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7/25/68

CHEMISTRY OF β-STREPTOMYCIN SALTS

A THESIS

Presented to

The Faculty of the Graduate Division

by

Rajender Kumar Chawla

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GLOSSARY OF ABBREVIATIONS

βDHS	crude β-dihydrostreptomycin
αDHS	α-dihydrostreptomycin
N-Cbz	*N*-carbobenzoxy
n.m.r.	nuclear magnetic resonance
i.r.	infrared
SE-30	Silicone gum rubber (methyl)
TMS	Tetramethylsilane
TLC	Thin-Layer Chromatography
K	Distribution coefficient

SUMMARY

The physical properties of crystalline β-streptomycin salts differ from those of the corresponding amorphous α-streptomycin salts, although both forms show no spectral evidence for the aldehyde group in the streptose portion of the molecule. It had been suggested previously that this aldehyde group is linked to various -OH groups in a hemiacetal formation in the α-salts to the nitrogen of the aminomethyl group of the N-methyl-L-glucosamine part of the molecule in the β-salts. No experimental evidence had been recorded to substantiate this suggestion. The purpose of this research was to establish the nature of the hemiacetal linkage of the potentially-free aldehyde group in the β-streptomycin salts.

The first approach to determine the structure of β-streptomycin salts by X-ray diffraction studies had to be abandoned because these salts were found to exist as liquid crystals and hence were unsuitable for X-ray diffraction studies.

Earlier work on the structure of dihydrostreptobiosamine had shown that the potentially-free aldehyde group in that molecule was linked with the nitrogen of the aminomethyl group of N-methyl-L-glucosamine. This was demonstrated by hydrogenolysis of dihydrostreptobiosamine. The resulting product, after acetylation, was found to be a pentaacetyl basic compound, instead of a heptaacetyl neutral compound. This was explained on the basis that the N-hemiacetal bond was hydrogenolyzed, yielding a morpholine derivative. Extending this analogy to

β-streptomycin salts, one would expect an azoxepine type of structure on reduction of β-streptomycin. This could be differentiated from the ordinary α-dihydrostreptomycin by the N-carbobenzoxylation of the aminomethyl nitrogen under suitable conditions. The product obtained on such a reduction of β-streptomycin trihydrochloride showed a 25-50 percent N-carbobenzoxylation under conditions that gave 100 percent N-carbobenzoxylation of α-dihydrostreptomycin. This showed that there was 75-50 percent conversion to β-dihydrostreptomycin by reduction of crystalline β-streptomycin salts. The exact amount of this conversion varied from preparation to preparation.

Various chromatographic techniques to purify the crude β-dihydrostreptomycin (from α-dihydrostreptomycin and N-carbobenzoxy-α-dihydrostreptomycin) did not give a pure compound. Countercurrent distribution of the mixture of N-carbobenzoxy-α-dihydrostreptomycin and β-dihydrostreptomycin in 1-butanol and 5 percent aqueous p-toluenesulfonic acid gave pure β-dihydrostreptomycin in the lower phase. This compound was characterized by cleavage to streptidine on acid hydrolysis, by its specific rotation, and by its R_F values on chromatography (from those of the α-dihydrostreptomycin). Beta-dihydrostreptomycin was also found to be biologically inactive.

Thus, it has been established that in β-streptomycin the potentially free aldehyde in the streptose fragment is linked to the aminomethyl nitrogen of the N-methyl-L-glucosamine part of the streptomycin molecule.

CHAPTER I

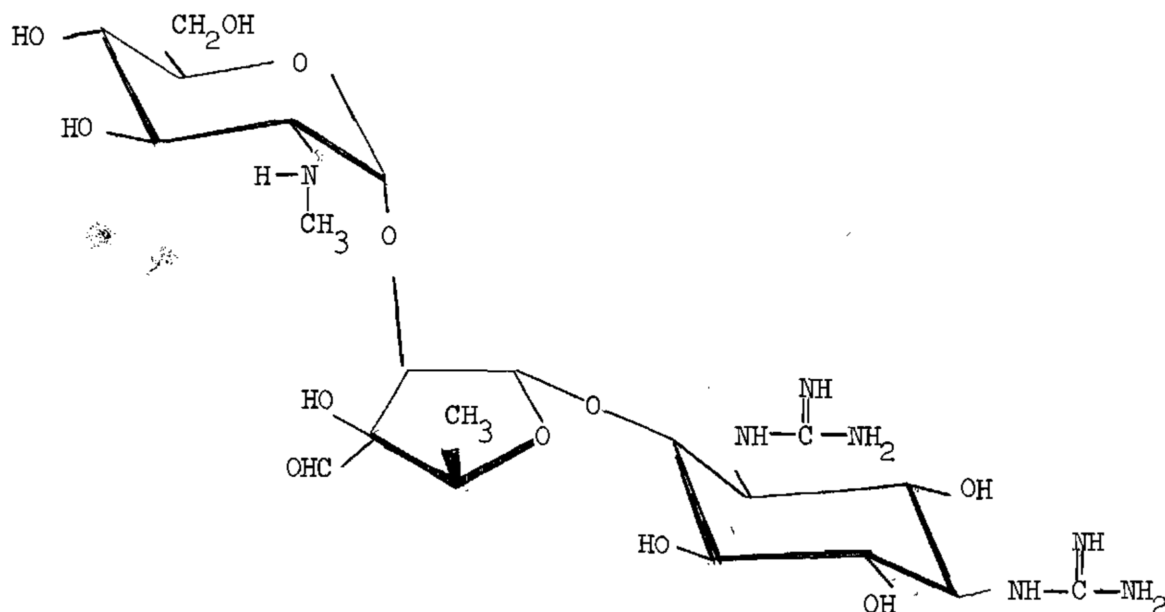
INTRODUCTION

Streptomycin was isolated in 1944 by Waksman and co-workers from cultures of *Streptomyces griseus*, an organism of the order *Actinomycetales* (1). The antibiotic was found to have low toxicity, potent therapeutic action, and was the object of intensive research soon after its discovery (2). The substance was found to be effective against a wide variety of microorganisms and was the first drug used effectively against tuberculosis. Streptomycin has also been employed successfully in the treatment of diseases such as leprosy, tularemia, typhoid, and brucellois (2,3). The clinical aspects of this antibiotic have been reviewed (2).

Purification of crude streptomycin was a major problem and was achieved by precipitation with picric acid and conversion of the picrate to the hydrochloride (4). Also, crude streptomycin was chromatographed over acid-washed alumina using methanol as the eluant (5). Conversion of streptomycin to the trihelianthate salt and then reconverting that salt to the trihydrochloride salt also achieved purification (4,5,6).

Determination of Structure

Extensive research established the structure of streptomycin as that shown by structure I (8). Many excellent reviews of the literature on the structure of streptomycin exist (2,7,8). The molecular formula of streptomycin has been shown to be $C_{21}H_{39}N_7O_{12}$. It is a levorotatory,



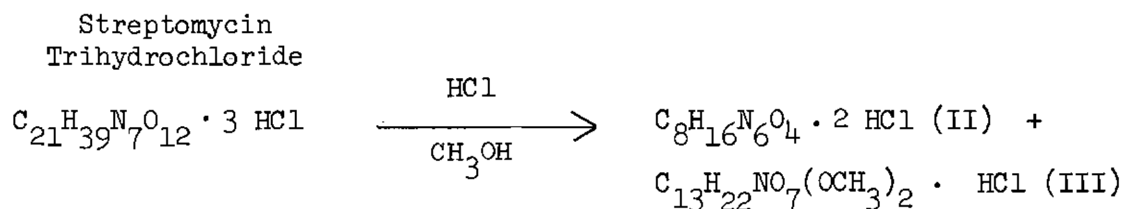
I

tribasic compound that is unstable in aqueous solution. It was readily converted to maltol on warming in the presence of dilute alkali. On hydrogenation at atmospheric pressure, using platinum or palladium as the catalyst, streptomycin trihydrochloride absorbed one equivalent of hydrogen. The product, dihydrostreptomycin trihydrochloride, differed but slightly from streptomycin trihydrochloride in its biological activity, a very unusual occurrence, since even a slight change in the structure of most antibiotics result in marked variation in their activities. Later, dihydrostreptomycin was isolated as a major fermentation product of <u>streptomyces humidus</u> (9).

Streptomycin formed oxime and semicarbazone derivatives, which

were biologically inactive; dihydrostreptomycin, however, gave no such derivatives. Because streptomycin showed no spectral evidence for the presence of a free carbonyl group, a potentially free carbonyl group was indicated from the above evidence (2,10).

Degradation of streptomycin with methanolic hydrogen chloride yielded two compounds, which were streptidine dihydrochloride (II) and methyl streptobiosaminide dimethyl acetal hydrochloride (III) (11,12).

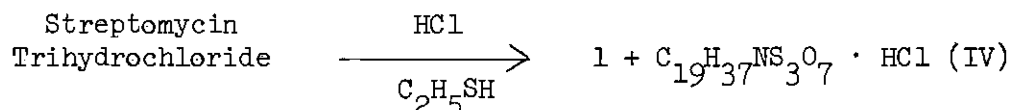


Streptidine was shown to be 1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane; all the functional groups were equatorial (2,13,14,15). The C_4 hydroxyl group of streptidine was attached to the streptobiosamine moiety. Streptidine has been synthesized (15), and the absolute stereochemistry of the six asymmetric carbon atoms of streptidine in the streptomycin molecule has been established (16). The absence of a free or potentially free carbonyl group in streptidine indicated that the carbonyl group resides in the streptobiosamine moiety and is converted to a dimethyl acetal derivative upon acidic methanolysis.

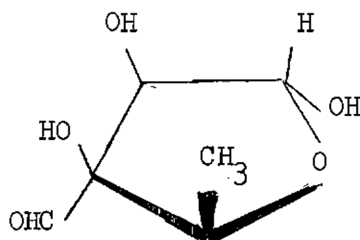
Using bromine water, streptomycin can be oxidized to an acid called streptomycenic acid, which has the same number of carbon atoms as streptomycin (5). This fact led to the inference that the potentially free carbonyl function in streptomycin is an aldehyde.

The mercaptolysis of streptomycin gave streptidine and another

product, which was identified as ethyl thiostreptobiosaminide dithioacetal (IV) (17). This also confirmed the presence of a potential aldehyde group in the streptobiosamine fragment and also indicated that streptidine is linked glycosidically to another aldehyde function in the streptobiosamine fragment (2,18).



When methyl streptobiosaminide dimethyl acetal hydrochloride (III) was treated with aqueous acid and the resulting crude product was acetylated, a compound resulted that had the molecular formula $C_{17}H_{25}NO_5$. The formula was consistent for a pentaacetyl N-methylhexosamine, which by subsequent tests and independent synthesis was identified as the pentaacetyl derivative of N-methyl-L-glucosamine (38). This further led to the conclusion that the potentially free aldehyde group was present in the streptose moiety. Streptose has the molecular formula $C_6H_{10}O_5$ (19), and has been shown to have structure V, which has been confirmed by an independent synthesis (20).



V

Tautomeric Forms of Streptomycin

The potentially free aldehyde group in streptose has been suggested to be linked with various hydroxyl, amino, and guanidino functional groups. Titus and Fried (21), in their countercurrent distribution studies of streptomycin, observed that the antibiotic existed as a mixture of "tautomeric" modifications, the relative proportions of which depended upon pH. They indicated that such "tautomers" could at least partially be separated by chromatography on alumina. The tautomeric shift was dependent on the pH; an alkaline pH favored conversion to that "tautomer" that had a high distribution constant.

In 1953, Heuser and co-workers (22) reported their studies on purification and crystallization of streptomycin. Streptomycin precipitates with such general reagents as methyl orange, Reinecke acid, picric acid, and phosphotungstic acid. Until Heuser's observation no practical method had been found for obtaining crystalline streptomycin salts of simple mineral acids. These workers found that such commercially available wetting agents as Duponols and Tergitols could precipitate the basic antibiotics, and the metathesis of such precipitated salts provided an excellent means for the preparation of crystalline mineral acid salts of streptomycin. Tergitol-7, a sodium sulfate derivative of 3,9-diethyl-tridecan-6-ol was found to give the best results for precipitating streptomycin at suitably controlled pH. These precipitates (hereafter called "tergitates"), after aging at 5° for a few days, could be crystallized. The crude tergitate was readily soluble in solvents like benzene, ether, chloroform, various alcohols, and aqueous acetone. In contrast, the crystalline tergitate was found to be difficultly soluble

in most organic solvents. The solubility was, of course, increased by addition of a small amount of water.

Streptomycin of higher purity could be recovered from these alkyl sulfate salts of streptomycin by a variety of methods (23). Some of these methods were unsatisfactory in that some of the antibiotic decomposed and some other undesirable impurities were added. One of the best methods to effect metathesis was by the addition of an inorganic acid to a solution of the streptomycin tergitate at a pH controlled by anion exchange resins. This method increased the biological potency of a given sample of crude streptomycin from an original 100-300 γ/mg to 650-700 γ/mg.

Heuser, et al.(22) also obtained crystalline trihalides, trinitrate, sulfate, and phosphate salts of streptomycin from the methanolic solution of aged streptomycin tergitates. The trihydrochlorides and trinitrates were obtained by adding a dilute solution of the appropriate acid or calcium salt to the methanolic solution of the tergitates. Thus, in the reaction of methanolic calcium chloride with a methanolic solution of aged streptomycin tergitate, the crystalline trihydrochloride was obtained. These workers did not observe a double salt of calcium chloride and streptomycin as has been reported by Folkers (24). The trihydrohalides were monoclinic crystals, which could be recrystallized from methanol with great difficulty. However, better yields in recrystallization were reported when a highly concentrated aqueous solution of the crystalline trihydrochloride, for example, was diluted with methanol or methanolic calcium chloride. If the aqueous solution of the crystalline salt was allowed to stand before diluting, the yield of the crystals

became considerably lower.

Elemental analyses and ultraviolet and infrared spectral properties of the crystalline material were reported similar to the pure amorphous salts.

The crystalline sulfate differed strikingly from the amorphous sulfate in solubility behavior. While the amorphous sulfate is readily soluble in water, the crystalline form is only sparingly soluble. The sulfate was crystallized by dissolving streptomycin tergitate in 1-butanol and mixing this solution with an aqueous solution of guanidine sulfate. The crystalline form of this streptomycin sulfate is rectangular prisms; analysis showed it to be a sesquisulfate.

All the crystalline organic salts of streptomycin, like the crystalline tergitate, were obtained in optimum yield only after aging the crude tergitate for about one week at 5°. Furthermore, it was very difficult to recrystallize a once-crystallized salt that had been dissolved in aqueous solution for some time. These facts clearly indicated that these were "tautomeric forms" of streptomycin. Thus, the earlier observations of Titus and Fried (21) were confirmed. Heuser and co-workers (22) further confirmed this hypothesis by a comparative study of the distribution constants of amorphous and crystalline streptomycin trihydrochloride in the system butanol-aqueous-p-toluenesulfonic acid. They observed the instability of the crystalline "tautomer" in aqueous solution and also that hydrochloric acid stabilized it. If the sample of the crystalline streptomycin was dissolved directly in the sulfonic acid phase before introduction into the butanol-_p_-toluenesulfonic acid system, a marked stabilization was observed. The amorphous form dis-

played essentially the same value for K, as shown in Table 1.

Table 1. Distribution Constants of Streptomycin Trihydrochloride Tautomers

K for	Solvent		
	1-Butanol-Water	1-Butanol-0.01 N Hydrochloric Acid	1-Butanol-5 Percent Aqueous p-toluene-sulfonic acid
crystals	1.30	11.1	15.0
amorphous	0.97	1.05	1.01

The rate of change in these solvents from the crystalline form to the amorphous form was studied by allowing the respective solutions to stand. Samples were withdrawn periodically for the determination of K. It was shown that conversion was almost instantaneous in a neutral aqueous medium.

Additional evidence of "tautomerism" and "tautomeric" change with the passage of time was provided by polargrams recorded from solutions of crystalline and amorphous streptomycin salts. The clearest evidence (25) that this phenomenon was not due to solvolysis or allotropy was that a solution of crystalline streptomycin trihydrochloride precipitated crystalline streptomycin sulfate on the addition of sulfate ion.

Young repeated and extended Heuser's work and designated the crystalline form as β-streptomycin salts (26), using α-streptomycin to indicate the amorphous form. He obtained the biphenyl-4-sulfonate, the

5-cyclohexyl-2-hydroxy-3-methylbenzoate, and the 2-hydroxy-5-isobutyl-3-methylbenzoate salts of β-streptomycin. In the case of the first two of these, the α-streptomycin salts are also crystalline. However, the α- and β-pairs of these salts had different X-ray diffraction patterns. These three new salts were further converted into β-streptomycin sulfate.

Young (26) also studied the conversion of β- to α-streptomycin salts polarimetrically and studied the effect of pH on this conversion. A methanol solution of β-streptomycin tergitate (50 mg/ml) showed a change of optical rotation at 20° from -19.8° to -26.4° in a few days. In 0.01 N-methanolic sodium hydroxide, the change in rotation was more rapid. However, in 0.01 N-methanolic sulfuric acid, no measurable change in optical rotation was observed for 18 hours; in 0.1 N-methanolic acetic acid the change in optical rotation showed only 3 percent conversion into the α-form in 24 hours. The β-tergitate salt of streptomycin could be "stabilized" when recrystallized from methanol-water containing a trace of sulfuric acid, in that it showed no appreciable change of optical rotation in methanol solution. It did revert to the α-form if a trace of sodium hydroxide was added; this change was second-order in all cases.

Similar changes were observed for β-streptomycin 5-cyclohexyl-2-hydroxy-3-methylbenzoate. The change was rather rapid and small; $[\alpha]_D^{22}$ was -82.3 10 minutes after preparation (50 mg/ml) and changed to a final value of -79.1° (24 hours). Gravimetric assay of the β-form by precipitation as the sulfate salt gave identical results. Here again, the change was distinctly retarded by 0.001 N-hydrochloric acid.

Young also observed that the β-salts were solvated. The sulfate salt lost the equivalent of seven molecules of water when dried; the

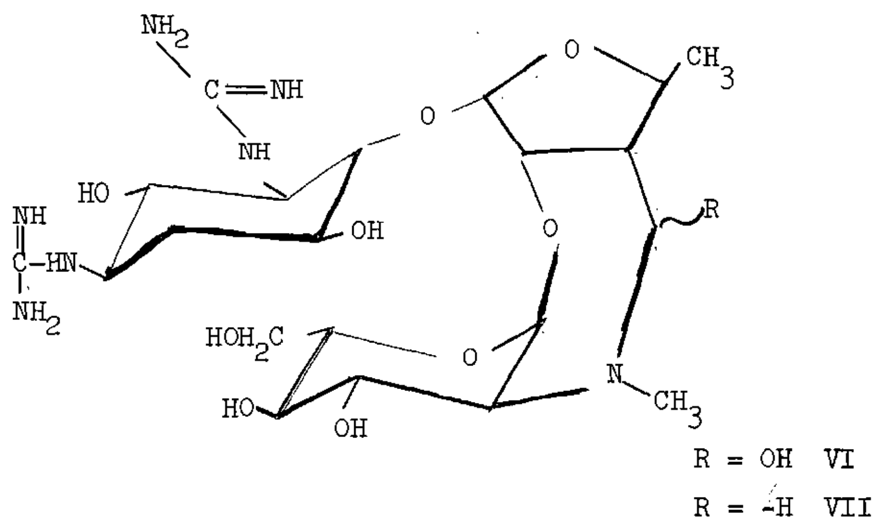
dried salt was readily soluble in water, but the hydrated sulfate precipitated soon. The trihydrochloride was solvated with two molecules of methanol. Thus, the β-streptomycin cation was shown to have the same composition as the corresponding α-streptomycin cation. These forms have sufficiently mobile interconversion to justify the term "tautomerism."

The only other salt which on aging yields the crystalline β-salts is the related 4-ethyl-1-isobutyloctyl sulfate (Tergitol-4) (26).

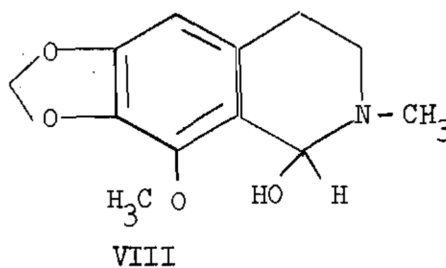
It was also observed that an aqueous solution of α-streptomycin salts exhibited no detectable amount of the β-form in equilibrium. There was no change in rotation in solutions of α-streptomycin salts; no β-sulfate could be precipitated from the solutions of α-streptomycin by the addition of an excess of sulfate ions. It was therefore concluded that conversion to the β-form could not occur in a solid phase in contact with a solvent containing appreciable amounts of dissolved streptomycin. The tergitate had the unique combination of properties, that is, it is insoluble in water but the presence of water is required to convert it into the β-"tautomer." The driving force of this reaction was attributed presumably to the crystallization of the hydrate.

Mannosido streptomycin shows the same phenomenon, but dihydrostreptomycin and streptomycin oxime do not, which implies that the aldehyde group is involved in the structural change. Young (26) confirmed this by showing that β-streptomycin tergitate, in methanolic 0.1 N acetic acid, reacted more slowly than the α-salt with hydroxylamine and 4-phenylsemicarbazide. The reaction in both instances was followed polarimetrically. Thus, the aldehyde group in the β-salts is combined in such a way as to be less reactive. The distinct influence of bases

on the $\alpha \rightleftharpoons \beta$ conversion is manifested at the neutralization point of the least basic ionizable group of the streptomycin cation. Young suggested that the weakest basic group in the streptomycin molecule, i.e., the methylamino group, is concerned in this structural change. This group may combine with the aldehyde group to form a seven-membered ring that is sterically favored in the twist chair form (VI). This kind of



structure has an analogy in cotarnine, VIII (27). No serious non-bonded interaction would be expected from the structure suggested for β-streptomycin.

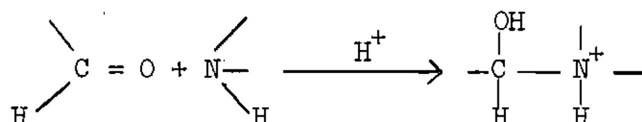


VIII

The alternative proposal that the aldehyde group could be linked with one of the guanidino nitrogens as a hemiacetal can be ruled out on the basis of steric hindrance.

Purpose of the Research

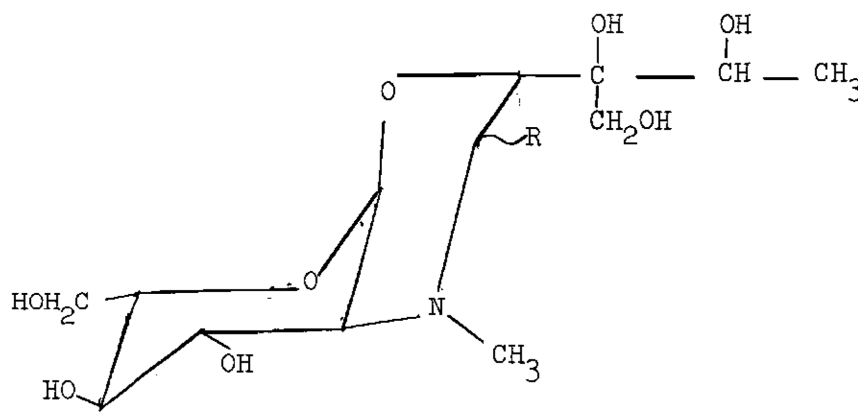
It was proposed to establish unequivocally the structure of β-streptomycin salts. The stability of the β-salts in acidic medium seemed to indicate that these salts might have an N-hemiacetal structure:



The positive charge on nitrogen would hinder the approach of a hydronium ion at carbon, the first step in the hydrolysis of acetals and hemiacetals. Earlier work of Fried and co-workers (30) had shown that hydrogenation of dihydrostreptobiosamine hydrochloride resulted in the uptake of one mole of hydrogen. The acetylation of the product yielded a crystalline derivative that was not heptaacetyltetrahydrostreptobiosamine, but a base hydrochloride $C_{23}H_{20}O_8N(CH_3CO)_5 \cdot HCl$. Prior to acetylation, the product was non-reducing and did not give N-methyl-L-glucosamine on hydrolysis. These workers showed that the reduction of dihydrostreptobiosamine had given rise to the morpholine structure, IX, called tetra-anhydrostreptobiosamine. Dihydrostreptobiosamine was shown to possess structure X.

On the basis of this analogy, if β-streptomycin salts have the N-hemiacetal structure VI, on catalytic hydrogenolysis it would be expected to yield a compound with structure VII, a tertiary amine. An

examination of Dreiding models reveals that no strain or unusual non-bonding interaction exists in such a compound. Such a structure would not be expected to yield N-methyl-L-glucosamine on hydrolysis, but would give streptidine.



R = H IX

R = -OH X

On the other hand, if the aldehyde group of the streptose moiety formed a hemiacetal linkage with a nitrogen of the C_1 or C_3 guanidine units of streptidine (as suggested by Bricker and Vail (28), although subsequently discounted on the basis of steric hinderance (10)), the catalytic hydrogenolysis would be expected to give a derivative with a disubstituted guanidine group. Upon acid hydrolysis of this compound, one would expect cleavage of N-methyl-L-gluosamine rather than of streptidine.

It was proposed to subject the β-streptomycin salts to catalytic hydrogenation and to establish the nature of amino group in the resulting compound. This would, in turn, confirm the structure of β-streptomycin salts.

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

Unless otherwise stated, all concentrations and evaporations were performed using a Rinco rotating evaporator at temperatures below 50° and at water aspirator vacuum. Anhydrous magnesium sulfate (Mallinckrodt AR 6070) was used in drying chloroform extracts. Anhydrous methanol was prepared as described elsewhere (31). Redistilled benzene was dried using sodium ribbon. Anhydrous ether (Fisher Scientific Company, Reagent Grade E-138) was further dried using sodium ribbon. Petroleum ether (b.p. 30-60°) was redistilled, as was acetone, before use. Ethyl acetate was dried by distillation from powdered phosphorous pentoxide. Pyridine was purified by distillation from potassium hydroxide pellets until the distillate did not become pale yellow on storage over potassium hydroxide pellets. Acetic anhydride was redistilled, as was acetic acid. Tergitol anionic 7 (sodium heptadecyl sulfate) was purchased from Union Carbide.

Streptomycin and dihydrostreptomycin sulfate used in this work were provided by Merck Sharp and Dohme Company, and Charles Pfizer and Company.

Melting points were observed using a Köfler hot stage melting point apparatus and are corrected, unless otherwise stated. Microanalyses were performed by Bernhardt Laboratories, Mülheim, West Germany.

A Perkin-Elmer Model 137 Infracord recording spectrophotometer was used for all infrared spectra. The pH values were monitored using a Leeds and Northup pH meter, Model 7401. A Bellingham and Stanley polarimeter (Model 397619) equipped with a General Electric Sodium Lab-Arc lamp as a source of the sodium D-line was used for the determination of optical rotations.

Nuclear magnetic resonance (n.m.r.) spectra were determined using a Varian Model A-60D spectrometer. The magnet temperature was essentially constant at some value around 30° during the determination of a given spectrum. Unless otherwise indicated, the chemical shift values are reported in τ units ($\tau = 10 - \delta$). Tetramethyl silane (TMS) and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were used as internal standards for organic solvents and water, respectively. The area of a peak was measured using a Gelman Instrument Company (Model 39231) planimeter.

Thin-layer chromatography was performed as described elsewhere (7). Silicic acid column chromatography was performed by making a slurry of the specified amount of silicic acid (100 mesh, Mallinckrodt AR-2847) with chloroform. The slurry was poured in a suitable column fitted with a coarse sintered glass disc at the lower end. The slurry was allowed to settle, excess chloroform was drained, and the column was vibrated by tapping with a rubber stopper until the adsorbant was firm.

The techniques employed for gas-liquid chromatography have been described elsewhere (7). A Glowall Corporation Chromalab (Model A-110) instrument equipped with a Minneapolis Honeywell continuous recorder was employed. The dimensions of the column and the nature of the packing

material are described in the text.

Paper chromatography was performed as described elsewhere (32). The ion-exchange resins used were regenerated as by the method of Hayes (32). The volume of the resin was determined by allowing the resin to settle to a constant volume in a graduated cylinder. Amberlite anion exchange resin 45 in the chloride phase [IRA 45 (Cl^-)] was used after washing to a pH of 3-3.5.

The Sephadex used in this work was purchased from Pharmacia Fine Chemicals, Inc.; the material was soaked in 0.01 N formic acid overnight before packing a column. An automatic fraction collector (GM Instrument Company) was used in all column chromatographic separations.

Conversion of Streptomycin Sulfate to Streptomycin Trihydrochloride

A solution of 14.6 g (0.02 mole) of streptomycin sulfate (Charles Pfizer and Company) in 60 ml of distilled water was mixed with a solution of 6.640 g of barium chloride (0.03 mole) in a 500-ml beaker. The precipitate formed was filtered using a Celite mat in a sintered glass funnel. A sample of the filtrate yielded no precipitate with a few drops of the barium chloride solution. The filtrate was concentrated under vacuum and then dried overnight *in vacuo* using a vacuum pump at room temperature. The dried residue, light pink in color, was dissolved in 150 ml of anhydrous methanol. About 200 ml of redistilled acetone was added to the solution; the resulting precipitate was filtered using a medium sintered glass funnel. The residue was dried *in vacuo* and reprecipitated from its methanol solution using acetone.

The weight of dried hydroscopic residue was 10.607 g (79 percent).

Conversion of Streptomycin Trihydrochloride to Streptomycin Tergitate

The crystalline streptomycin tergitate was prepared according to the method of Heuser, et al. (22) as modified by Young (26).

To a solution of 10 g of streptomycin trihydrochloride in 3.3 l of distilled water (concentration of streptomycin: 3000 μg/ml) was gradually added 57 ml of Tergitol-7 anionic over a period of 20 minutes. During the addition the mixture was stirred using a magnetic stirrer, and the pH was maintained between 7.0 and 7.5 by periodic addition of 1 N-hydrochloric acid. The white precipitate that formed was allowed to settle, and the suspension was kept in a refrigerator for five days. After this period, most of the slightly turbid supernatant liquid was decanted. The residue was filtered using hardened filter paper and was allowed to dry in air. The white residue was transferred to a flask and lyophilized. The dried residue weighed 18.986 g (56 percent).

An amount of 2.017 g of the crude tergitate was dissolved in 10 ml of anhydrous methanol. The insoluble material was filtered. To the filtrate, 0.02 ml of 1 N sulfuric acid was added. The solution was made slightly cloudy by the addition of about 1 milliliter of water. After cooling overnight, white needle shaped crystals were obtained that melted at 142-143° and had an optical rotation of -23.4° (c 1.0, methanol) [lit (22) mp 141-142°, $[\alpha]_D^{25}$ -25° (c 1, methanol)].

Preparation of β-Streptomycin Trihydrochloride

This salt was prepared according to the method of Heuser (22). A solution of 20 g of crude, aged, and dried streptomycin tergitate was mixed with 100 ml of anhydrous methanol. The insoluble material was filtered. To the filtrate, 70 ml of 10 percent calcium chloride in

methanol (containing 0.7 ml of concentrated hydrochloric acid) was added. White crystals began to appear at once. After keeping the mixture in the refrigerator for 16 hours, the crystals were filtered and dried under vacuum; the yield was 3.305 g (33 percent). The crystals were recrystallized from anhydrous methanol and had $[\alpha]_D^{22}$ -89.6° (*c* 1, water), [*lit*. (22) -86.1 (*c* 1.0, water)]. The final weight of the crystals after drying was 2.4766 g.

Studies on the Quantity of β-Streptomycin Trihydrochloride Recovered by Aging of Streptomycin Tergitate

The streptomycin tergitate, prepared as described earlier, was aged at 5° for periods of one to seven days. The lyophilized crude streptomycin tergitate was converted to β-streptomycin trihydrochloride by metathesis with calcium chloride solution as described earlier. The percentage yield of β-streptomycin trihydrochloride based on the starting amount of α-streptomycin trihydrochloride is tabulated in Table 2.

Table 2. Effect of Aging Upon Yields of β-Streptomycin

Duration of Aging	Percentage Yield of β-Streptomycin Trihydrochloride
One day	28
Four days	35.5
Five days	42
Six days	43.5
Seven days	44

Preparation of β-Streptomycin Trihydrobromide

This salt was prepared as described by Heuser (22). A 2.3170 g sample of crystalline β-streptomycin tergitate was mixed with 12 ml of anhydrous methanol. The suspended material was filtered. The filtrate was mixed with 12 ml of a freshly prepared 10 percent solution of anhydrous calcium bromide in methanol. On standing in the refrigerator overnight, white crystals separated. These were filtered and dried; the yield was 0.350 g (26 percent). The material had no sharp melting point and had $[\alpha]_D^{22}$ -69° (*c* 1.2, water) (*lit.* -72.1°).

Attempted Preparation of β-Streptomycin Trihydroiodide Using Calcium Iodide

The method followed for this preparation was based on the procedure that had been used (22) for other trihalides.

The calcium iodide required for this preparation was available commercially only as the polyhydrate (City Chemical Corporation, New York, N. Y.; U.B. 821). It was dried by boiling a solution of 12.132 g of calcium iodide hydrate and 12 g of 2,2-dimethoxypropane under reflux for 16 hours. The solvent and water were evaporated and the resulting solid (9.576 g) was dissolved in methanol to make a 10 percent solution.

Twenty grams of crude aged streptomycin tergitate was mixed with 100 ml of methanol. The suspended material was filtered. The filtrate was mixed with 145 ml of a 10 percent solution of anhydrous calcium iodide in methanol. On standing at 5° for 16 hours, no crystals appeared. On prolonged storage for seven days only a slight cloudiness resulted.

Other preparations were attempted using 12 percent and 15 percent solutions of calcium iodide in methanol, but no crystals appeared. Even

when crystalline β-streptomycin tergitate was used, no crystals resulted.

Attempted Preparation of β-Streptomycin Trihydroiodide Using Sodium Iodide

A solution of 295 mg (0.36 millimole) of β-streptomycin tergitate in 1.5 ml of methanol was mixed with a solution of 164 mg of sodium iodide in 1 ml of acetone. The solution was stored at 5° for four months. No precipitate or crystals appeared.

Reduction of β-Streptomycin Trihydrochloride

Fried and Wintersteiner's method (5) for reducing α-streptomycin was used for this reduction except that 5 percent platinum on carbon was the catalyst instead of platinum oxide.

In a 250-ml hydrogenation bottle, 70 ml of redistilled acetic acid (Fisher Reagent) was employed to suspend 5.2 g of 5 percent platinum on carbon (Englehard Industries, Newark, New Jersey, Lot 5634). The apparatus was flushed five times with nitrogen and five times with hydrogen and left in an atmosphere of hydrogen for 16 hours for equilibration.

Then, 2.477 g (3.6 millimoles) of β-streptomycin trihydrochloride dissolved in 20-ml of water was added to the hydrogenation bottle. The uptake of hydrogen after 16 hours was 79 ml (3.6 millimoles). The catalyst was filtered using a Celite mat,and the filtrate was lyophilized. The weight of the residue was 2.142 g after drying overnight under vacuum. The melting point was 239-241° and the preparation had $[\alpha]_D^{22} = -77^\circ$ (c 1.0, water).

Conversion of α-Dihydrostreptomycin Sulfate to the β-Naphthalenesulfonate Salt

This salt was made by a modified procedure (43) of Regna and Carboni (33).

In a 50-ml Erlenmeyer flask, 2.581 g (3.5 millimoles) of dihydrostreptomycin sulfate was dissolved in 10 ml of distilled water. In another flask, 2.523 g of sodium β-napthalenesulfonate (Eastman, white label) was dissolved in 11 ml of warm water. The two solutions were mixed and kept at room temperature overnight. Small white crystals appeared in the flask. These were filtered, dried, and recrystallized from hot water. The yield was 0.873 g, mp 203-204° (corr.) [lit. 184-186°], $[\alpha]_D^{21} = -57°$ (c, 1.0, water) [lit. -52.1 (33)].

Conversion of Crude β-Dihydrostreptomycin Trihydrochloride to the β-Naphthalenesulfonate Salt

Beta-Dihydrostreptomycin trihydrochloride (0.256 g; 0.34 millimole) was dissolved in 1 milliliter of water and mixed with a solution of 0.260 g of sodium β-naphthalenesulfonate (Eastman, white label) in 1.3 ml of water. On standing for 16 hours at room temperature, white crystals appeared. These were filtered, dried, and recrystallized twice from hot water. The yield was 21 mg, mp 175.8-176.8° (corr.).

Preparation of α-Dihydrostreptomycin Trihelianthate Salt

This preparation was attempted using the method of Fried and Wintersteiner (5). A solution of 0.156 g (0.22 millimole) of α-dihydrostreptomycin trihydrochloride in 2.2 ml of water was mixed with a solution of 0.264 g of methyl orange in 5 ml of 50 percent methanol. Crystals began to form immediately. After standing 2 hours at room temperature,

the crystals were filtered and recrystallized from 50 percent methanol. The yield was 270 mg. No sharp melting point was observed, although the crystals appeared to start decomposing at 220°.

Preparation of the Trihelinthate Salt of Crude β-Dihydrostreptomycin

This salt was also prepared by the same procedure as the one for α-dihydrostreptomycin. A solution of 0.142 g (0.20 millimole) of crude β-dihydrostreptomycin trihydrochloride in 2 ml of water was mixed with a solution of 0.261 g of methyl orange in 5 ml of 50 percent methanol. On standing, crystals appeared which were collected by filtration, recrystallized from dilute methanol, and dried. The yield was 0.246 g; mp 217-219° (dec).

Orange II Salts of α- and Crude β-Dihydrostreptomycins

A sample of 95 mg (0.12 millimole) of α-dihydrostreptomycin trihydrochloride was dissolved in 1 milliliter of water. Another solution of 87 mg (0.11 millimole) of β-dihydrostreptomycin trihydrochloride in 1 milliliter of water was also prepared.

A solution of 0.9001 g of Orange II (Fisher Scientific Company, O-266, 721371) was prepared in 10 ml of water and 1 milliliter of absolute ethanol. Two milliliters of the Orange II solution was added to each of the dihydrostreptomycin solutions. On keeping for 16 hours at 5°, scarlet crystals appeared in both the mixtures. These crystals were filtered, recrystallized from hot water, and dried.

The salt form α-dihydrostreptomycin trihydrochloride weighed 110 mg and had a mp 222-223° (dec). The salt from crude β-dihydrostreptomycin weighed 113 mg and had a sharp mp of 216-217°.

Attempted Purification of the Crude β-Dihydrostreptomycin

A solution of 3.6491 g (5.3 millimoles) of β-dihydrostreptomycin trihydrochloride preparation in 50 ml of water was mixed with a solution of 6.2538 g of methyl orange in 250 ml of 50 percent methanol.

Crystals began to appear immediately. After standing 2 hours these crystals were filtered and recrystallized twice from 60 percent methanol. The final weight of the trihelinthate salt was 5.039 g.

In a 200-ml Erlenmeyer flask, 2.436 g (1.7 millimoles) of the trihelinthate was dissolved in 100 ml of 50 percent methanol and stirred for 6 hours with 50 ml of ion exchange resin IRC 45 (OH^- phase). The resin was filtered and the filtrate was passed over a column of 50 ml of ion exchange resin IRC 45 (Cl^- phase; washed to pH 3.0-3.5). The column was washed with 500 ml of water. After concentration and lyophilization, 0.847 of β-dihydrostreptomycin trihydrochloride was recovered.

N-Carbobenzoxylation of Crude β-Dihydrostreptomycin Trihydrochloride

A modified version of the method described by Polglase (35) was used for the N-carbobenzoxylation at the methylamino nitrogen.

In a typical preparation, 0.610 g (0.9 millimole) of the β-dihydrostreptomycin trihydrochloride preparation was dissolved in 10 ml of water. The pH of the solution was adjusted to 8.5 and maintained between pH 7.0 and 9.5 by occasional addition of ion-exchange resin (Amberlite IRA 400 (OH^- phase) washed to pH 9.5-10.0.

The solution was agitated using a magnetic stirrer, and 790 mg (4.5 millimoles) of carbobenzoxy chloride was added dropwise over a period of about 20 minutes. Stirring was continued for a period of

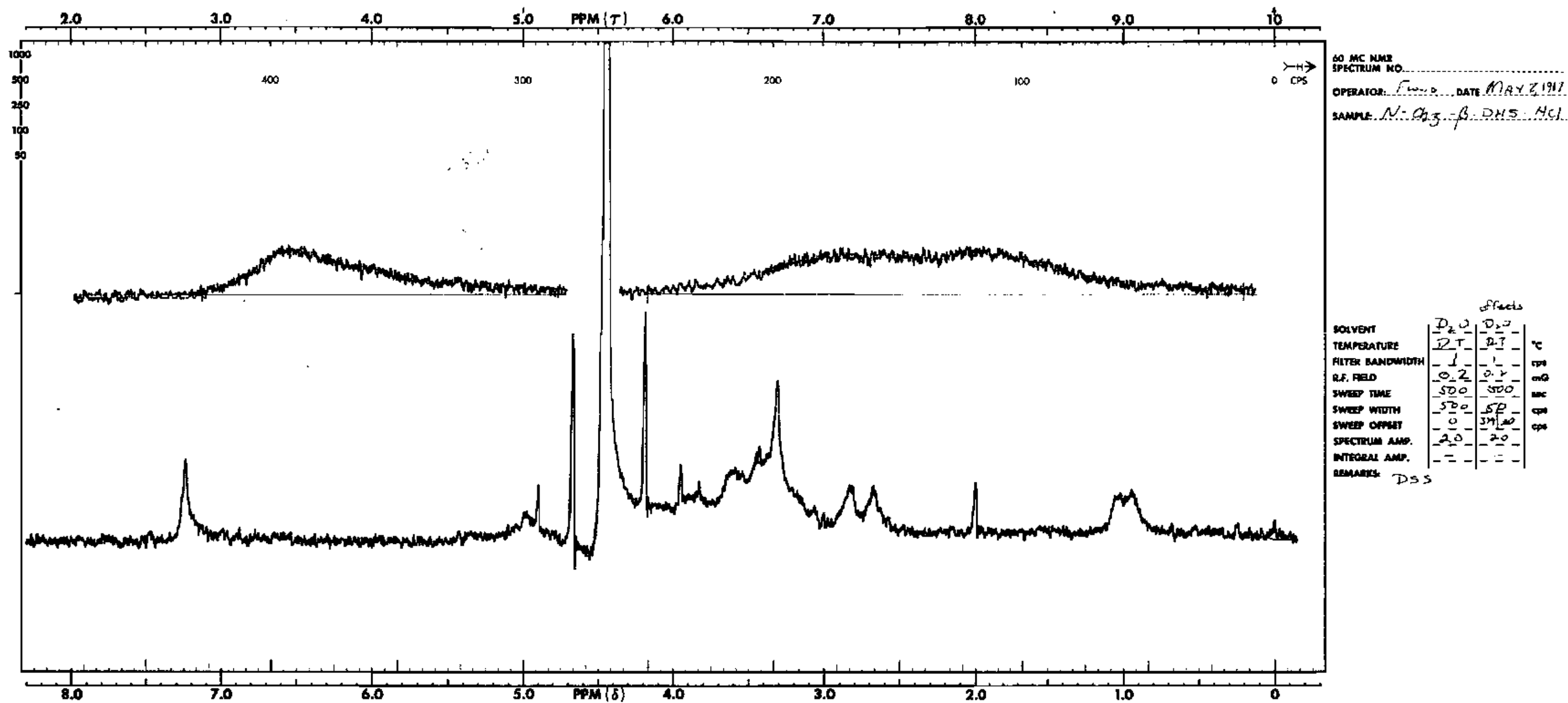


Figure 1. N.m.r. Spectrum of the N-Carbobenzoxylated Derivative of Crude β-Dihydrostreptomycin

about 2 hours during which the pH was maintained between the above-mentioned limits. After this period, the resin was filtered, and the filtrate was washed with three 50-ml portions of ether. The aqueous portion was concentrated to remove dissolved ether and was passed over a column of 50 ml of ion-exchange resin IRC-45 (Cl^- phase). The column was further eluted with 100 ml of distilled water. The eluants were pooled and lyophilized. The final weight of the recovered carbobenzoxylated preparation was 0.440 g.

The n.m.r. spectrum of this preparation in deuterium oxide revealed the following pertinent peaks: (i) a singlet at 2.7 τ corresponding to C-phenyl protons; (ii) a complex multiple between 5.0 and 7.0 τ, corresponding to various ring protons; (iii) two singlets at 7.15 and 7.25 τ corresponding to the N-CH_3 protons; (iv) a doublet at 8.9 τ, corresponding to C-CH_3 protons.

The peaks at (i) and (iv) were expanded at 50 cps and gave a respective area of 168 squares and 329 squares.

In subsequent experiments, unless otherwise specified, the crude β-dihydrostreptomycin used was found to contain at least 70 percent β-dihydrostreptomycin by n.m.r. analysis; the remainder was α-dihydrostreptomycin.

A solution of 150 mg (0.24 millimole) of the "purified" β-dihydrostreptomycin trihydrochloride in 10 ml of water was treated with 5 ml of carbobenzoxy chloride in the presence of ion exchange resin IR 400 (OH^- phase) to maintain the pH between 8.5-9.0. The workup was identical to that described previously.

The final n.m.r. analysis revealed that 23 percent of N-carbenzoxy-

α-dihydrostreptomycin was present.

Methanolysis of α-Dihydrostreptomycin Trihydrochloride

The procedure adopted was the same as described elsewhere (5).

A sample (8.407 g, 12. 2 millimoles) of α-dihydrostreptomycin trihydrochloride was dried over phosphorus pentoxide at 60° under high vacuum for a period of two hours. It was dissolved in 173 ml of 1.0 N hydrogen chloride in dry methanol and allowed to stand at room temperature for 48 hours.

At the end of this period, 370 ml of dry ether was added to the solution with constant stirring. The white precipitate of streptidine hydrochloride was filtered and dried under vacuum. The weight of the streptidine was 4.697 g (98 percent). The filtrate was concentrated to 25 ml; the cloudy solution was centrifuged for 20 minutes. The supernatant solution was poured into 250 ml of dry ether. It was kept at 5° for 14 hours. The oily layer that resulted was separated from the supernatant solution by decantation and was washed with dry ether (3 x 50 ml). The oily layer was poured into a crystallizing dish and was dried under vacuum over potassium hydroxide and phosphorus pentoxide for 16 hours. The white solid methyl dihydrostreptobiosamine weighed 3.861 g (72 percent), started decomposing at 182° [*lit.* mp 182-183° (dec)], and had $[\alpha]_D^{22} = -130°$ (*c* 1.20, methanol, (*lit.* -135°) (5).

Methanolysis of β-Dihydrostreptomycin Trihydrochloride

A sample of dried crude β-dihydrostreptomycin trihydrochloride (0.794 g, 1.17 millimoles) in 17 ml of 1 N hydrogen chloride in dry methanol was kept at room temperature for 48 hours. At the end of this

period, 36 ml of dry ether was poured into this solution. The white precipitate that resulted was filtered, dried, and weighed, 0.410 g (99 percent). The precipitate was identified as streptidine dihydrochloride. Identification was established by preparing a solution of 98 mg of this streptidine dihydrochloride in 2 ml of water and mixing this with a solution of 50 mg of anhydrous sodium sulfate in 3 ml of water. On standing in the refrigerator, white crystals appeared. These crystals were the known streptidine sulfate.

The ether-methanol filtrate from the collection of streptidine dihydrochloride was concentrated *in vacuo* to about 3 milliliters. The mixture was centrifuged to remove suspended material and then concentrated and dried *in vacuo* over potassium hydroxide and calcium chloride. A highly hygroscopic, white glassy solid formed which was further dried at 60° over phosphorus pentoxide for 1 hour *in vacuo*. It had no sharp melting point and started decomposing at 185°; it weighed 365 mg and had $[\alpha]_D = -99.2^{\circ}$ (*c* 1.29, methanol)([*lit*. for methyl α-dihydrostreptobiosaminide, $[\alpha]_D$ -135° (*c* 1.15, methanol).

Gas-Liquid Chromatographic Analysis of Crude Methyl β-Dihydrostreptobiosaminide

The analysis was carried out on the *O*-silyl derivative of the crude methyl β-dihydrostreptobiosaminide prepared using the method of Sweeley and co-workers (37). In a glass vial with a plastic cap 10 mg of the methyl β-dihydrostreptobiosaminide prepared above was dissolved in 1 milliliter of dry pyridine. To this solution, 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane were added. Dense white fumes appeared. The vial was capped and shaken well for 30 seconds. A

white precipitate appeared. The supernatant liquid was analyzed on a gas chromatographic apparatus using a 6-foot column of SE-30 at 200°, detector at 145° and sampler at 250°. Helium was used as the carrier gas at the rate of 25 ml per minute. Two major peaks were observed that had retention times of 11 minutes and 33 minutes; the areas were in the ratio of 1:3, respectively. The O-silylderivative of methyl α-dihychostreptobiosaminide gave only one peak (11 minutes).

Reduction of β-Streptomycin Trihydrochloride Using a Variety of Catalysts

The reduction of β-streptomycin trihydrochloride was carried out on a small scale (ca. 0.5 to 1.0 millimole quantity), using rhodium (5 percent on carbon), palladium (5 percent on carbon), platinum oxide, and platinum (5 percent on carbon). In each case the workup was the same as described previously. The lyophilized β-dihydrostreptomycin trihydrochloride was found to give a negative Fehling test and a negative maltol test. Each was subjected to carbobenzoxylation according to the procedure described previously. The resulting products gave the results by an n.m.r. analyses shown in Table 3.

Mercaptolysis of Crude β-Dihydrostreptomycin Trihydrochloride

The procedure followed has been described previously by Kuehl and co-workers (38).

A sample of dried (under vacuum, at room temperature) crude β-dihydrostreptomycin trihydrochloride (1.760 g; 2.5 millimoles) was stirred with 18 ml of ethanethiol in a three-necked round bottomed flask fitted with a mechanical stirrer. The flask was kept in an ice bath and

Table 3. Effect of Catalysts on the α-Dihydrostreptomycin Content of Reduced β-Streptomycin

Catalyst	Reaction Period	Percentage of α-Dihydro-streptomycin from Carbo-benzoxylation
Palladium on charcoal	14-16 hours	100
Rhodium on charcoal	14-16 hours	100
Platinum oxide	14-16 hours	30
Platinum on charcoal	7 days	30
Platinum on charcoal	5 days	21

hydrogen chloride bubbled through the contents for 1 hour to insure saturation. The mixture was then stirred for 24 hours at room temperature.

The solvent was evaporated under vacuum with constant stirring to avoid any bumping. The glassy, hygroscopic pale yellow solid was dissolved in about 3 ml of methanol; 50 ml of 2-propanol was added to precipitate the streptidine hydrochloride which weighed 0.865 g (99 percent). The filtrate, after removal of the streptidine hydrochloride, was concentrated _in vacuo_; the resulting brownish powder weighed 0.797 g. Attempts to crystallize this material in a variety of solvents did not succeed. Chromatography over a column of acid washed alumina (1 cm x 55 cm) using 50 percent methanol in benzene as the solvent gave no

fractions that could be crystallized.

Using thin layer chromatography and employing alumina plates, the R_F values listed in Table 4 were obtained. R_F values obtained for α-dihydrostreptobiosaminide thioacetal (prepared by Torline by the same procedure) (36) are also listed.

Table 4. Thin-Layer Chromatographic Analysis of α- and β-Dihydrostreptobiosaminide Thioacetal

	R_F Values	
Solvent System	α-Dihydrostreptobiosaminide Thioacetal	β-Dihydrostreptobiosaminide Thioacetal (Crude)
1-Propanol:Acetic Acid: Water (2:1:1)	0.5	0.5
1-Butanol:Acetic Acid: Water (2:1:1)	0.6	0.8

The spots were visualized using iodine vapors.

Acetylation of Crude β-Dihydrostreptomycin Trihydrochloride

A modified procedure of Comrie and co-workers was used (39).

A sample (0.907 g, 1.3 millimoles) of crude β-dihydrostreptomycin trihydrochloride was stirred with 16 ml of redistilled acetic anhydride. After the addition of 1.120 g of sodium acetate an additional 16 ml of acetic anhydride was added and the reaction mixture was kept at room temperature for 48 hours. It was next stirred at 50° for 5 hours; thereafter the solvents were removed under vacuum and the semisolid residue

was mixed with 50 ml of dry benzene to remove insoluble sodium acetate by filtration. The benzene solution, when slowly mixed with 200 ml of petroleum ether with constant stirring, gave a light-brown precipitate, which was washed with petroleum ether (3 x 25 ml), and weighed 1.036 g.

The i.r. spectrum showed peaks at λ_{max} 5.65 and 6.15μ, among others.

This preparation, along with authentic peracetyl-α-dihydrostreptomycin (prepared elsewhere (36)) was subjected to paper chromatographic analysis. The results are given in Table 5.

Table 5. Paper Chromatographic Analysis of Peracetyl Crude β- and α-Dihydrostreptomycin

	R_F Values	
Solvent System	Peracetyl Crude-β-Dihydrostreptomycin	Peracetyl-α-Dihydrostreptomycin
5 Percent Methanol in Benzene	0.0, 0.22	0.15, 0.23
5 Percent Methanol in Chloroform	0.0, 0.3, 0.5	0.0, 0.3, 0.4, 0.55
9 Percent Ethylacetate in Benzene	0.0	0.0
Acetone	0.0, 0.15, 0.2	0.0, 0.15

Whatman No. 1 filter paper was used in this analysis. The spots were identified by spraying with Weber reagent.

Attempted Separation of Crude β-Dihydrostreptomycin Trihydrochloride on a Sephadex G-15 Column

A column with an inner diameter of 1.9 cm was packed to a height of 475 cm with a slurry of Sephadex G-15 that had been soaked for 16 hours in 0.01 M formic acid. A 5 ml solution containing 1.632 g (2.5 millimoles) of crude β-dihydrostreptomycin trihydrochloride was carefully poured onto the top of the column and was washed on with 0.01 M formic acid solution.

Each fraction(15 ml) was evaporated to dryness and analyzed by ascending paper chromatography using Whatman No. 1 paper and t-butyl alcohol:acetic acid:water (2:1:1) as the solvents. The major fraction and several tailing fractions gave two spots each with R_F 0.19 and 0.63. The spots were visualized by spraying with Weber reagent. On identical analysis, α-dihydrostreptomycin trihydrochloride gave only one spot of R_F 0.17.

Chromatography of Crude β-Dihydrostreptomycin Trihydrochloride on a Cellulose Column

This chromatographic analysis was carried out on the basis of the procedure described elsewhere (40).

To 50 g of powdered cellulose was added 100 ml of the solvent mixture t-butyl alcohol: acetic acid: water (2:1:1), and the slurry was filtered using hardened filter paper. The cellulose powder was transferred to a large evaporating dish to dry in air for nine days. The powder was occasionally stirred during this period. A column (1.8 cm in internal diameter and 50 cm long) was dry-packed using a heavy glass rod with one end flattened to tamp down each of the 80 portions

of the cellulose.

A solution of 1.322 g (1.9 millimoles) of the preparation in 1 milliliter of water was slurried with about 3 g of cellulose. The mixture was thoroughly mixed and dried under vacuum over potassium hydroxide. The dried powder was ground, subjected to the same procedure two more times and dry-packed on the top of the column. Two milliliters more of cellulose were packed on the top. The eluting solvent used was *t*-butyl alcohol:acetic acid:water (2:1:1) and the column was run under a pressure of 3 psi. Fractions measuring 25 ml were collected and lyophilized.

Fractions 1 and 2 gave two spots with R_F values of 0.3 and 0.83; the latter one was faint. The other fractions gave only one spot at R_F values between 0.25 and 0.3. When these fractions were subjected to carbobenzoxylation, the usual partial *N*-carbobenzoxylation was observed showing that about 30 percent α-dihydrostreptomycin was present. The total weight recovery from this column was 1.456 g (110 percent).

Thin-Layer Chromatographic Analysis of the Compound Resulting from *N*-Carbobenzoxylation of Crude β-Dihydrostreptomycin

The crude β-dihydrostreptomycin trihydrochloride that had been subjected to *N*-carbobenzoxylation was analyzed both on silicic acid HF 254 and on normal cellulose plates along with a sample of the crude β-dihydrostreptomycin. The spots were visualized both by ultraviolet light and Weber reagent. The results are shown in Table 6.

Table 6. Thin-Layer Chromatographic Analysis of Crude β-Dihydrostreptomycin and the Compound Resulting from Its N-Carbobenzoxylation

Solvent System	R_F Values	
	N-Cbz of β-DHS*	β-DHS**
(Adsorbant: Silicic Acid HF 254 Plates)		
Ethanol:Acetic Acid:Water (3:1:1)	0.3	0.3
Pyridine:Water (1:1)	0.67	0.66
t-Butyl Alcohol:Acetic Acid:Water (2:1:1)	0.24 and 0.53 (faint)	0.21
0.13 M Formic Acid	0.75	0.77
(Adsorbant: Normal Cellulose)		
Ethanol:Acetic Acid:Water (3:1:1)	0.73	0.73
t-Butyl Alcohol:Acetic Acid:Water (2:1:1)	0.81 and 0.09 (faint)	0.73 and 0.09
0.13 M Formic Acid	0.9	0.9

* N-Cbz of β-DHS signifies the product resulting from the N-carbobenzoxylation of crude β-dihydrostreptomycin.

** β-DHS signifies crude preparation of β-dihydrostreptomycin.

Thin-Layer Chromatographic Analyses of β-Streptomycin Trihydrochloride and Related Streptomycin Derivatives on Silicic Acid Plates

This analysis was carried out using TLC plates made of silicic acid HF 254 by a previously described procedure (10). The solvents employed were 1 percent ammonium formate, 4 percent ammonium formate, and 1 percent sodium acetate. The developing time was about 30 minutes and the spots were visualized using ultraviolet light and by Weber reagent spray. The results are given in Table 7.

Table 7. Thin-Layer Chromatographic Analysis of β-Streptomycin and Other Streptomycin Derivatives

Compound	R_F Values		
	1 % Ammonium Formate	4 % Ammonium Formate	1 % Sodium Acetate
Streptidine Hydrochloride	0.80	0.92	0.71
α-Streptomycin Trihydrochloride	0.60	0.80	0.43
β-Streptomycin Trihydrochloride	0.60	0.90	0.43 and 0.71 (faint)
α-Dihydrostreptomycin Trihydrochloride	0.52	0.78	0.35
Crude β-Dihydrostreptomycin Trihydrochloride	0.56	0.80	0.43
N-Cbz of Crude β-Dihydrostreptomycin*	0.76	0.88	0.6
N-Cbz-α-Dihydrostreptomycin Hydrochloride	0.52	0.80	0.36

* Signifies the product resulting from the N-carbobenzoxylation of crude β-dihydrostreptomycin.

Thin-Layer Chromatographic Analyses of β-Streptomycin and Related Compounds Using Microcrystalline Cellulose

Microcrystalline cellulose (purchased from Applied Science Laboratories) was used to prepare the plates. A slurry of 100 g of this cellulose with 430 ml of water was mixed in a Waring blender for 30-40 seconds and spread on clean glass plates to a thickness of 0.25 mm. The plates were dried at room temperature for 4 hours and stored over indicating silica gel desiccant at room temperature. The solvent system used was _t_-butyl alcohol: acetic acid: water (4:1:5). The spots were visualized by spraying with Weber reagent. The R_F values obtained are shown in Table 8.

Table 8. Thin-Layer Chromatographic Analyses of β-Streptomycin and Related Compounds on Microcrystalline Cellulose Plates

Solvent: _t_-butyl alcohol:acetic acid:water (4:1:5)

Compound	R_F Value
Streptidine Hydrochloride	0.63
α-Streptomycin Trihydrochloride	0.58
β-Streptomycin Trihydrochloride	0.58
α-Dihydrostreptomycin Trihydrochloride	0.63
Crude β-Dihydrostreptomycin Trihydrochloride	0.63
_N_-Cbz of β-Dihydrostreptomycin Trihydrochloride*	0.58 and 0.60
_N_-Cbz-α-Dihydrostreptomycin Dihydrochloride	0.73

* Signifies the product resulting from the _N_-carbobenzoxylation of crude β-dihydrostreptomycin.

In the solvent system 1-butanol:acetic acid:water (4:1:5), the following R_F values obtained are shown in Table 9.

Table 9. Thin-Layer Chromatographic Analysis of Crude β-Dihydrostreptomycin and Related Compounds on Microcrystalline Cellulose Plates

Solvent: 1-butanol:acetic acid:water (4:1:5)

Compound	R_F Value
Crude β-Dihydrostreptomycin Trihydrochloride	0.46
N-Cbz of β-Dihydrostreptomycin Chloride*	0.48, 0.67, and 0.41 (faint)
α-Dihydrostreptomycin Trihydrochloride	0.55
N-Cbs-α-DHS**	0.61

* Signifies the compound resulting from the N-carbobenzoxylation of crude β-dihydrostreptomycin.

** Signifies N-carbobenzoxy-α-dihydrostreptomycin

Attempted Separation of the Compound Resulting from the N-Carbobenzoxylation of Crude β-Dihydrostreptomycin Using Microcrystalline Cellulose Plates

The cellulose plates were made from a slurry of microcrystalline cellulose and water as described above, except that the cellulose layer was 1 millimeter thick.

A solution of 109 mg of the N-carbobenzoxy product of crude β-dihydrostreptomycin in 2 ml of 50 percent ethanol was spotted on the plates (two 10 cm x 20 cm and one 20 cm x 20 cm). The eluting solvent

mixture consisted of 1-butanol:acetic acid:water (4:1:5). The developing time was 4 hours. The plates were then air-dried and a small oblong area was sprayed with Weber reagent. The spots observed were diffused and overlapped with each other; thus, no separation for practical purposes was realized.

Paper Chromatography of Crude β-Dihydrostreptomycin Trihydrochloride and Related Compounds

This analysis was performed in accordance with the procedure described elsewhere (41) and its modification.

A borate buffer was prepared by dissolving 0.0621 g of boric acid and 0.021 g of borax in water and making up the solution to 100 ml. This buffer was used to make a 0.5 percent solution of sodium chloride. In 100 ml of n-pentyl alcohol (Matheson-Coleman Bell 7232, P x 4) was added 1 milliliter of di-2-ethylhexyl phosphate (Stauffer Chemical Company). Equal volumes (100 ml each) of the sodium chloride solution and n-pentyl alcohol were mixed and the pH of the mixture was adjusted to 8.0 while stirring, with a few drops of 10 percent sodium hydroxide. Stirring was continued for another 30 minutes. After this period the mixture was allowed to settle in a separatory funnel and the two phases were separated. A suitably sized sheet of Whatman No. 20 chromatography paper was dipped in lower or aqueous phase, avoiding any contact with fingers. The paper was blotted between two sheets of Whatman No. 20 paper and the aqueous solutions of the samples referred to in Table 10 were applied onto the blotted sheet in small quantities. The sheet was developed by the descending method using the upper or organic phase. The developing time was about 8 hours. The sheet was removed from the

Table 10. Results of Descending Paper Chromatography Using Whatman No. 20

Compound	R_F Value
Crude β-Dihydrostreptomycin Trihydrochloride	0.63
Compound Resulting from N-Carbobenzoxylation of Crude β-Dihydrostreptomycin Trihydrochloride	0.72 and 0.61 (faint)
α-Dihydrostreptomycin Trihydrochloride	0.58 (streak)
Streptidine	0.79

tank, air-dried, and sprayed with alkaline α-naphthol-diacetyl color reagent (51). Brown-colored spots appeared that had R_F values listed in Table 10.

Chromatography of the Compound Resulting from N-Carbobenzoxylation of Crude β-Dihydrostreptomycin Using Sephadex G-15

Sephadex G-15 was soaked in an excess of 0.01 N formic acid for 16 hours before packing the column. The 16-foot column (internal diameter 1.9 cm) was slowly packed with the Sephadex slurry and allowed to settle for 16 hours. A solution of 2.830 g of partially carbobenzoxylated β-dihydrostreptomycin in 5 ml of 0.01 N formic acid was poured onto this column and the column was washed with the same solvent. Each fraction (10 ml) was tested with Weber reagent to detect any streptomycin derivative.

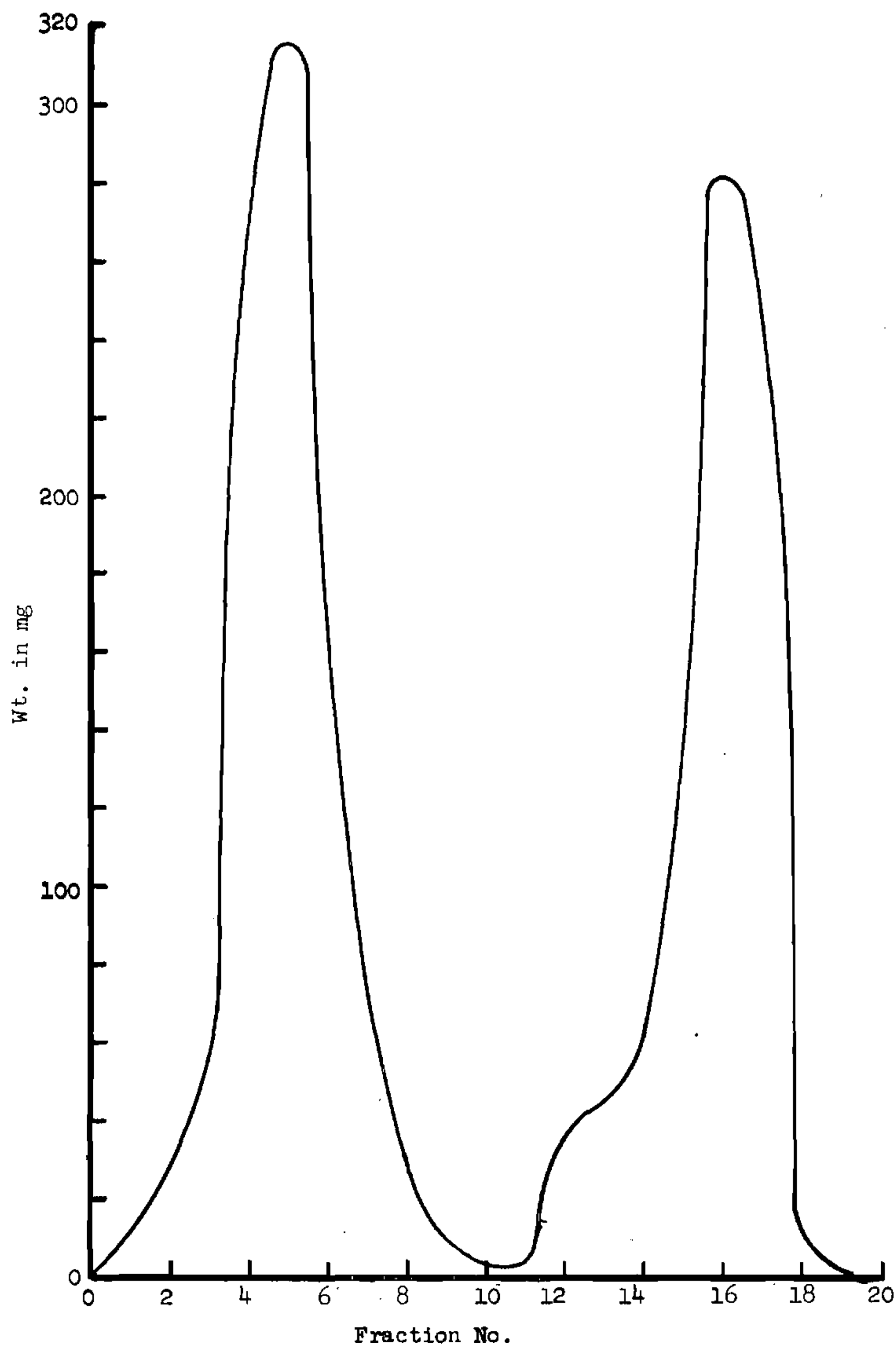


Figure 2. Plot of Fraction Number Versus Weight for Ion-Exchange Column Chromatography of Polybenzoyl Preparation Resulting from the N-Carbobenzoxylation of Crude β-Dihydrostreptomycin

Selected fractions were analyzed using microcrystalline cellulose plates in *t*-butyl alcohol:acetic acid:water (4:1:5). The results are shown in Table 11.

Table 11. TLC Analyses of Samples from Sephadex Column Chromatography of *N*-Carbobenzoxylated Crude β-Dihydrostreptomycin

Fraction Number	R_F Value
74	0.61, 0.85
79	0.61, 0.85
84	0.61, 0.85
89	0.69, 0.77, 0.85 (faint)
98	0.54, 0.69
103	0.46, 0.77
107	0.46, 0.77
114	0.46, 0.73
117	0.46, 0.73, 0.83 (faint)
122	0.46, 0.73, 0.83 (faint)

The n.m.r. spectra of random fractions revealed the presence of aromatic protons in all of them.

Benzoylation of the Compound Resulting from the N-Carbobenzoxylation of Crude β-Dihydrostreptomycin

The procedure for the benzoylation of α-dihydrostreptomycin has been described elsewhere (43). A modified version was used.

To 0.910 g (1.1 millimoles) of the N-carbobenzoxylated crude β-dihydrostreptomycin was added 3 ml of anhydrous pyridine. The mixture was stirred at 0° for about 20 minutes and then 2.310 g (16.5 millimoles) of benzoyl chloride was slowly added. Stirring was continued for an additional hour at ice bath temperature. Thereafter the slurry was stirred for 16 hours at room temperature, during which time it became brick-red in color.

The mixture was diluted with 0.5 ml of water, and 75 ml of chloroform was added. The supernatant dark-colored solution was removed and the chloroform extract was washed with saturated solution of sodium bicarbonate (3 x 20 ml). The pooled sodium bicarbonate solution was backwashed with chloroform. On concentrating the pooled dry chloroform solutions, a dark-colored semisolid having the odor of pyridine was obtained. Attempts to crystallize this material using chloroform/petroleum ether gave only an oily precipitate. Other solvents (acetonitrile, benzene, ethanol, etc.) also failed to give any crystalline material.

Ion Exchange Chromatography of the Polybenzoylated Preparation Resulting from the N-Carbobenzoxylation of Crude β-Dihydrostreptomycin

The polybenzoylated compound prepared above was dried *in vacuo*. A sample of 1.188 g was weighed out.

IRC-50 (H^{+} phase) Ion-exchange resin was packed in a column

(35 cm x 2.5 cm). The sample (1.188 g) was applied to the column using a small quantity of 6:1 acetone-water. The eluting solvent used was 6:1 acetone:water followed by 0.05 N-hydrochloric acid in the same solvent mixture. The weight versus number of the fraction is given in Figure 3.

The fractions eluted using the neutral solvent showed no sharp melting point and the n.m.r. spectrum indicated extensive decomposition. The material obtained from the acidic solvent was whitish, insoluble in acetone, soluble in water, and when heated, began to decompose at 250°.

Attempted Separation of Polybenzoyl Crude β-Dihydrostreptomycin on a Column of Ion-Exchange Resin Dowex 50 (H^+)

A sample of crude β-dihydrostreptomycin trihydrochloride (0.731 g, 1 millimole) was stirred with 40 ml of anhydrous pyridine at 0° for 1 hour. Thereafter, 2 ml (2.340 g, 16.5 millimoles) of benzoyl chloride was slowly added over a period of 5 minutes. Stirring was continued for another hour at 0° for 18 hours at room temperature, when the solution became dark red in color. It was diluted with 30 ml of chloroform and the chloroform extract was washed with a saturated solution of sodium bicarbonate until effervescence ceased (3 x 30 ml required). The chloroform solution was concentrated to a syrup under vacuum and the syrup was mixed with less than 1 milliliter of chloroform followed by a few drops of petroleum ether (bp 40-60°). On cooling the turbid mixture an oil separated. The supernatant solution was pipetted into another flask. The oily residue, after drying, showed the absence of any streptomycin derivative by n.m.r. analysis.

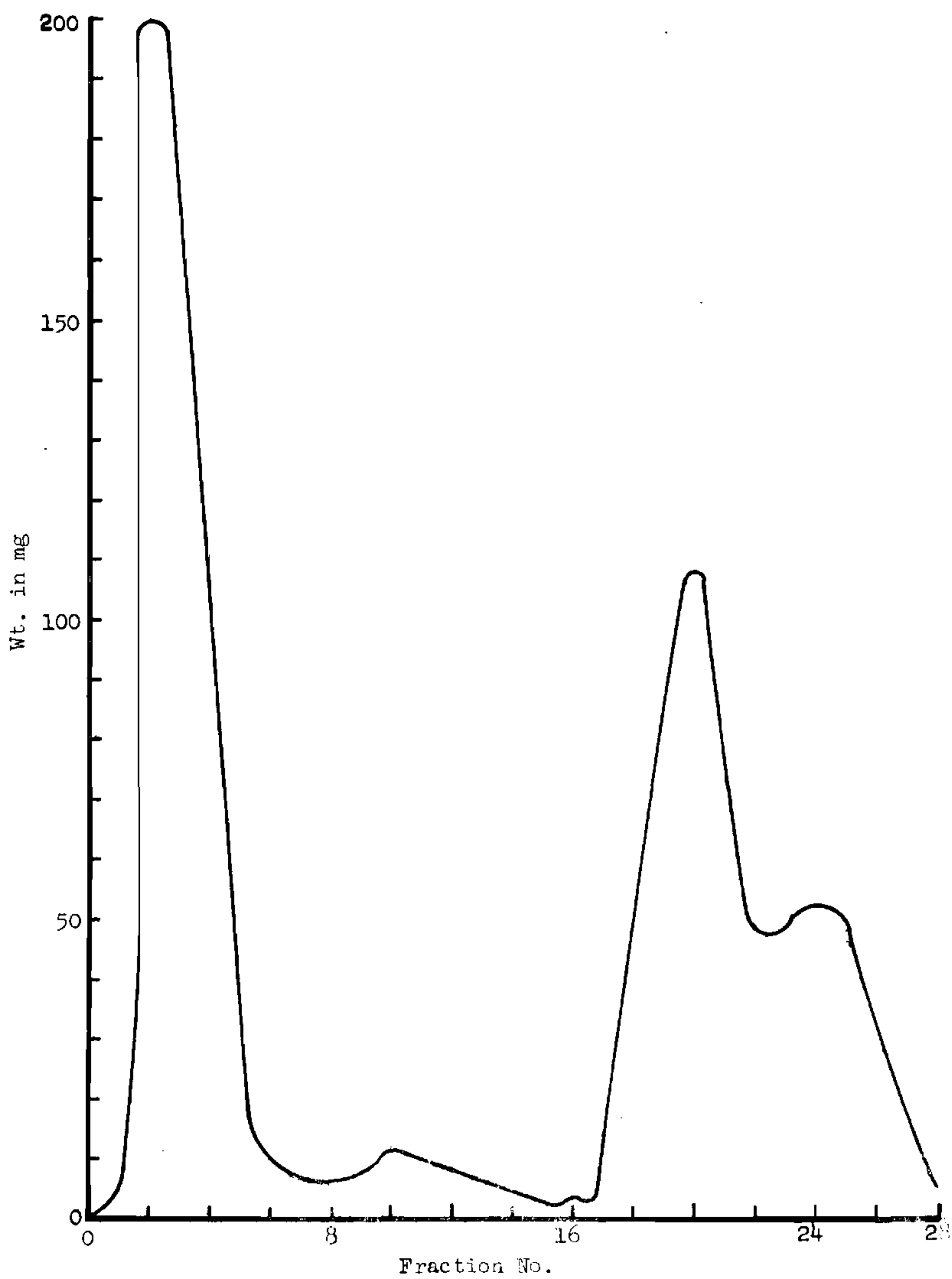


Figure 3. Plot of Fraction Number Versus Weight for Ion-Exchange Column Chromatography of Polybenzoyl Crude β-Dihydrostreptomycin

The supernatant solution dried in vacuo. The residue was dissolved in a mixture of actone and water (6:1) and passed over a column of ion-exchange resin IRC 50 (H^+ phase, 1.2 cm x 48 cm); a mixture of acetone and water (6:1) followed by 0.05 N hydrochloric acid in the same solvent mixture were the eluting solvents.

The graph (Figure 4) shows the relation between the weights and the fraction numbers.

The n.m.r. spectrum of fraction number 5 indicated the absence of any spectromycin derivative. The semi-crystalline material obtained after the evaporation of the neutral solvent melted at 120-124° (corr). The residue obtained from the evaporation of fractions eluted with acidified solvent showed non-conclusive n.m.r. spectrum.

The TLC analyses of random samples on silicic acid HF 254 using the solvents indicated are given in Table 12.

Table 12. TLC Analyses of Samples from Ion-Exchange Resin Chromatography of Polybenzoyl Crude β-Dihydrostreptomycin

	R_F Value in	
Fraction Number	Chloroform	Acetone
2	0.63, 0.37, 0.16 0.08, and 0.00	0.90, 0.75 and 0.00
5	0.63 (faint), 0.4 (faint), 0.16, 0.08, and 0.00	0.8, 0.37
10	0.16, 0.08, and 0.00	0.83, 0.16
19	0.08 and 0.00	0.2 (streak)
22	0.08 and 0.00	0.15, 0.00

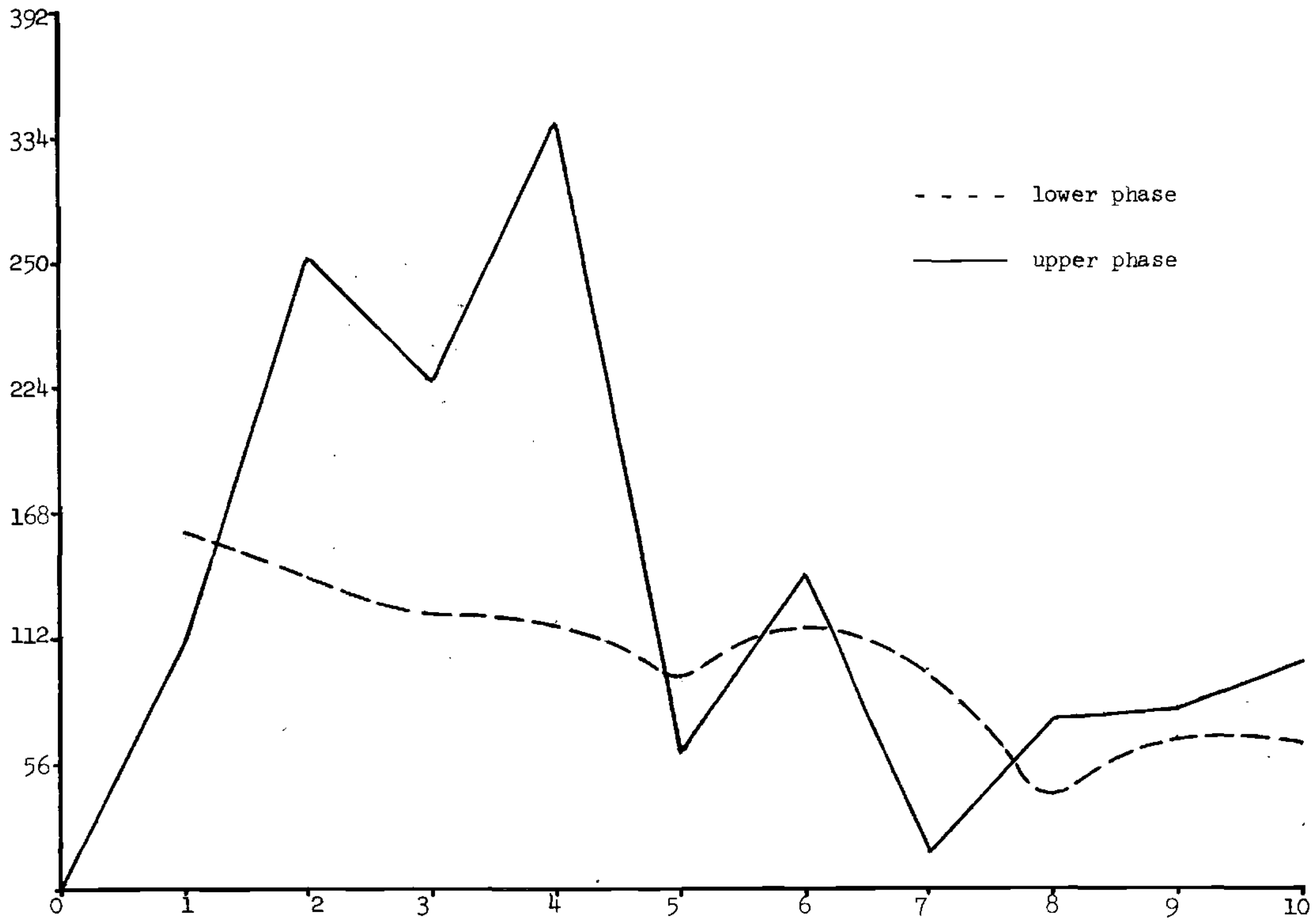


Figure 4. Plot of Fraction Number Versus Weight for Countercurrent Distribution of N-Carbobenzoxylated Crude β-Dihydrostreptomycin

Countercurrent Distribution Studies on the N-Carbobenzoxylated Crude β-Dihydrostreptomycin

Determination of the Distribution Coefficient

The solvent system used in this study consisted of 100 ml of redistilled 1-butanol and 100 ml of 5 percent p-toluenesulfonic acid monohydrate (Columbia Organic Chemicals) in distilled water. The two solvents were agitated for about 10 minutes in a 500-ml separatory funnel. A sample of 478 mg of the N-carbobenzoxylated crude β-dihydrostreptomycin was added to the separatory funnel and the mixture was once again thoroughly agitated. On standing the two layers separated. The lower aqueous layer was treated with ion-exchange resin IRC 45 (OH^- phase) to a pH of 5.6-6.0, the eluent was passed over a 50 ml column of IRC 45 (CI^- phase). The column was also washed with about 75 ml of water. The eluent was lyophilized and weighed 204 mg.

The upper (organic) phase was evaporated to about 10 ml and was treated with IRC 45 (OH^- phase) and IRC 45 (CI^- phase) as above. The lyophilized residue weighed 197 mg. The distribution coefficient was thus 0.97.

The n.m.r. spectrum of the organic layer residue showed only 15 percent carbobenzoxylation, compared with almost quantitative carbobenzoxylation of the residue from the water layer.

Separation of the N-Carbobenzoxylated Crude β-Dihydrostreptomycin Using Countercurrent Distribution

The solvent system consisted of an upper phase of 1 liter of 1-butanol equilibrated with 1 liter of 5 percent p-toluenesulfonic acid monohydrate. The two phases were separated.

A ten-tube countercurrent distribution apparatus (each tube

capacity 50 ml) was employed. A sample (2.615 g) of the N-carbobenzoxylated crude β-dihydrostreptomycin preparation was dissolved in the lower phase and extracted with the upper phase. Ten upper phase fractions, each measuring 50 ml, were collected and each fraction was concentrated to about 10 milliliters, neutralized with ion-exchange resin IRC 45 (OH^- phase), and finally passed over 25-30 ml columns of ion-exchange resin IRC 45 (CI^- phase). The fractions were lyophilized and the weights recorded. A graph relation between the number and the weight of the fraction is given in Figure 4.

The lower fractions were similarly concentrated, neutralized, and passed over ion-exchange resin IRC 45 (CI^- phase) columns, and lyophilized. The weights of the fractions are given in Figure 4.

The n.m.r. spectra of fractions 1 and 4 (lower phase) showed no aromatic protons, while fractions 2 and 8 (upper phase) showed quantitative N-carbobenzoxylation.

The TLC analysis of the fractions was performed using microcrystalline cellulose plates (0.25 mm thick) using a solvent system 1-butanol:acetic acid:water (4:1:5). Fractions 1, 3, 5, 7, and 9 of the lower and upper phases were analyzed. Two analyses were run for each fraction simultaneously, one of which was more highly loaded (three times the first application). The spots were visualized by spraying with Weber reagent. The R_F values are given in Table 13.

Characterization of Pure β-Dihydrostreptomycin Trihydrochloride

The β-dihydrostreptomycin trihydrochloride showed the following infrared absorptions (potassium bromide pellet).

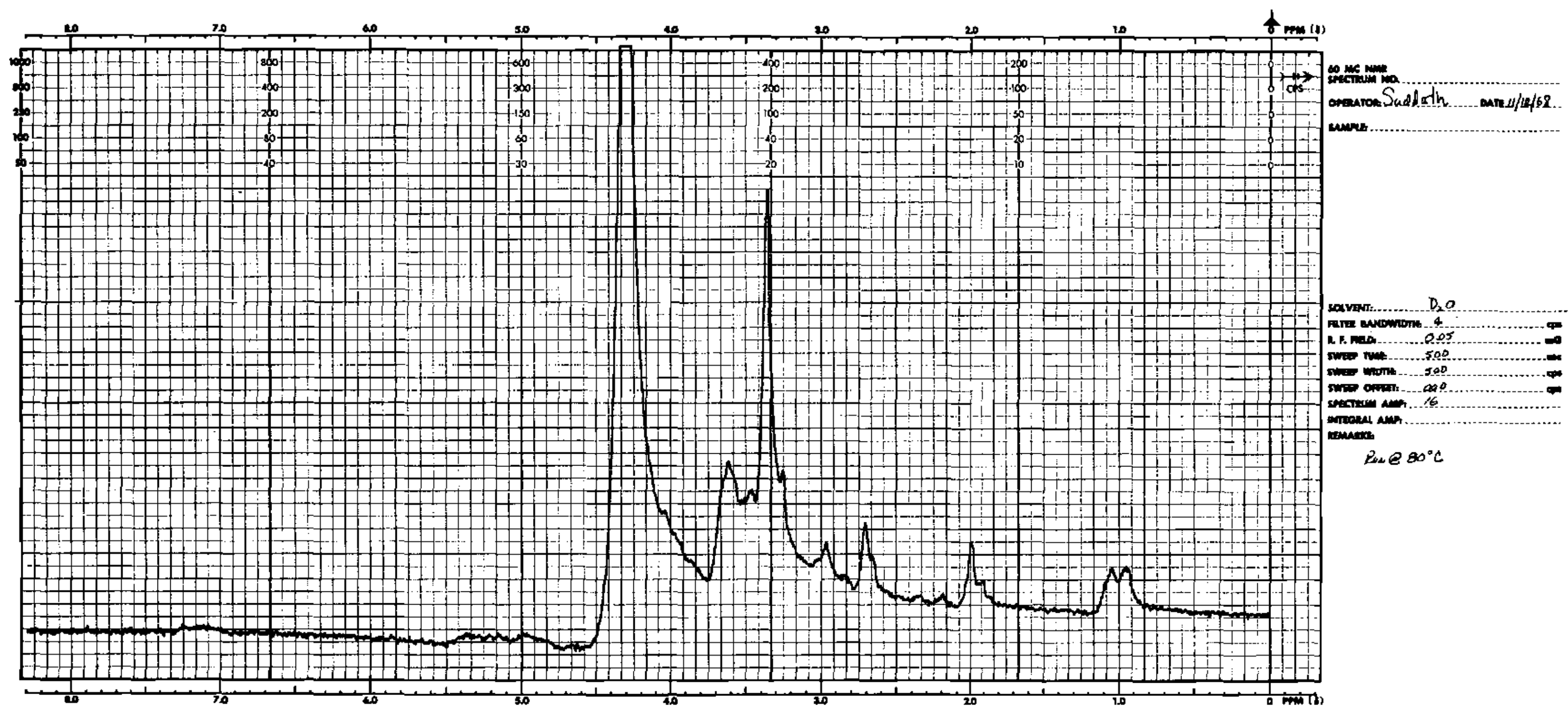


Figure 5. N.m.r. Spectrum of Pure β-Dihydrostreptomycin

Table 13. TLC Analyses of Fractions from Countercurrent Distribution of Preparation from N-Carbobenzoxylation of Crude β-Dihydrostreptomycin

N.B. A prime superscript indicated more highly loaded run.

Upper Phase

Fraction Number	R_F
1	0.25, 0.60
1′	0.21, 0.58
3	0.25, 0.53
3′	0.26, 0.58
5	0.27, 0.53
5′	0.23, 0.58
7	0.21, 0.52
7′	0.22, 0.52
9	0.25, 0.50
9′	0.22, 0.50

Lower Phase

Fraction Number	R_F
1	0.29
1′	0.29
3	0.31
3′	0.31
5	0.29
5′	0.29
7	0.28
7′	0.28
9	0.29
9′	0.24

λ_{max} 3.0μ (broad), 6.1μ(broad), 7.2μ (weak, broad), 7.95μ (sharp), 9.2μ, 9.8μ, and 12.5μ, among others.

The optical rotation was $[\alpha]_D^{25}$ = -23.3° (c 1.2, water) (lit. for α-dihydrostreptomycin trihydrochloride = -88.7° (c 1.0 water) (44).

Acid Hydrolysis of Pure β-Dihydrostreptomycin Trihydrochloride

A sample of β-dihydrostreptomycin trihydrochloride (141 mg; 0.2 millimole) was treated with 10 ml of 1N sulfuric acid at 45° for 8 hours. White crystals appeared at the end of this period. The mixture was cooled for 4 hours in the refrigerator. The crystals were filtered, dried, and weighed 69 mg (0.2 millimole). This material was identified as streptidine sulfate by comparison of its infrared spectrum with that of the authentic streptidine sulfate.

The filtrate, after neutralization with ion-exchange resin IRC 45 (OH^- phase) and elution over an ion-exchange resin, IRC 45 (Cl^- phase), was lyophilized immediately. The resulting pale yellow glassy solid showed no sharp melting point and weighed 68 mg. Using thin-layer chromatographic analysis on microcrystalline cellulose plate, employing a 1-butanol:acetic acid:water system in the ratio of 4:1:5, the solid gave a spot with an R_F of 0.35. A similar analysis with α-dihydrostreptobiosamine showed an R_F of 0.26.

CHAPTER III

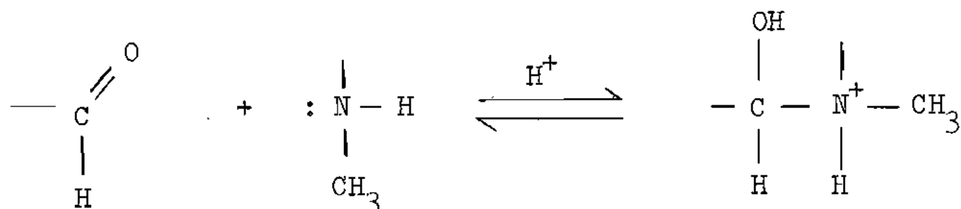
DISCUSSION

The purpose of this research was to elucidate the structural differences in the α- and β-streptomycin salts. The crystalline β-streptomycin salts have been obtained by precipitating streptomycin as the tergitate (4-ethyl-1-3′-ethylpentyloctyl sulfate; tergitol 7-anionic is its sodium salt). On "aging" at neutral pH and at 5° for an extended period of time, this precipitate could be crystallized. This salt has been converted to crystalline chloride and bromide salts. The differences in the solubility properties of the corresponding α- and β-streptomycin salts are striking. The β-streptomycin trichloride, unlike the α salt, is insoluble in methanol. The β-streptomycin sulfate is insoluble in water, whereas the α-streptomycin sulfate is readily water soluble. The aging of crude streptomycin tergitate at 5° proceeded satisfactorily and optimum yields of crystallized tergitate were obtained after five days. The tergitol-7 anionic did not produce any crystalline tergitates (β-tergitates) if it was added rapidly to the streptomycin sulfate solution. The tergitol-7 anionic also appeared to decompose when left at room temperature for five to six months, as indicated by the darkening of its color.

The potentially free aldehyde group in the streptose moiety of streptomycin cannot be detected spectroscopically, although the group has been reduced (to dihydrostreptomycin) and oxidized (to streptomycinic

acid (5,44). It had been suggested by earlier workers that this aldehyde group in streptomycin is involved in an internal hemiacetal linkage with one of the various hydroxyl groups or nitrogens of aminomethyl or guanidino functions. Young suggested that the α and the β forms of streptomycin are tautomers, and also that the most weakly basic group, i.e., the $-NH-CH_3$ group, may be concerned in the "tautomerism" (26). He, however, recorded no experimental evidence to substantiate this suggestion.

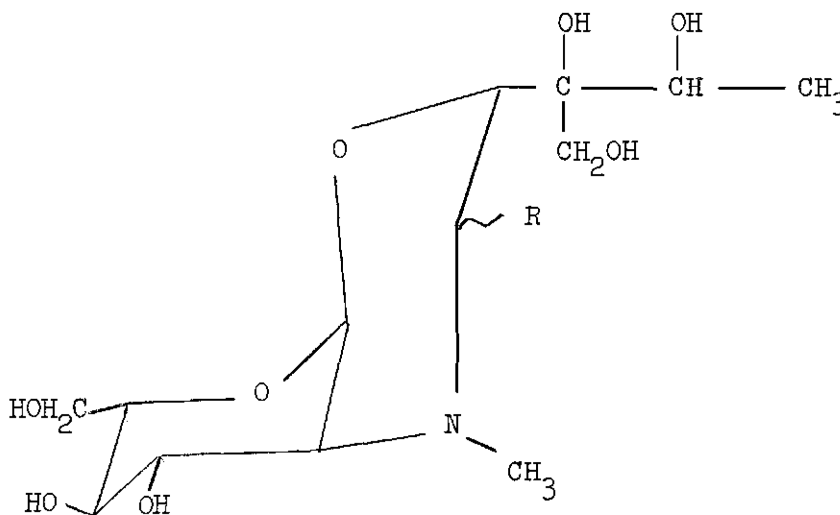
Since the β- salts are more stable in acidic medium, it seems reasonable to believe that they possess the N-hemiacetal structure as proposed by Young.



In acid solution, the approach of hydronium ion toward the hemiacetal carbon atom is hindered because of the positive charge on the nitrogen. This would prevent the hydrolysis of the N-hemiacetal linkage.

If such an N-hemiacetal structure was present in β-streptomycin

salts, catalytic hydrogenation studies should provide useful information. If the C-N bond were hydrogenolyzed, α-dihydrostreptomycin would result; if the C-O bond were hydrogenolyzed, a new tertiary amine (an oxazepine) would result. An example of this is the hydrogenation of dihydrostreptobiosamine hydrochloride which results in absorption of one mole of hydrogen. The product, when acetylated, did not give the expected heptaacetyltetrahydrostreptobiosamine, but a base hydrochloride with the molecular formula $C_{23}H_{20}O_8N(CH_3CO)_5 \cdot HCl$. The hydrogenated product, prior to acetylation, could not be hydrolyzed to N-methyl-L-glucosamine and was also found to be non-reducing (30). The reduced product was assigned the morpholine structure IX.

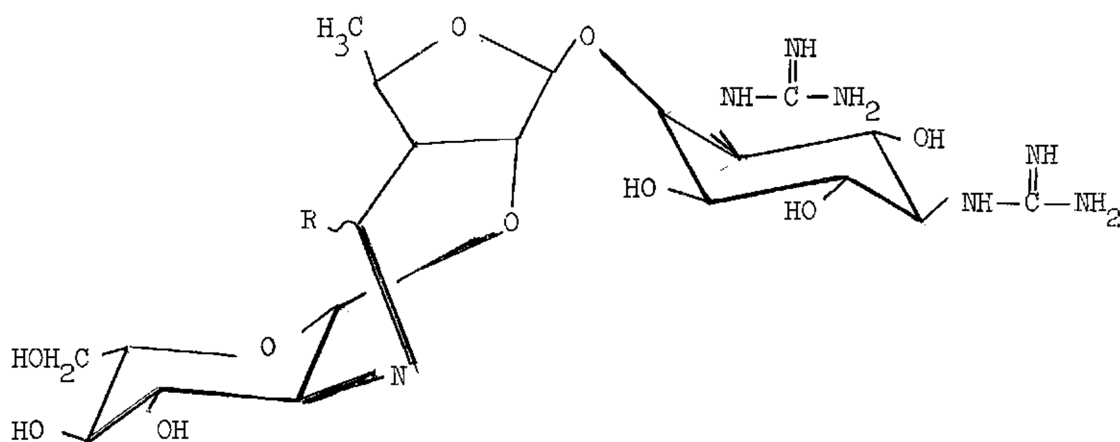


R = -H IX

R = -OH X

Prior to hydrogenolysis, the dihydrostreptobiosamine was assigned a similar structure X. The hydrogenolysis of the N-hemiacetal linkage proceeded selectively, cleaving the carbon-oxygen bond preferentially over the carbon-nitrogen bond.

On the basis of this analogy and the earlier suggestion by Young, if the β-streptomycin salts have the N-hemiacetal structure, on catalytic hydrogenation they would be expected to yield a tertiary amine. Examination of Dreiding models of the streptomycin molecule reveals that N-hemiacetal formation with any of the guanidino nitrogen atoms results in a strained ring with severe non-bonding interactions. N-Hemiacetal formation with the amino-methyl nitrogen results in no unusual interactions or strain; a seven-membered ring results. The structure of β-streptomycin salts after hydrogenolysis would be a tertiary amine (VII, R = H) and not dihydrostreptomycin.



R = OH VI

R = -H VII

When subjected to acid hydrolysis, streptidine and not _N_-methyl-_L_-glucosamine should be the hydrolysis product. Similarly, if there is a hemiacetal linkage between the aldehyde group of streptose and any nitrogen of the guanidino groups of streptidine (non-bonding interaction not withstanding), the catalytic hydrogenation should be expected to yield a compound containing a disubstituted guanidino group. On acid hydrolysis, such a compound would be expected to yield _N_-methyl-_L_-glucosamine.

The most conclusive approach toward the solution of this problem would be to establish the structure of a crystalline β-streptomycin salt by X-ray diffraction studies. Attempts were at first directed toward a trihydroiodide salt of β-streptomycin which had not been reported in the literature. Attempts to prepare this salt by mixing a methanolic solution of calcium iodide and a concentrated methanolic solution of crystalline β-streptomycin tergitate proved futile, even when the mixture was kept at 0° for up to six months. A slight variation, employing sodium iodide in place of calcium iodide, met with a similar fate. The trihydrobromide salt had been reported in the literature and was used for X-ray diffraction studies. This salt gave rise to a diffuse pattern and it has been suggested that these crystalline salts are, in effect, liquid crystals, and hence unsuitable for X-ray diffraction studies (45).

Hydrogenolysis of β-streptomycin trihydrochloride, using freshly regenerated platinum oxide or 5 percent platinum on carbon as the catalyst and 75 percent acetic acid as the solvent, gave rise to a glassy white material after absorption of one equivalent of hydrogen.

This substance had no sharp melting point, and its specific rotation varied from preparation to preparation. However, it exhibited no mutarotation, gave a negative maltol test, and a negative Fehling test. On acid hydrolysis it gave a quantitative amount of streptidine and a water soluble basic compound. The latter, in the form of its O- and N-trimethylsilyl ethers, by gas liquid chromatography, showed two major peaks in the ratio of about 1:3. The trimethylsilyl ethers of dihydro-streptobiosamine, obtained from α-dihydrostreptomycin on similar analysis showed only one major peak, which had the same retention time as the smaller peak in the first case.

Further attempts were directed at establishing the separate entity of the hydrogenolysed β-streptomycin, hereinafter referred to as the crude β-dihydrostreptomycin preparation.

The hypothesis of the N-hemiacetal formation between the aldehyde group and the aminomethyl nitrogen could be further confirmed if the hydrogenolyzed product was subjected to carbobenzoxylation under conditions where only the aminomethyl nitrogen of α-dihydrostreptomycin is carbobenzoxylated. The procedure for this N-carbobenzoxylation was established using α-dihydrostreptomycin. The pH of the reaction was maintained between 8.5-10.0 by addition of ion-exchange resin IR-400 (OH^- phase). This solution of α-dihydrostreptomycin was next treated with about a five-fold excess of carbobenzoxy chloride (solvent free) for about 2 hours. After filtering the resin and removing the excess carbobenzoxy chloride by extraction with ether, the aqueous solution was converted to the chloride form by means of ion-exchange resin IRC 45 (CI^- phase; washed to pH 3.5-4.0). After lyophilization, a glassy white

solid resulted. The extent of carbobenzoxylation was determined by analysis of the n.m.r. spectrum of the product. The area of the peak given by the protons in the aromatic region ($\tau 2.7$) and that of the C-methyl doublet ($\tau 8.9$) were in the ratio of 5:3, showing that essentially quantitative N-carbobenzoxylation of α-dihydrostreptomycin had resulted.

When the crude β-dihydrostreptomycin was subjected to an exactly similar procedure for N-carbobenzoxylation, the extent of this N-carbobenzoxylation varied from preparation to preparation. In the initial stages of this work, using 5 percent platinum on carbon as the catalyst and 70 percent acetic acid as the solvent, 18 to 32 percent N-carbobenzoxylation resulted from the crude β-dihydrostreptomycintrihydrochloride, thus, 82 to 68 percent of the β-streptomycin had been converted to β-dihydrostreptomycin. This result indicates that β-streptomycin has structure VI and β-dihydrostreptomycin has structure VII.

In the latter stages of this work, the catalyst (5 percent platinum on carbon) gave a product containing almost no β-dihydrostreptomycin (as indicated by almost quantitative N-carbobenzoxylation). Various other catalysts were used for this hydrogenolysis, but only platinum oxide proved satisfactory and gave 50 to 55 percent conversion to the β form. All these percentages were, of course, calculated by analyses of the n.m.r. spectrum of the carbobenzoxylated product by a comparison of the areas of the peaks corresponding to the C-methyl group and the protons in the aromatic region.

Various crystalline salts of this preparation were prepared, and their physical properties were studied and compared with those of the corresponding salts of α-dihydrostreptomycin. No significant differ-

ences were observed in the melting points or crystalline properties. Purification of the crude β-dihydrostreptomycin was attempted first by converting it to a crystalline trihelianthate salt and then converting it back to the trihydrochloride by treatment with suitable ion-exchange resins. This procedure also did not give a pure β-dihydrostreptomycin (by n.m.r. analysis).

Acetylation of the crude β-dihydrostreptomycin would be expected to yield a mixture containing fully acetylated (hence neutral) α-dihydrostreptomycin and fully acetylated (but basic, due to the unacetylated tertiary amine) β-dihydrostreptomycin. It thus might be possible to separate the two on a column of a suitable ion-exchange resin. The product obtained after acetylation was observed to be a complex mixture by TLC analysis. This approach was, therefore, abandoned.

Benzolyation of the preparation did yield a mixture that was comparatively less complex. This product on elution using water-acetone over a column of ion-exchange resin IRC-50 (H^+ phase) yielded a white residue, which on analysis by n.m.r. was found to be mainly benzoic acid.

Since the preparation of β-dihydrostreptomycin contains two components that are significantly different in structure, it might be possible to separate them using a sufficiently long Sephadex column. A portion of this preparation, when eluted over the Sephadex G-15 column (475 cm tall and 1.9 cm in diameter) with 0.013 M formic acid, did not show any separation. All the fractions showed protons in the aromatic region in their n.m.r. spectra. Only one peak was observed in the graph of mass versus fraction number. Another attempt was made with a similar column but using the N-carbobenzoxylated β-dihydrostreptomycin preparation.

The peak this time was observed to be broad; no separation of the components was achieved.

Various other chromatographic techniques were next used to attempt this separation. Using silica gel as the adsorbant and aqueous solutions of sodium acetate and ammonium acetate appeared to be a desirable solvent system (46). However, employing various concentrations of the salts resulted in no separation for the β-dihydrostreptomycin preparation or the N-carbobenzoxylated form of this preparation. Using paper chromatography (Whatman No. 1) and 1-butanol:acetic acid:water (2:1:1) as the solvent, the β-dihydrostreptomycin preparation showed two spots. When TLC plates using normal cellulose were used, the same solvent system again showed two spots. The separation of this preparation on a cellulose column under pressure was attempted. The mass versus fraction number graph did show two peaks but all fractions showed protons in the aromatic region in their n.m.r. spectra, thus indicating that no separation had been achieved. When the N-carbobenzoxy-β-dihydrostreptomycin preparation was chromatographed on a similar cellulose column, no separation was achieved.

Another attempt to separate this mixture was made using microcrystalline cellulose which had been reported to be very effective for separating disachharides, amino acids, etc. (47). Using 1-butanol:acetic acid:water (4:1:5), two spots were observed, one of which was more intense than the other. When this analysis was carried out on a preparative scale, the spots were not well separated and were diffuse. Both spots showed protons in the aromatic region of their n.m.r. spectra, although not to the same extent. This approach was abandoned.

Only partition methods had given any indication of separating to any extent the N-carbobenzoxylated form of crude β-dihydrostreptomycin preparation. A study of the partition coefficient of the above preparation between two immiscible liquid phases was undertaken. The mixture 1-butanol and 5 percent aqueous solution of p-toluenesulfonic acid (which had been pre-equiliberated) was selected as the liquid system for these studies. A sample that had only 15 percent conversion to the β-form when partitioned between these solvents showed a distribution coefficient of 0.97. A more significant observation was that the material recovered from the aqueous phase showed the presence of 75 percent of the β-form, while the material from the 1-butanol layer had no β-form at all.

When this partition was carried out using a Craig countercurrent distribution apparatus, the fractions from the lower phase showed no protons in the aromatic region by their n.m.r. spectra and showed only one spot by microcrystalline cellulose TLC plates using 1-butanol:acetic acid:water (4:1:5) as the solvent system. The butanol layer fractions showed protons in the aromatic region (n.m.r. spectra) and two spots by TLC analyses.

The pure β-dihydrostreptomycin obtained by this separation, upon acid hydrolysis, yielded streptidine sulfate which was identified by its solubility behavior, by comparison of its R_F value with that of authentic streptidine sulfate, and by a comparison of the infrared spectra of the two.

The other product of hydrolysis, β-dihydrostreptobiosamine, when compared with authentic α-dihydrostrepbiosamine, showed different R_F values in at least one solvent system. The optical rotation of pure

β-dihydrostreptomycin was observed to be -23.2° (c 1.2 water); α-dihydrostreptomycin has a specific rotation of -88° (c 1.0 water) (44). A sample of crude β-dihydrostreptomycin (n.m.r. assay of its N-carbobenzoxy form revealed 25 percent of α-dihydrostreptomycin) was found to be 30 percent as active as a streptomycin standard, against *Bacillus subtlis* in vitro. It suggested that the pure β-dihydrostreptomycin had a low order of antibacterial potency against *Bacillus subtlis*. Both streptomycin and dihydrostreptomycin have equivalent potency values against this organism (48). Pure β-dihydrostreptomycin, when tested against a sensitive strain of *B. subtlis* and *S. pyrogenes* showed no biological activity. Alpha-dihydrostreptomycin showed marked activity against these bacteria (49).

Suggestions for Further Work

The X-ray diffraction pattern of β-streptomycin sulfate has been reported to be different from that of the corresponding bromide. It is suggested that a selenate of β-streptomycin be used for X-ray diffraction studies of this molecule. This study would also confirm the stereochemistry of N-H bond in the oxazepine part of the molecule.

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VITA

Rajender Kumar Chawla was born on November 26, 1938 in Lahore, Pakistan. He attended various elementary and secondary schools in Lahore, Pakistan and New Delhi, India. He graduated from Doaba College, Punjab University, Jullundur, India in 1955. He worked at the Central Research Institute, Kasauli, India as a technical supervisor for three years and at the Ministry of Education and Scientific Research, Government of India, New Delhi, India for another three years. In September, 1962 he entered the School of Chemistry of the Graduate Division at the Georgia Institute of Technology, Atlanta, Georgia, and was awarded the degree of M.S. in June, 1965. Since September, 1968, he has been working as an Assistant Professor of Chemistry at Clark College, Atlanta, Georgia.

He is married and has two children.