Hydroxystreptomycin, a New Antibiotic from *Streptomyces Griseocarneus*[2]

By Frank H. Stodola, Odette L. Shotwell, Anne Marie Borud, Robert G. Benedict and Arthur C. Riley, Jr.

A new member of the streptomycin series has been degraded to streptidine, N-methyl-L-glucosamine and 2-hydroxymethyl-3-hydroxy-1,4-pyrone. These cleavage products indicate that the new antibiotic differs from streptomycin only in having a hydroxymethyl group instead of a methyl in the streptose portion of the molecule.

In a preliminary note[3] we reported the isolation of a new member of the streptomycin series of the composition $C_{21}H_{39}N_7O_{13}$ produced by a new species of *Streptomyces*, *S. griseocarneus*, obtained from a Japanese soil. Since the new antibiotic differed from streptomycin by only one hydroxyl group, it was given the name "hydroxystreptomycin." In the present paper are given the details of that work along with some data on the paper chromatography of the streptomycins.

The cleavage of dihydrohydroxystreptomycin with methanolic hydrogen chloride gave streptidine and a disaccharide isolated as the hexaacetate (I). Under similar conditions dihydrostreptomycin yields streptidine and a pentaacetate. The extra hydroxyl group indicated by the acetyl determina-
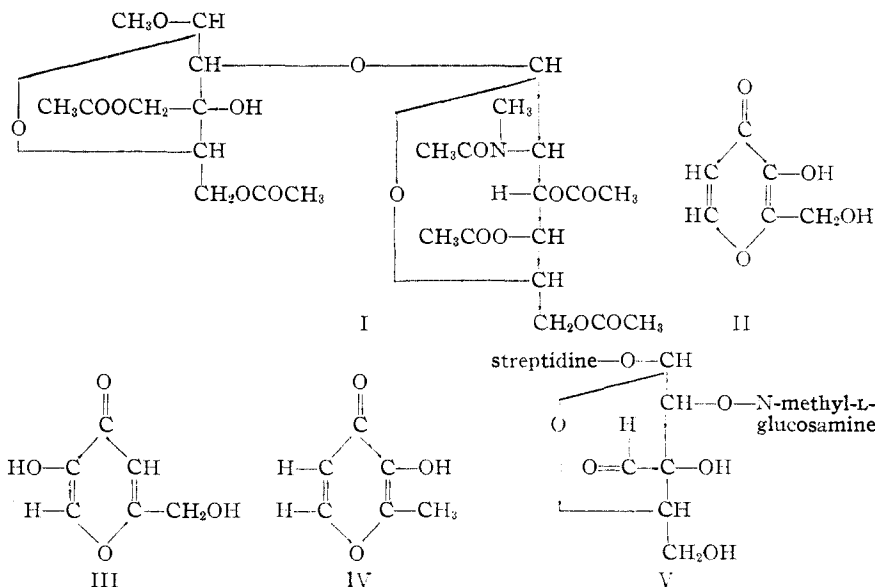
On the basis of these studies the formula (V) is indicated for hydroxystreptomycin.

As paper chromatography proved to be so useful in detecting the new streptomycin we show in Figs. 1 and 2 some separations of the various streptomycins to assist future workers in the characterization of the members of this growing group. In the figures streptomycin is designated as A, mannosidostreptomycin as B and hydroxystreptomycin as C.

In a recent note, Grundy and co-workers[4] have described the isolation of a streptomycin which appears to be identical with hydroxystreptomycin. Their antibiotic was produced by a *Streptomyces* isolated from a soil sample collected at North Chicago, Illinois.

Experimental

Isolation of Crude Hydroxystreptomycin.—The method of purification was essentially that developed by earlier workers on streptomycin.[5] To each liter of filtered culture liquor was added 11 g. of Nuchar C-250 N, the mixture stirred for 30 minutes, the carbon removed by filtration, and washed in succession with one liter of water, 100 cc. of 50% ethanol and 100 cc. of absolute methanol. The air-dried carbon was stirred for five minutes with sufficient methanol–hydrochloric acid mixture (0.5 cc. concd. HCl per liter of methanol) to permit stirring. After filtration the filtrate was concentrated *in vacuo* to one-fourth volume and added to ten volumes of acetone. The precipitate was separated by centrifugation and washed twice with acetone. Drying *in vacuo* gave a somewhat hygroscopic tan powder assaying about 30–40% hydroxystreptomycin.

This crude product (25 g.) was dissolved in 50 cc. of water, the pH adjusted to 5.8, the insoluble material removed by centrifugation and the solution placed on a column (6.5 × 45 cm.) of acid-washed Harshaw alumina (adjusted to pH 4.7). For development 80% methanol was used. One hundred cubic centimeter portions of the eluate gave about 75% of the activity in fractions 9 through 16. The methanol in these fractions was removed *in vacuo* and the resulting aqueous solutions lyophilized to white powders the purities of which ranged from 80–90%.

Preparation of the Helianthate.—Crude hydroxystreptomycin (1.54 g.) of about 80% purity was dissolved in 44 cc. of 50% methanol. To this solution was added 2.2 g. of

tion on the hexaacetate was shown not to be in the glucosamine portion of the molecule by the isolation of a pentaacetyl N-methyl-α-L-glucosamine identical with the pentaacetyl derivative from streptomycin. That the extra hydroxyl is located in the streptose moiety was demonstrated by alkaline degradation of hydroxystreptomycin to the pyrone (II) which is an isomer of kojic acid (III). The corresponding product from streptomycin is maltol (IV).

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented before the Division of Agricultural and Food Chemistry at the 118th Meeting of the American Chemical Society, Chicago, Illinois, September, 1950.

(3) R. G. Benedict, F. H. Stodola, O. L. Shotwell, A. M. Borud and J. A. Lindenfelser, *Science*, 112, 77 (1950).

(4) W. E. Grundy, J. R. Schenck, R. K. Clark, Jr., M. P. Hargie, R. K. Richards and J. C. Sylvester, *Arch. Biochem.*, 28, 150 (1950).

(5) A. Schatz, *et al.*, *Proc. Soc. Exp. Biol. Med.*, 55, 66 (1944); H. E. Carter, *et al.*, *J. Biol. Chem.*, 160, 337 (1945); F. A. Kuehl, Jr., *et al.*, *Science*, 102, 34 (1945); R. L. Peck, *Ann. N. Y. Acad. Sci.*, 49, 235 (1948).

Separations of Streptomycins A, B, and C

B A — 65 hours   C A — 65 hours   B C — 65 hours   B C — 6 days
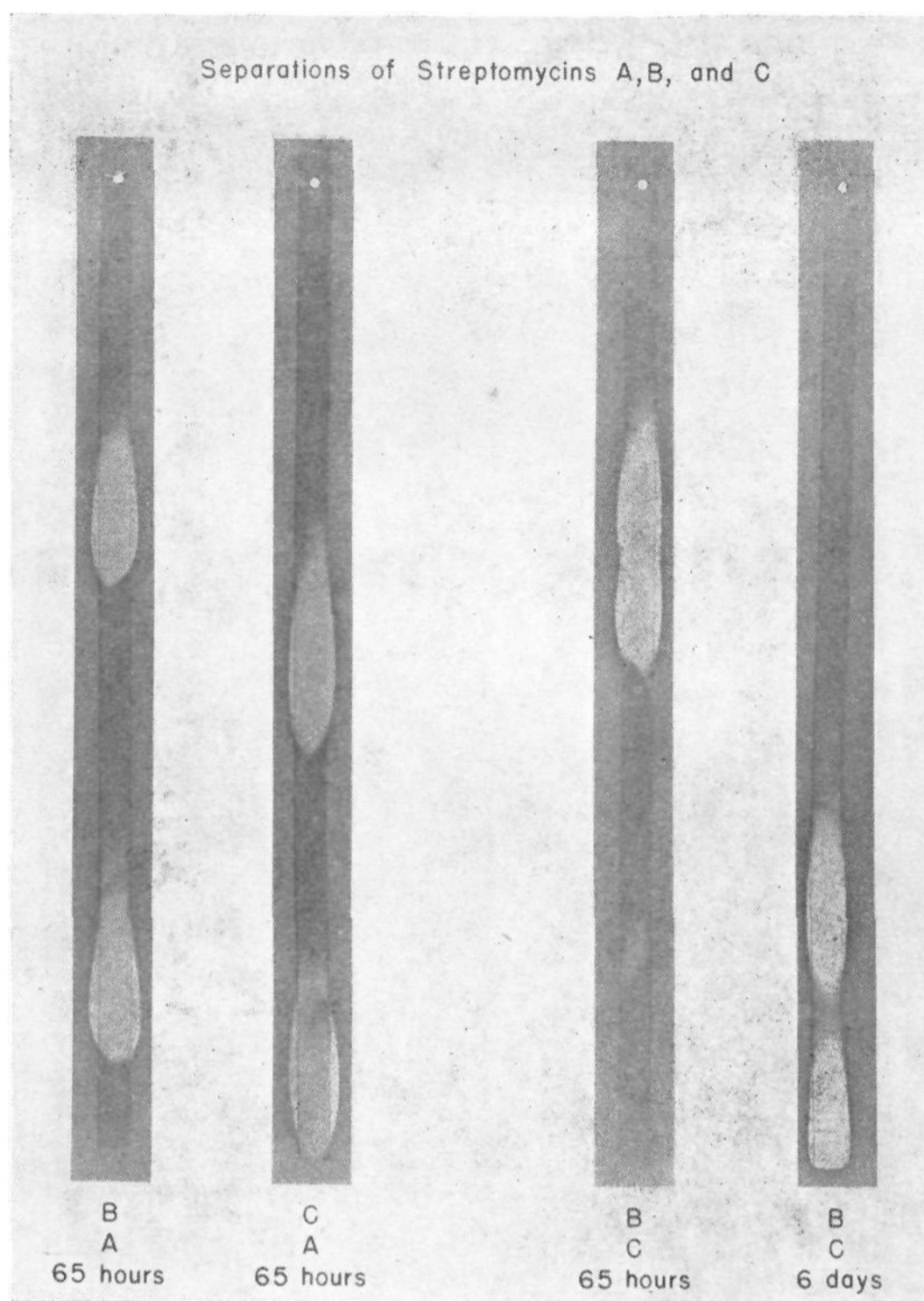
Fig. 1.

methyl orange dissolved in 150 cc. of water at 55°.[6] The reaction mixture was cooled in the refrigerator overnight and the crystals then separated by centrifugation and washed twice with water. Crystallization from 33% methanol gave 1.8 g. of reddish-brown crystals of helianthate which were dried at 100° *in vacuo* to constant weight for analysis. The helianthate started to darken at about 220° and slowly charred without melting. Its X-ray diffraction pattern could scarcely be distinguished from that of streptomycin helianthate.

Anal. Calcd. for $C_{21}H_{39}N_7O_{13}\cdot3C_{14}H_{15}N_3O_3S$: C, 49.99; H, 5.59; N, 14.81; S, 6.36. Found: C, 50.1, 50.2; H, 5.75, 5.71; N, 14.6; S, 6.40.

Preparation of Pure Hydroxystreptomycin Trihydrochloride.—The pure helianthate (700 mg.) was ground vigorously for one minute with 11 cc. of a mixture of methanol and hydrochloric acid (1 cc. concd. HCl in 50 cc. of methanol) and the solution filtered through a pad of Darco G-60.[7] The colorless filtrate was added dropwise to 150 cc. of ether and the white precipitate separated by centrifugation. After two washings with ether, the hydrochloride (268 mg.) was dried *in vacuo* at 80° for analysis. The trihydrochloride showed a rotation of $[\alpha]^{28}D$ −91° (*c*, 1 in water). When

(6) F. A. Kuehl, Jr., R. L. Peck, C. E. Hoffhine, Jr., R. P. Graber and K. Folkers, This Journal, **68**, 1460 (1946).

(7) F. A. Kuehl, Jr., R. L. Peck, A. Walti and K. Folkers, *Science*, **102**, 34 (1945).

assayed against *Bacillus subtilis*, the hydrochloride was equivalent to 784 micrograms of streptomycin base per milligram. The corresponding value for streptomycin trihydrochloride is 842 µg. per mg.

Anal. Calcd. for $C_{21}H_{39}N_7O_{13}\cdot3HCl$: C, 35.68; H, 5.99; N, 13.87; Cl, 15.05. Found: C, 35.8, 35.4; H, 6.12, 5.79; N, 13.9; Cl, 14.8.

Cleavage of Dihydrohydroxystreptomycin with Methanolic Hydrogen Chloride.—Hydroxystreptomycin hydrochloride (500 mg.) in aqueous solution was reduced in the presence of platinum oxide for six hours. Approximately one mole of hydrogen was absorbed and the lyophilized product gave a negative maltol test. The reduced product was dissolved in 30 cc. of 0.79 N methanolic hydrogen chloride (anhydrous).[8] After 48 hours at room temperature, 60 cc. of dry ether was added which gave a white precipitate of crude streptidine hydrochloride. The precipitate (300 mg.) was separated by centrifugation and characterized as described below. The decantate was neutralized with a methanol solution of sodium hydroxide and the precipitated sodium chloride removed by centrifugation. The decantate was then concentrated to 200 mg. of white powder which was acetylated with acetic anhydride in pyridine. Crystallization from ethyl acetate–petroleum ether gave needles (82 mg.) of α-methyl hexaacetyldihydrohydroxystreptobios-

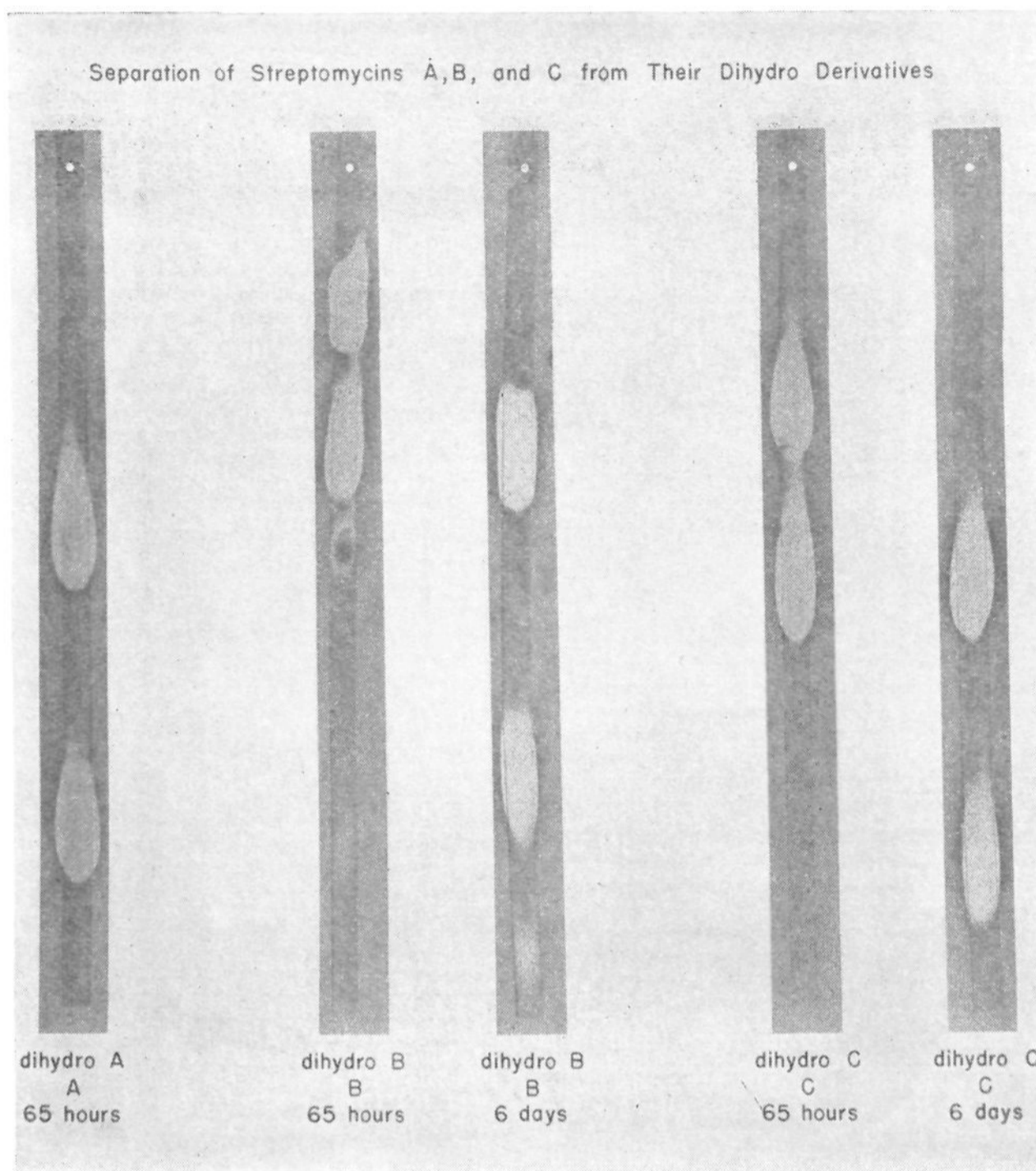(8) N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn and K. Folkers, This Journal, **68**, 2557 (1946).

Fig. 2.

aminide (I) melting at 124–125° (capillary; uncorrected); $[\alpha]^{24}D$ −108° (c, 1 in chloroform).

Anal. Calcd. for $C_{13}H_{18}O_4(NCOCH_3)(OCOCH_3)_5$-(OCH₃): C, 50.24; H, 6.33; N, 2.25; OCH₃, 4.99; total acetyl, 41.55; O-acetyl, 34.62. Found: C, 50.2, 49.8; H, 6.30, 6.33; N, 2.16; OCH₃, 5.13; total acetyl, 40.8; O-acetyl, 34.6.

From the crude streptidine hydrochloride fraction could be prepared a crystalline picrate and sulfate which were shown by X-ray diffraction patterns to be identical with authentic samples obtained from streptomycin.

Hydrolysis of Hydroxystreptomycin to N-Methyl-L-glucosamine.—Hydroxystreptomycin trihydrochloride (1.27 g.) in 0.79 N methanolic hydrogen chloride (anhydrous) was kept at room temperature for 48 hours. Addition of dry ether precipitated the streptidine hydrochloride which was separated by centrifugation. The decantate was neutralized with sodium hydroxide in methanol and the sodium chloride removed by filtration. The filtrate was concentrated to dryness and the residue refluxed for two hours with concd. HCl. The reaction mixture was extracted with chloroform to remove black decomposition products. The aqueous solution was decolorized and concentrated to a solid which was acetylated with acetic anhydride and pyridine. The acetyl derivative was chromatographed on alumina as described by Brink, *et al.*,[8] to give needles melting at 156° **which were shown by mixed melting point test and X-ray diffraction patterns to be identical with a sample of penta-acetyl N-methyl-L-glucosamine prepared from steptomycin.**

The derivative from hydroxystreptomycin gave $[\alpha]^{25}D$ −95° (c, 1 in chloroform); the value reported by Kuehl, *et al.*,[9] for the product from streptomycin was −100°.

Action of Alkali on Hydroxystreptomycin.—Hydroxystreptomycin trihydrochloride (2.0 g.) was dissolved in 40 cc. of 1 N sodium hydroxide and heated at 100° for three minutes.[10] After acidification with hydrochloric acid the solution was extracted for five hours with ether. Concentration of the ether gave gummy crystals which were purified by sublimation and crystallization from toluene-methanol. The compound melted at 152–153° and was shown by mixed melting point tests and X-ray diffraction patterns to be different from both maltol and kojic acid. Its strong reddish-violet color with ferric chloride and its adsorption at 2,740 Å. ($E^{1\,cm.}_{1\%}$ = 666) are in agreement with formula II for 2-hydroxymethyl-3-hydroxy-1,4-pyrone.

Anal. Calcd. for $C_6H_6O_4$: C, 50.71; H, 4.26. Found: C, 50.2; H, 4.43.

Paper Chromatography of the Streptomycins.—The procedure was essentially that developed by Winston and Eigen.[11]

The streptomycin and hydroxystreptomycin were analytically pure samples and the mannosidostreptomycin, kindly supplied by Dr. J. Fried of the Squibb Institute for

(9) F. A. Kuehl, Jr., E. H. Flynn, F. W. Holly, R. Mozingo and K. Folkers, THIS JOURNAL, **68**, 536 (1946).

(10) J. R. Schenck and M. A. Spielman, *ibid.*, **67**, 2276 (1945).

(11) Winston and Eigen, *ibid.*, **70**, 3333 (1948).

Medical Research, contained a small amount of strepto-mycin impurity. The dihydro derivatives were prepared from these samples by catalytic reduction.

A solution of 5 μg. of streptomycin trihydrochloride or hydroxystreptomycin trihydrochloride (25 μg. of the manno-sido compounds) in 0.005 cc. of water was applied with a micropipet to a strip of Whatman No. 4 paper 0.5″ by 17.5″. The developing solvent was wet butanol containing

2% p-toluenesulfonic acid and 2% piperidine. After de-velopment the solvent was removed by washing the paper with ether and drying at room temperature. The strip was then placed on an agar layer inoculated with *Staphylococcus aureus*. Ten minutes was allowed for the streptomycin to diffuse into the agar. The strips were then removed and the trays incubated overnight at 37°.

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Pinacolic Rearrangements of Epimeric Aminocyclanols[1]

BY G. E. MCCASLAND

cis-2-Aminocyclohexanol on treatment with nitrous acid undergoes a pinacol rearrangement, yielding both cyclohexanone and cyclopentylmethanal. The *trans* epimer yields almost exclusively cyclopentylmethanal. Possible mechanisms are discussed in relation to configuration and conformation. Aminocyclanols often give anomalous high Van Slyke amino nitro-gen values, which cannot be accounted for by reaction of an intermediate carbonyl compound, diol or epoxide with excess nitrous acid.

The formation of a ketone (I), aldehyde (II), epoxide (III) or diol (IV) from a 2-aminoalkanol by reaction with nitrous acid is conveniently explained in terms of the displacement of N_2 from the pre-sumed diazonium intermediate (V or VI) by a mi-grating or entering electron-rich group.[2] The products I–IV are shown in protonated form. Ke-tone and aldehyde result from the migration of H: and R: respectively. Epoxide may result from internal displacement of N_2 by the carbinol oxygen, and diol by external displacement of N_2 by a mole-cule of the solvent (water).
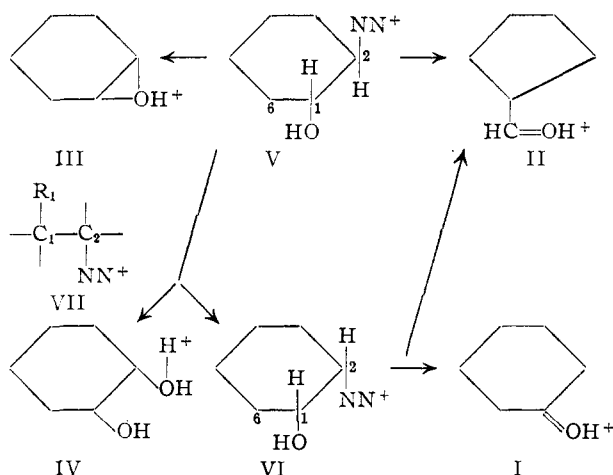
Influence of Configuration and Conformation.—

Pollak and Curtin[3] have recently discussed the ef-

fect of configuration on migrations in diastereo-meric 2-aminoalkanols. As they suggest, a 1–2 shift of R_1 should be favored by that conformation of the molecule in which R_1 is *trans and coplanar* with respect to the diazonium ion group and carbon atoms 1 and 2 (formula VII). The fraction of molecules having this favorable *conformation* is in turn influenced by the *configuration*.

The application of this hypothesis to the 2-amino-cyclohexanols requires a consideration of the equa-torial and polar conformations[4] of the cyclohexane ring. Here the ring itself at carbon 6 constitutes one migrating group, and the hydrogen atom at car-bon 1 constitutes the other.

In the case of *trans*-2-aminocyclohexanol, hydro-gen 1 is *cis* to the diazonium ion group (V). Such 1,2-*cis* cyclohexane derivatives exist as a non-re-solvable mixture of two antimeric equatorial-polar (*e,p*) conformations. When the diazonium ion group is *p*, hydrogen 1 must be *e*, and *vice versa*. Examination of models shows that when the diazo-nium group has the *e* position, ring carbon 6 is in the favored (*i.e. trans* coplanar) position for migra-tion, but hydrogen 1 is not. When the diazo-nium group has the *p*-position, *neither* ring carbon 6 nor hydrogen 1 is in a favorable position for migration.

In *cis*-2-aminocyclohexanol, however, hydrogen 1 is *trans* to the diazonium group. Therefore, either hydrogen 1 and the diazonium ion group are both polar (*p,p*), or both are equatorial (*e,e*). Examina-tion of models reveals that the (*p,p*) conformation favors migration of hydrogen 1, while the (*e,e*) fa-vors migration of ring carbon 6. The relative extent of each migration might then depend on the rela-tive proportion of (*p,p*) and (*e,e*) molecules.

On this basis it would be predicted that the car-bonyl product formed by rearrangement of *trans*-2-aminocyclohexanol would consist exclusively of the aldehyde II (ring-contraction); while the *cis* epimer would yield a mixture containing both this aldehyde and the uncontracted ketone (I).

Experimentally, treatment of *trans*-2-aminocyclo-hexanol with nitrous acid gave a high yield of cyclo-pentylmethanal, confirming a similar finding by

(1) For related publications see: (a) G. E. McCasland, THIS JOURNAL, **73**, 2295 (1951); (b) McCasland and Smith, *ibid.*, **72**, 2190 (1950); (c) McCasland, Clark and Carter, *ibid.*, **71**, 637 (1949); (d) Carter, Clark, Lytle and McCasland, *J. Biol. Chem.*, **175**, 683 (1948); (e) Carter, Belinskey, Clark, Flynn, Lytle, McCasland and Robbins, *ibid.*, **174**, 415 (1948).

(2) The reaction of acyclic amino-alcohols has been extensively studied, in particular by McKenzie and co-workers, *e.g.*, *Ber.*, **63**, 904 (1930). Whitmore played an important role in formulating the mecha-nism here favored (*e.g.*, see THIS JOURNAL, **61**, 1324 (1939)). For further discussion and citation of the numerous references see Chap. 12 in G. W. Wheland, "Advanced Organic Chemistry," (John Wiley and Sons, Inc., New York, N. Y., 1949) and Chap. 12 by E. S. Wallis in Gilman's "Advanced Treatise," John Wiley and Sons, Inc., New York, N. Y., 2nd ed., 1944.

(3) Pollak and Curtin, THIS JOURNAL, **72**, 961 (1950).

(4) Pitzer and Beckett, *ibid.*, **69**, 977 (1947).