomposition, after darkening and softening 186–190°; $[\alpha]^{22}D$ +62°, 0.4% in water. *Anal.* Found:[5] C, 50.71; H, 6.95; N, 9.45; acetyl, 32.4. *Neomycin C N-acetate*, needles from aqueous acetone; m.p. similar to that of neomycin B N-acetate, but showing a depression of 10° in mixed melting point determination; $[\alpha]^{22}D$ +90°, 0.4% in water. *Anal.* Found: C, 51.09; H, 7.47; N, 9.24; acetyl, 32.7). These data suggest that the two bases may be isomeric. All the nitrogen atoms are present as primary amino groups (Van Slyke).

Methanolysis of the hydrochlorides afforded the following two fragments:

(1) An amorphous hydrochloride, identical from both neomycin B and C, which was devoid of reducing power and yielded all its nitrogen as amino nitrogen in the Van Slyke determination. The empirical formula, $C_9H_{19}O_5N_3 \cdot 3HCl$, may be derived from the analyses of the following crystalline derivatives. *N-acetate*, needles from methanol; decomposes *ca.* 300° without melting, after sintering at 260°; $[\alpha]^{22}D$ +88°, 0.3% in water. *Anal.* Calcd. for $C_9H_{16}O_5N_3(COCH_3)_3$: C, 48.00; H, 6.71; N, 11.20; acetyl, 34.4. Found: C, 47.76, 47.94; H, 6.95, 7.76; N, 11.46; acetyl, 36.5. *N-Benzoate*, needles from aqueous methanol, decomposes to black tar at 299–300° after darkening at 270°; $[\alpha]^{22}D$ +70°,0.5 % in methanol. *Anal.* Calcd. for $C_9H_{16}O_5N_3(COC_6H_5)_3$. C, 64.16; H, 5.57; N, 7.48. Found: C, 64.54; H, 6.02; N, 7.43. *Heptaacetate*, prisms from acetone, dried sample softens at 165°, undergoes transition 190–215°, liquifies at 260–262°; the cooled and solidified material (needles) then melts (without transition) at 262–265°; $[\alpha]^{22}D$ +49°, 0.5% in methanol. *Anal.* Calcd. for $C_9H_{12}O_5N_3(COCH_3)_7$ (mol. wt., 5.44): C, 50.83; H, 6.12; N, 7.73; total acetyl,

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

(2) The crude neomycin preparations used were produced by the Divisions of Microbiological and Chemical Development, E. R. Squibb & Sons, New Brunswick, N. J.

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

(4) R. Peck, C. E. Hoffhine, P. Gale and K. Folkers, *ibid.*, **71**, 2590 (1949).

(5) All analytical determinations were carried out by Mr. J. F. Alicino, Microanalytical Laboratory, E. R. Squibb & Sons, New Brunswick, N. J.

55.4; O-acetyl, 31.7. Found: C, 49.45, 49.71; H, 6.51, 6.61; N, 7.65; total acetyl, 53.4; O-acetyl, 30.2; mol. wt. (Rast, in camphor), 543, 512.

(2) Non-identical methyl glycosidic moieties, which for reasons given below have been provisionally termed methyl neobiosaminides B and C ($[\alpha]_D$ of the amorphous hydrochlorides, 1.0% in water, +22° and +78°, respectively). The analysis of a chromatographically purified amorphous *polyacetate of methyl neobiosaminide B* was best compatible with the composition $C_{11}H_{16}O_6N_2(OCH_3)(COCH_3)_5$: $[\alpha]^{22}D$ +2°, 0.5% in ethanol. *Anal.* Calcd.: C, 50.96; H, 6.61; N, 5.40; CH_3O, 5.98; 5 acetyl, 41.5. Found: C, 49.62; H, 6.70; N, 5.41; CH_3O, 5.16; acetyl, 41.9.

On vigorous hydrolysis with hydrochloric acid, methyl neobiosaminide C yielded the dihydrochloride of a reducing diamine, $C_6H_{14}O_3N_2.2HCl$, platelets from methanol–ether; m.p. 182–185° with decomposition after shrinking and darkening 155–175°, $[\alpha]^{22}D$ +69°, equilibrium value, 0.4% in water. *Anal.* Calcd.: C, 30.65; H, 6.86; N, 11.92; Cl, 30.16. Found: C, 30.79; H, 7.18; N, 12.52; Cl, 30.51. The reducing diamine from methyl neobiosaminide B has not yet been obtained in crystalline form but mutarotation and paper chromatographic data indicate that it is not identical with that from neomycin C. The remainder of the neobiosaminide fragment seems to be accounted for by a pentose, as evidenced by the formation, on acid hydrolysis, of furfural from both neomycin B and C and the corresponding methyl neobiosaminides. This property has been utilized for the chemical assay of the two antibiotics.

The average weight ratio, in several methanolyses, of non-glycosidic fraction to methyl biosaminide fraction was 1.85; on the other hand, the ratio of the molecular weight of the C_9 base hydrochloride (358) to that of the methyl biosaminide hydrochloride (381) is 0.94. Hence it appears that actually two moles of the C_9 base are liberated, conceivably from acetalic linkage, by the methanolysis. On this basis the neomycins B and C would appear to have the composition $C_{29}H_{58}O_{16}N_8$.

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A TERMINAL AMINO ACID RESIDUE OF LYSOZYME AS DETERMINED WITH 2,4-DINITROFLUOROBENZENE

Sir:

Studies on lysozyme to determine which amino acid residues occupy terminal positions at the amino ends of the polypeptide chains have shown that one of the lysine residues occupies such a position in the protein. This conclusion is drawn from the fact that α,ε-di-DNP-lysine was the only DNP-amino acid isolated chromatographically from the ether extract of an acidic hydrolysate of DNP-lysozyme. No attempt has yet been made to determine whether the hydrolysate contains basic DNP-amino acids other than ε-DNP-lysine which are not extracted with ether.

DNP-Lysozyme was prepared from lysozyme (Armour and Company Lot 805L1) in a manner similar to that described by Sanger[1] for insulin. A sample of the DNP-lysozyme was refluxed with 6 *N* hydrochloric acid for 8 hours and the ether extract of the hydrolysate was examined chromatographically according to a scheme which has recently been developed in these Laboratories.[2] This scheme for the separation of 16 ether-soluble DNP-amino acids by adsorption chromatography on 2:1 silicic acid–Celite is considerably faster than that described by Sanger,[1] Porter and Sanger,[3] and Blackburn,[4] and gives satisfactory qualitative results on a large number of samples of silicic acid. The ether extract of the hydrolysate gave two yellow zones on a silicic acid column. The chromatographic characteristics of the lower zone were identical with those of α,ε-di-DNP-lysine with three different solvent systems as developers, even after a second hydrolysis. The upper zone, which did not behave like any known DNP-amino acid, yielded α,ε-di-DNP-lysine on further hydrolysis and, therefore, contained a DNP-lysyl peptide (or peptides), highly resistant to hydrolysis, which is now being investigated. The changes in development behavior of α,ε-di-DNP-lysine from one developer to another are greater than those of other similarly adsorbed DNP-amino acids so that its chromatographic behavior is an excellent criterion for its identity.

In preliminary quantitative studies it has been found that the ether extract of an 8-hour hydrolysate contained α,ε-di-DNP-lysine equivalent to about 0.35 terminal amino groups per molecule (mol. wt. 13,900[5]), and in addition a considerable amount of DNP-lysyl peptide(s). After hydrolysis for 24 hours, α,ε-di-DNP-lysine equivalent to 0.75 terminal amino groups per molecule was isolated. This value, which includes corrections for 7% loss during chromatographic operations and for 15% destruction of the lysine derivative during hydrolysis, is considerably less than one terminal group per molecule and further improvements in the analytical method are now being sought. There was still evidence of a small amount of DNP-lysyl peptide(s) after 24 hours hydrolysis but probably not enough to account for the low yield of lysine derivative. The absence of free lysine when the amino acid content of the hydrolysate was determined by starch chromatographic methods indicates the completeness of the reaction between the protein and dinitrofluorobenzene. From the results obtained thus far it seems unlikely that more than one lysine residue occupies an amino terminal position in lysozyme.

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(1) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
(2) F. C. Green and L. M. Kay, to be published.
(3) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).
(4) S. Blackburn, *ibid.*, **45**, 579 (1949).
(5) K. J. Palmer, M. Ballantyne and J. A. Galvin, THIS JOURNAL, **70**, 906 (1948).