benzamido - 2 - benzoxy - 8 - bromo - 6 - methylquinoline at room temperature in preference to the benzamido group.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

Bis-(α-hydroxystreptomycyl)-amine, a Toxic Derivative of Streptomycin[1]

BY I. A. SOLOMONS AND PETER P. REGNA

During investigation of the composition of residues remaining after streptomycin processing and purification, a streptomycin derivative was isolated that proved to be highly toxic when injected intravenously into mice. The present paper deals with the isolation of this compound, its identification as bis-(α-hydroxystreptomycyl)-amine (II), and its subsequent synthesis. The substance was of interest in connection with its possible bearing on observed minor variations in the toxicity of different preparations of streptomycin and dihydrostreptomycin.

Initial purification of bis-(α-hydroxystreptomycyl)-amine was accomplished by converting the streptomycin residues to the corresponding hydrochlorides and vacuum freeze-drying the product. Trituration with small quantities of methanol removed a major portion of the streptomycin trihydrochloride and other extraneous substances. From the residue, a reineckate was crystallized and this was converted to amorphous bis-(α-hydroxystreptomycyl)-amine sulfate.

When dilute aqueous solutions of bis-(α-hydroxystreptomycyl)-amine sulfate were prepared, the compound was found to hydrolyze predominantly to streptomycin which was ascertained by increases in maltol analysis,[1a] biological potency and the minimum lethal dose in mice. Moreover, it was observed that the hydrolysis is accelerated in acid solutions and retarded in alkaline solutions. Bis-(α-hydroxystreptomycyl)-amine sulfate and its hydrolysis products were compared with streptomycin by paper chromatography, using the method of Winsten and Eigen.[2] The position of the components on the paper after development were detected by pressing the paper chromatograms on agar plates seeded with *B. subtilis*. In addition, the spots were detected in the same position by spraying the paper with sodium nitroprusside–potassium ferricyanide reagent after a method of Horne and Pollard[3] which detects the streptidine moiety of the streptomycin molecule. It was found (Fig. 1) that the intact material does not move from the point of application on the paper under the conditions used, while after mild hydrolysis the only chromatographically detectable component is streptomycin. These results showed that streptomycin was the only biologically active or streptidine-containing component in the toxic compound.

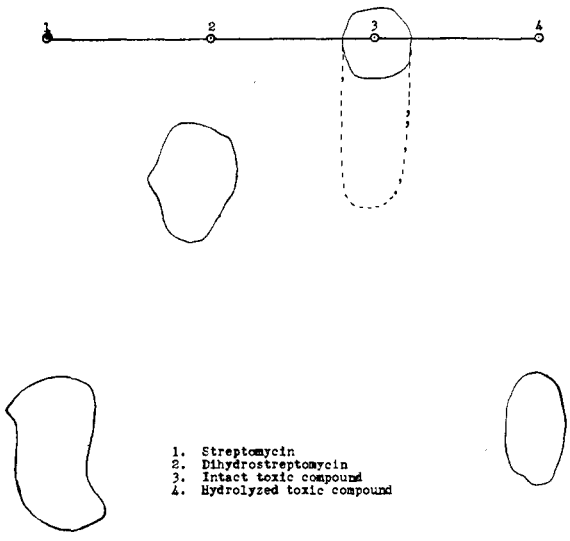


Fig. 1.—Chromatographed on Whatman #4 paper, descending system, using *n*-butanol–*p*-toluene sulfonic acid–piperidine solvent system; developed overnight; spots identified with streptidine spray.

Infrared and ultraviolet absorption spectra of bis-(α-hydroxystreptomycyl)-amine are very similar, in general, to those of streptomycin. On the other hand, polarographic analysis shows it to be significantly different. Whereas the presence of a free aldehyde group in streptomycin causes a definite break in the curve at a half-wave potential ($E_{1/2}$) of −1.45 volts,[4] an analysis of the toxic compound shows no break. Since it had been established by paper chromatography that bis-(α-hydroxystreptomycyl)-amine contains streptomycin, this indicated that the streptomycin was joined to another component through its carbonyl group.

Since acid is consumed during the hydrolysis of bis-(α-hydroxystreptomycyl)-amine, a measure of the consumption was used in calculating an empirical formula weight of 1450 for the compound. The hydrolysis in this experiment was performed

(1) Presented before the Division of Medicinal Chemistry at the Meeting of the American Chemical Society at Atlantic City, September, 1949.

(1a) G. E. Boxer, V. C. Jelinek and P. M. Leghorn, *J. Biol. Chem.*, **169**, 153 (1947).

(2) W. A. Winsten and E. Eigen, THIS JOURNAL, **70**, 3333 (1948).

(3) R. E. Horne, Jr., and A. L. Pollard, *J. Bact.*, **55**, 231 (1948).

(4) G. B. Levy, P. Schwed and J. W. Sackett, THIS JOURNAL, **68**, 528 (1946).

under such conditions that streptomycin itself is stable.[5] Inasmuch as the molecular weight of streptomycin sulfate is 729, the value determined for bis-(α-hydroxystreptomycyl)-amine is about twice that of streptomycin. Direct potentiometric titration of bis-(α-hydroxystreptomycyl)-amine showed that it contained six basic groups: twice that of the streptomycin molecule. This titration curve could be superimposed on a curve obtained from a similar titration of streptomycin. It was concluded from these data that the basic groups in the streptomycin moiety are free and thus not involved in the formation of bis-(α-hydroxystreptomycyl)-amine, and that the latter was probably made up of two streptomycin molecules joined by a low molecular weight molecule or radical of a basic nature.

When streptomycin is hydrogenated over platinum catalyst at 25° and atmospheric pressure, one mole of hydrogen is consumed.[6] Bis-(α-hydroxystreptomycyl)-amine does not react with hydrogen under the same conditions. This difference offered additional evidence that the carbonyl groups in bis-(α-hydroxystreptomycyl)-amine are not free. However, after mild acid treatment, bis-(α-hydroxystreptomycyl)-amine, under the same hydrogenation conditions, consumed one mole of hydrogen per mole of streptomycin formed in the hydrolysis. Therefore, the hydrolysis ruptures the bond between the carbonyl groups and the molecule joining the two streptomycin molecules. Hydrogen was consumed when the bis-(α-hydroxystreptomycyl)-amine was hydrogenated over platinum at or above 30 lb./sq. in. pressure. The hydrogenated product is not affected by mild dilute acid hydrolysis and was shown by paper chromatography to be composed of two substances (Fig. 2), dihydrostreptomycin and streptomycylamine.[7]

It has been previously reported that streptomycin can be reductively coupled with various amines to form N-substituted streptomycylamines.[8] Ammonia has been similarly treated with streptomycin to yield streptomycylamine in which the carbonyl group is converted to an amino methyl group.[7] Paper chromatography of this latter compound shows that it does not migrate from the point of application (Fig. 2) during development. However, streptomycylamine (a primary amine) can be treated with benzaldehyde under reducing conditions to form benzylstreptomycylamine,[7] which is readily detected by its rapid migration during chromatography.

In order to prove the presence of streptomycylamine, formed during the hydrogenation of bis-

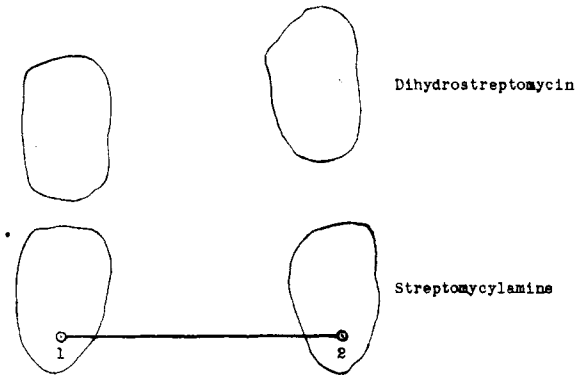


Fig. 2.—1, Toxic compound hydrogenated 30 lb./sq. in. over platinum; 2, streptomycylamine (dihydrostreptomycin as by-product); chromatographed on Whatman #4 paper, ascending system using *n*-butanol–*p*-toluenesulfonic acid–piperidine solvent system; developed 3 days; spots identified with streptidine spray.

(α-hydroxystreptomycyl)-amine, the latter was reduced over platinum catalyst at 30 lb./sq. in., and subsequently reductively coupled with benzaldehyde. The resulting hydrogenation mixture showed the presence of benzylstreptomycylamine on chromatographing it on paper alongside of an authentic sample of the same substance. In addition, the paper chromatograms (Fig. 3) disclosed the presence of dihydrostreptomycin, a product of the initial hydrogenation, and unreacted streptomycylamine. Therefore, it was concluded that the higher pressure hydrogenation of bis-(α-hydroxystreptomycyl)-amine produced dihydrostreptomycin and streptomycylamine.

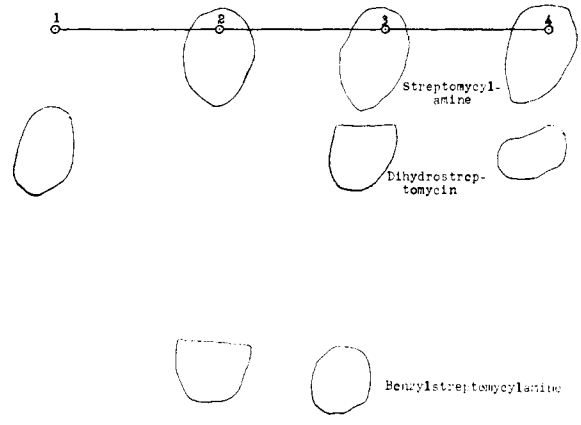


Fig. 3.—1, Dihydrostreptomycin; 2, benzylstreptomycylamine (streptomycylamine present as by-product); 3, toxic compound hydrogenated 30 lb./sq. in. then hydrogenated with benzaldehyde; 4, streptomycylamine (dihydrostreptomycin present as by-product); chromatographed on Whatman #4 paper, descending system using *n*-butanol–*p*-toluenesulfonic acid–piperidine solvent system; developed overnight; spots identified with streptidine spray.

In order to verify these conclusions, bis-(α-hydroxystreptomycyl)-amine was synthesized by

(5) P. P. Regna, L. A. Wasselle and I. A. Solomons, *J. Biol. Chem.*, **165**, 631 (1946).

(6) R. L. Peck, C. E. Hoffhine, Jr., and K. Folkers, THIS JOURNAL, **68**, 1390 (1946).

(7) C. I. Jarowski, F. X. Murphy and W. A. Lazier, to be published.

(8) W. A. Winsten, Abstracts, 114th Meeting, American Chemical Society, Washington, D. C., 1948, p. 1C.

warming a concentrated solution of streptomycin hydrochloride containing ammonia. The isolated product had properties identical with bis-(α-hydroxystreptomycyl)-amine. In a control experiment without ammonium chloride, none of the compound was formed.

Figure 3 shows the chromatogram obtained when benzylstreptomycylamine was prepared from the bis-(α-hydroxystreptomycyl)-amine isolated from residues. Figure 4 compares the products obtained when the same series of reactions is carried out with the synthetic bis-(α-hydroxystreptomycyl)-amine.

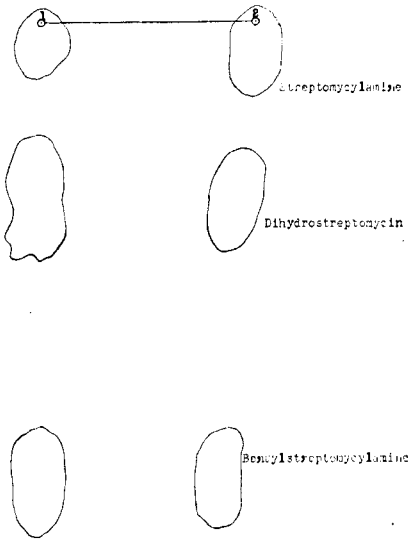


Fig. 4.—1, Synthetic toxic compound hydrogenated 30 lb./sq. in. then hydrogenated with benzaldehyde; 2, isolated toxic compound hydrogenated 30 lb./sq. in. then hydrogenated with benzaldehyde; chromatographed on Whatman #4 paper, descending system, using *n*-butanol–*p*-toluenesulfonic acid–piperidine solvent system; developed overnight; spots identified with streptidine spray.

Attempts to prepare similar compounds using methylamine, 2-aminohexane, ethanolamine and aniline yielded only the starting materials. Also, attempts to react ammonia with dihydrostreptomycin were unsuccessful, indicating that the carbonyl group in streptomycin is that which reacts with ammonia.

Methods

Paper Chromatography.—Either ascending[9] or descending solvent flow was utilized in the chromatographic development with the butanol–*p*-toluenesulfonic acid system described by Winsten and Eigen.[2] All figures represent sketches of the paper chromatograms.

Biological Assays.—The plate assays were carried out essentially by the method of Schmidt and Moyer[10] which was used for penicillin, but with the following exceptions: (a) the medium used was Difco Bacto-streptomycin assay agar, originally employed by Skell for the assay of streptothricin; (b) *Bacillus subtilis*, P. C. I. 219, was used as the test organism; (c) a 1% phosphate buffer at *p*H 7.9 to 8.0 was used.

Mouse Toxicity.—The toxicity of the various preparations was determined according to the procedure recommended by the U. S. Food and Drug Administration[11] as follows: Inject intravenously each of five mice, within the weight range of 18 to 25 g. with 0.5 ml. of a solution containing 2 mg./ml. of streptomycin. The injection should be made over a period of not more than 5 seconds. In this test, the term LD_0 is used to denote the maximum number of micrograms of streptomycin (in terms of free base) that can be injected into a selected number of mice and cause no deaths.

Experimental

Isolation of Bis-(α-hydroxystreptomycyl)-amine.—Streptomycin sulfate residues (150 g.) were dissolved in 500 ml. of water and treated with an aqueous solution of 60 g. of barium chloride·$2H_2O$. The barium sulfate was filtered, and the filtrate was vacuum freeze-dried. The hydrochloride salts (100 g.), assaying: maltol, 460 γ/mg., streptidine, 730 γ/mg., *B. subtilis*, 360 γ/mg., were shaken for several hours in 300 ml. of methanol. The alcohol-insoluble material was filtered, washed with methanol, and dried *in vacuo* to yield 34 g. of a light tan product assaying: maltol, 350 γ/mg., streptidine, 740 γ/mg., and *B. subtilis*, 230 γ/mg. The latter (33 g.) was dissolved in 200 ml. of water, and treated with 1000 ml. of a warm aqueous solution containing 60 g. of reinecke salt. The resulting mixture was heated to 55°, and after storing overnight at 25°, it was filtered and washed with water. Recrystallization was carried out from 4000 ml. of water to yield 15 g. of the reineckate as very small plates, and these were dried *in vacuo* at 100°.

Anal. Calcd. for $C_{66}H_{123}N_{51}S_{24}O_{24}Cr$: C, 25.60; H, 4.00; N, 23.07; Cr, 10.08. Found: C, 25.63; H, 3.97; N, 23.01; Cr, 9.97.

Conversion of Bis-(α-hydroxystreptomycyl)-amine Reineckate to Sulfate.—The reineckate (4.5 g.) was dissolved in 700 ml. of warm methanol, and, after filtering, the sulfate was precipitated by the addition of an excess of methanolic triethylamine sulfate, leaving the methanol-soluble triethylamine reineckate in solution. The precipitate was filtered over filter aid, washed thoroughly with methanol and then suspended in water, stirred, and filtered from the filter aid. A trace of reineckate remaining in the filtrate was removed with silver sulfate. On vacuum freeze-drying, 2.3 g. of bis-(α-hydroxystreptomycyl)-amine sulfate was obtained: maltol, 200 γ/mg.; streptidine, 750 γ/mg.; *B. subtilis*, 80 γ/mg.; LD_0 12 γ.

Anal. Calcd. for $C_{42}H_{37}N_{15}O_{36}S_3$: C, 34.21; H, 5.95; N, 14.25; SO_4, 19.56. Found: C, 34.42; H, 6.03; N, 14.15; SO_4, 19.89.

Hydrolysis of Bis-(α-hydroxystreptomycyl)-amine.—Bis-(α-hydroxystreptomycyl)-amine sulfate (2.0 g.) was dissolved in 50 ml. of water, adjusted to *p*H 2.5 with dilute sulfuric acid and allowed to stand at 20° overnight. The excess acid was removed with aqueous barium hydroxide, filtered, and the filtrate was vacuum freeze-dried to yield a white powder composed principally of streptomycin: maltol, 690 γ/mg.; streptidine, 750 γ/mg.; *B. subtilis*, 650 γ/mg.; LD_0 1250 γ.

Hydrogenation of Hydrolysis Product of Bis-(α-hydroxystreptomycyl)-amine Sulfate.—An aqueous solution containing 1.006 g. of hydrolyzed bis-(α-hydroxystreptomycyl)-amine sulfate was hydrogenated over 0.200 g. of previously reduced platinum oxide catalyst at atmospheric pressure and 25° for twenty-four hours; 27 ml. of hydrogen (S. T. P.) was consumed. After filtering and vacuum freeze-drying, the resulting product had the following properties: maltol, 0 γ/mg.; streptidine, 760 γ/mg.; *B. subtilis*, 650 γ/mg. By paper chromatography, the product was shown to have the same R_f value as dihydrostreptomycin.

(9) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

(10) W. H. Schmidt and A. J. Moyer, *J. Bact.*, **47**, 199 (1944).

(11) U. S. Food and Drug Administration Federal Register and Code of Regulations Title 21, Part 1, Section 141.

Estimation of Empirical Formula Weight of Bis-(α-hydroxystreptomycyl)-amine.—Bis-(α-hydroxystreptomycyl)-amine sulfate (192.9 mg.) was dissolved in 25.00 ml. of 0.01163 *N* hydrochloric acid and allowed to stand at 25° overnight. The excess hydrochloric acid was titrated potentiometrically with 14.00 ml. of 0.01120 *N* sodium hydroxide. The sample consumed 0.134 equivalent of hydrochloric acid giving a calculated value of the empirical formula weight of approximately 1450.

Hydrogenation of Bis-(α-hydroxystreptomycyl)-amine Sulfate.—Bis-(α-hydroxystreptomycyl)-amine sulfate (566 mg.) was dissolved in 25 ml. of water containing 50 mg. of Adams platinum oxide catalyst and hydrogenated in a Parr hydrogenation apparatus at room temperature and 30 lb. pressure for twenty-four hours. After filtering and vacuum freeze-drying, the reduced product, composed essentially of dihydrostreptomycin and streptomycylamine, showed: maltol, 0 γ/mg.; streptidine, 750 γ/mg.; *B. subtilis*, 360 γ/mg.

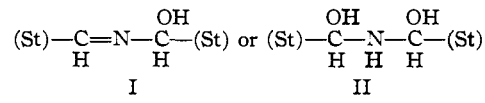
Benzylstreptomycylamine from Bis-(α-hydroxystreptomycyl)-amine.—The reduction product of bis-(α-hydroxystreptomycyl)-amine sulfate (250 mg.) was dissolved in 10 ml. of water and converted to the hydrochloride with barium chloride. The barium sulfate was filtered, and the filtrate was vacuum freeze-dried. The product was dissolved in 12.5 ml. of methanol containing 0.35 ml. of benzaldehyde and hydrogenated over 50 mg. of platinum oxide catalyst at 25° and 30 lb. pressure for twelve hours. The catalyst was removed by filtration, and benzylstreptomycylamine, one of the reaction products, was identified by comparison with an authentic sample by paper chromatography.

Synthesis of Bis-(α-hydroxystreptomycyl)-amine Sulfate.—A solution of 10 g. of streptomycin trihydrochloride (*B. Subtilis*, 750 γ/mg.) in 30 ml. of 1:1 methanol–water, containing 370 mg. of ammonium chloride, was adjusted to *p*H 7.5 with triethylamine and heated at 50–60°. The *p*H dropped slowly on heating, and it was maintained between *p*H 7.0–7.5 by additions of triethylamine. After three hours, the solution was cooled, adjusted to *p*H 5.5 with hydrochloric acid, and added to sufficient methanol to make the resulting solution 90% methanol. The streptomycin was fractionally precipitated as the sulfate to yield a first fraction weighing 1.3 g.: maltol, 215 γ/mg.; streptidine, 650 γ/mg.; *B. subtilis*, 130 γ/mg.; LD_0 50 γ. Bis-(α-hydroxystreptomycyl)-amine was isolated from this crude preparation as the reineckate and converted to the sulfate. Comparison with the compound isolated from residues showed them to be identical.

Discussion

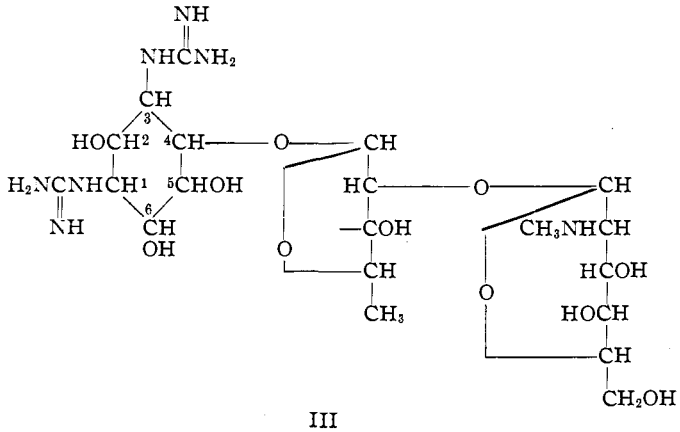
Glycosylamines[12] and diglycosylamines[13] have been prepared by treating sugars containing a free or potentially free aldehyde group with one or one-half mole, respectively, of ammonia either in water or methanol. The preparation and properties reported for these compounds are similar to those observed with the toxic compound.

In the proposed structure for streptomycin,[14] the carbonyl group of the streptose moiety exists as a free aldehyde. Therefore, there are only two possible formulations for the toxic compound



I II

where (St) is streptomycin less –CHO (III).



III

Since the toxic compound shows little or no absorption in the ultraviolet in contrast to streptomycyloxime which contains a —C=N group, the probable structure is that of a dialdehyde ammonia as shown in II. Such a formula accounts satisfactorily for the observed behavior and properties of bis-(α-hydroxystreptomycyl)-amine. It is of course possible that the two forms may be in equilibrium in aqueous solution.

Acknowledgment.—The authors wish to acknowledge the continued interest of Dr. W. A. Lazier during this investigation. Acknowledgment is also gratefully extended to Dr. J. Means and his group for the microanalyses, to Dr. B. Sobin and his group for the microbiological assays, and to Dr. G. Hobby and her group for the toxicity studies.

Summary

Bis-(α-hydroxystreptomycyl)-amine was isolated from streptomycin residues as the crystalline reineckate and converted to the amorphous sulfate. This latter salt was found to be unstable in dilute aqueous solution; on standing, it liberates streptomycin sulfate and ammonia. Hydrogenation yields dihydrostreptomycin sulfate and streptomycylamine sulfate. These products were compared on paper chromatograms with authentic samples. Bis-(α-hydroxystreptomycyl)-amine sulfate was synthesized from streptomycin trihydrochloride and ammonia and shown to be identical with the material isolated from streptomycin residues.

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(12) C. A. Lobry de Bruyn and A. P. N. Franchimont, *Rec. trav. chim.*, **12**, 286 (1893).

(13) C. A. Lobry de Bruyn and F. H. Van Leent, *ibid.*, **15**, 81 (1896).

(14) F. A. Kuehl, R. L. Peck, C. E. Hoffhine, E. W. Peel and K. Folkers, THIS JOURNAL, **69**, 1234 (1947).