[CONTRIBUTION FROM THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH]

Streptomycin Purification and Crystallization[1]

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Methods have been developed for the purification of streptomycin through salt formation with wetting agents of the sodium alkyl sulfate type. The simple mineral acid salts of streptomycin have been obtained in crystalline form through metathesis of these wetting agent salts with the appropriate mineral acid which constitutes the only known practical method for their crystallization. An analysis of the methods necessary for crystallization together with certain properties of the crystalline salts has led to the conclusion that streptomycin exists in distinct tautomeric forms.

Introduction

Many general reagents such as picric acid, phosphotungstic acid and Reinecke acid[2] have been used as precipitants in the purification of the basic antibiotics. Although some of these reagents form crystalline salts with streptomycin, no practical method has been reported for their conversion to crystalline streptomycin salts of the simple mineral acids. It has now been found that commercially available wetting agents such as the Duponols and Tergitols may also be used as precipitants in the purification of basic antibiotics; and it has been found that metathesis of these wetting agent salts provides an excellent means for the preparation of crystalline streptomycin mineral acid salts. A comparison of the physical and chemical properties of the amorphous and crystalline forms of streptomycin salts is presented. One of the crystalline salts, the trihydrochloride, has been announced by us in a previous communication.[3]

Precipitation of Streptomycin Alkyl Sulfate.—Of the various alkyl sulfates tested in the purification of streptomycin Tergitol-7, a sodium sulfate derivative of 3,9-diethyltridecanol-6 in 25% aqueous solution,[4] has been found most suitable for our purposes. Although difficulty was experienced in obtaining a precipitate in consistently good yields from filtered fermentation broths, partially purified broths gave satisfactory results. At a *p*H below 8.5, preferably at 7.0–7.5, the antibiotic was found to be precipitated as its trialkyl sulfate. The amount of wetting agent necessary for complete precipitation was found to be somewhat critical and varied with the purity of the streptomycin solution. An excess of precipitant was avoided because of its solubilizing effect upon the precipitate. Normally, 1 ml. of commercial Tergitol-7 solution was required per 100,000–150,000 micrograms[5] of streptomycin, the end-point being determined by sampling and testing for complete precipitation with additional reagent or by chemical assay. The course of the precipitation also could be followed by conductivity or surface tension measurements.

The crude Tergitol-7 salt of streptomycin, which we shall henceforth call the tergitate, was readily soluble in benzene, ether, chloroform, alcohols and aqueous acetone.[6] After standing a few days it could be crystallized from acetone, ethyl acetate, or aqueous methanol.

With the exception of the alcohols, the crystalline tergitate, in contrast to the crude material, was insoluble or difficultly soluble in most organic solvents. The solubility could be increased by the addition of 5–10% water.

Recovery of Streptomycin from Streptomycin Alkyl Sulfate.—A variety of methods have been developed for the preparation of streptomycin hydrochloride and sulfate from the alkyl sulfate salts.[7]

Some of these methods, although yielding streptomycin of high purity, either required conditions for metathesis resulting in some decomposition of the streptomycin or added traces of undesirable impurities. It soon appeared that one of the simplest and best procedures to effect metathesis was by the addition of the inorganic acid to a solution of streptomycin alkyl sulfate in the presence of an anion-exchange resin which served to control the acidity of the system, thus permitting metathesis at a more favorable *p*H. Precipitation of crude streptomycin in aqueous solution and recovery by this method was found to increase potency from an original 100–300 γ/mg. to 650–750 γ/mg.

Crystallization of Streptomycin Salts.—Further experimentation on the recovery of streptomycin from its tergitate led to the preparation of crystalline salts. The trihydrochloride, trihydrobromide and trinitrate could be prepared by dissolving the tergitate in methanol and adding a dilute methanolic solution of the appropriate acid or calcium salt.[8]

In the reaction of methanolic calcium chloride upon streptomycin tergitate in methanol only streptomycin trihydrochloride was obtained. There was no evidence of the formation of the calcium chloride double salt obtained by others[9] by the action of methanolic calcium chloride upon streptomycin helianthate.

The trihydrohalides crystallized as monoclinic prisms and the trinitrate as regular tetrahedrons. These salts were recrystallized from methanol in

(1) Presented, in part, before the American Chemical Society at Chicago, September 4, 1950.

(2) J. Fried and O. Wintersteiner, *Science*, **101**, 613 (1945).

(3) L. J. Heuser, M. A. Dolliver and E. T. Stiller, THIS JOURNAL, **70**, 2833 (1948).

(4) Manufactured by Carbide and Carbon Chemicals Corporation, New York, N. Y.

(5) The bioassays in this paper are based on the F.D.A. working standard for streptomycin.

(6) The relative organophilic properties of streptomycin and mannosidostreptomycin tergitate were utilized in their separation through countercurrent distribution by A. E. O'Keeffe, M. A. Dolliver, and E. T. Stiller, THIS JOURNAL, **71**, 2452 (1949).

(7) W. A. Lott, J. Bernstein and L. J. Heuser, U. S. Patents 2,537,933 and 2,537,934 (1951).

(8) Mannosidostreptomycin trihydrochloride also was crystallized by these methods.

(9) R. L. Peck, N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn, A. Walti and K. Folkers, THIS JOURNAL, **67**, 1866 (1945).

low yield because of the narrow solubility differential in hot and cold solvent. Recoveries of 70–90% were realized when the crystalline trihydrochloride, for example, was dissolved in water at high concentration and the solution diluted with methanol or methanolic calcium chloride. It was noted, however, that if the aqueous solutions were heated or allowed to stand, even at 5°, before dilution, a negligible yield of crystals was obtained.

The pure halides and nitrate showed only a gradual decomposition when heated on a hot stage. In analyses, ultraviolet and infrared absorption spectra, and optical rotation, the crystalline materials were similar to the pure amorphous salts. The bio-activity of a several times recrystallized sample of the trihydrochloride, in a series of 66 assays, was found to be 923 γ/mg. which is equivalent to 1090 γ/mg. for the free base.

Crystalline streptomycin sulfate and phosphate, which are insoluble in methanol, were prepared by dissolving streptomycin tergitate in butanol and mixing the rich solvent with an aqueous solution of the appropriate acid or of an amine salt of that acid such as guanidine sulfate or phosphate. Crystalline streptomycin sulfate, being insoluble in water saturated with butanol, appears immediately in the aqueous phase of the mixture. The phosphate required the addition of methanol to the aqueous phase for crystal formation.

Streptomycin sulfate and phosphate could be recrystallized from aqueous solutions containing excess sulfate or phosphate ions. The crystalline sulfate, difficultly soluble in water alone, was readily soluble in water maintained at a neutral *p*H but could not be recrystallized after acidification of this neutral solution. A slurry of the crystalline sulfate in water showed no equilibrium between the crystalline form and the dissolved salt, the crystals gradually dissolving with time as shown in Fig. 1.

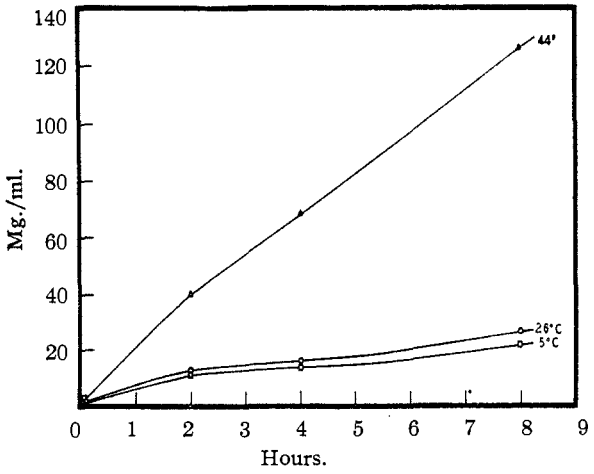

Fig. 1.—Solubility of crystalline streptomycin sulfate in water.

The crystalline form of streptomycin sulfate and phosphate depended on the method used for crystallization. Usually, the sulfate was obtained as rectangular prisms and the phosphate as hexagonal prisms. Analyses of the crystalline salts agreed with the values calculated for the sesquisulfate and sesquiphosphate. Optical rotation and absorption spectra in ultraviolet and infrared were the same as for the pure amorphous salts.

The crystalline inorganic salts of streptomycin, like the crystalline tergitate, were obtained in good yield from crude tergitate only after aging. As shown in Fig. 2, an aging period of at least one week at 5° was necessary for the maximum yield of crystalline streptomycin sulfate. The optimum aging period was shortened when it was carried out at higher temperatures.

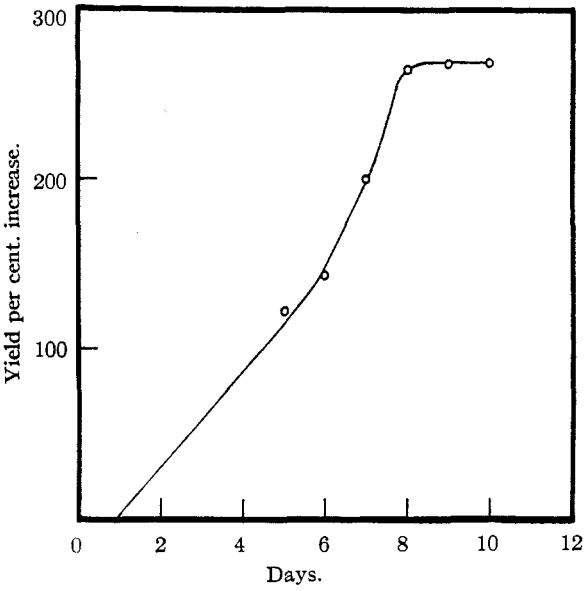

Fig. 2.—Effect of tergitate aging on the yield of crystalline streptomycin sulfate.

Tautomerism of Streptomycin Salts.—The failure to obtain crystalline streptomycin salts from crude tergitate, unless aged, and the inability to recrystallize a once crystallized salt which had stood in aqueous solution led us to believe that we were dealing with tautomeric forms of streptomycin.

Titus and Fried[10] have found evidence of tautomers in countercurrent distribution studies of amorphous streptomycin in the system butanol–aqueous *p*-toluenesulfonic acid. They demonstrated that the tautomeric shift was influenced by *p*H, an alkaline *p*H favoring conversion to that tautomer having a high distribution constant.

A comparative study of the distribution constants of amorphous and crystalline streptomycin trihydrochloride in the system butanol–*p*-toluenesulfonic acid supports the hypothesis of tautomerism. The configurational instability of the crystalline tautomer in aqueous solution, the stabilizing effect of hydrochloric acid, and the still greater stabilizing effect of the sulfonic acid are shown (Table I) by the increasing value of K[11] for the crystalline trihydrochloride as the solid is first dissolved in water or 0.01 N hydrochloric acid before introduction into the butanol–toluenesulfonic acid system, or dissolved directly in the sulfonic acid phase. It is apparent that the amorphous

(10) E. Titus and J. Fried, *J. Biol. Chem.*, **174**, 57 (1945).

(11) Determined by spectrophotometric assay similar to that published by G. P. Mueller, THIS JOURNAL, **69**, 195 (1947).

TABLE I

DISTRIBUTION CONSTANTS OF STREPTOMYCIN TRIHYDROCHLORIDE TAUTOMERS

	Solvent		
K for	H_2O	0.01 *N* HCl	5% *p*-$CH_3C_6H_4SO_3H$
Crystals	1.30	11.1	15.0
Amorphous	0.97	1.05	1.01

form, retaining essentially the same value for *K*, is stable under these conditions.

The rate of change in these solvents from the crystalline form to the amorphous form was established by allowing the respective solutions to stand and withdrawing samples periodically for the determination of *K*. The results (Fig. 3) showed that complete conversion occurred in water within the time necessary for solution and distribution. The stabilizing effect of the sulfonic acid and the lesser effect of hydrochloric acid are clearly indicated.

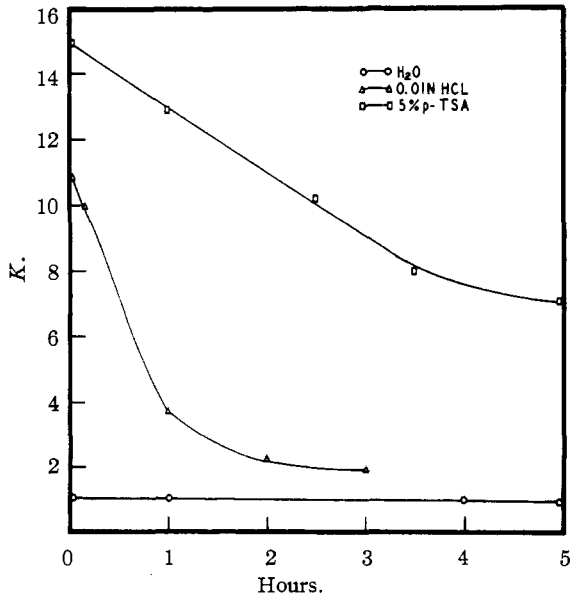

Fig. 3.—Effect of solvent on the rate of change in *K* for crystalline streptomycin trihydrochloride in the system butanol–*p*-toluenesulfonic acid at 26°: O—O, H_2O; Δ—Δ, 0.01 *N* HCl; □—□, 5% *p*-TSA.

Further evidence of tautomerism and tautomeric change with time together with an insight into the nature of the tautomerism was obtained by a comparison of the polarograms recorded from solutions of crystalline and amorphous streptomycin trihydrochlorides (Fig. 4). The salts were dissolved at 10^{-4} molar concentrations in 90% methanol containing 1% lithium chloride as a supporting electrolyte. Polarograms, taken at the time intervals indicated, show first a single wave, then the development of a second wave and a gradual approach to the double-wave polarogram recorded for the amorphous tautomer. Crystalline calcium chloride double salt of streptomycin trihydrochloride gave the double-wave polarogram immediately upon solution.

The reverse change, from a double-wave to a single-wave polarogram, was encountered in a study of the tergitate. Polarograms of freshly precipitated tergitate, of the same tergitate after aging at 5°, and of crystalline tergitate were recorded from a 2% solution in 95% methanol containing 1% lithium chloride (Fig. 5). The trend toward the single wave obtained for the crystalline material is definite but not complete.

There appears to be a demonstrable equilibrium in this transformation since even after an eleven day aging period, the tergitate still showed a slight second polarographic wave indicating incomplete change, within the time of observation, to the

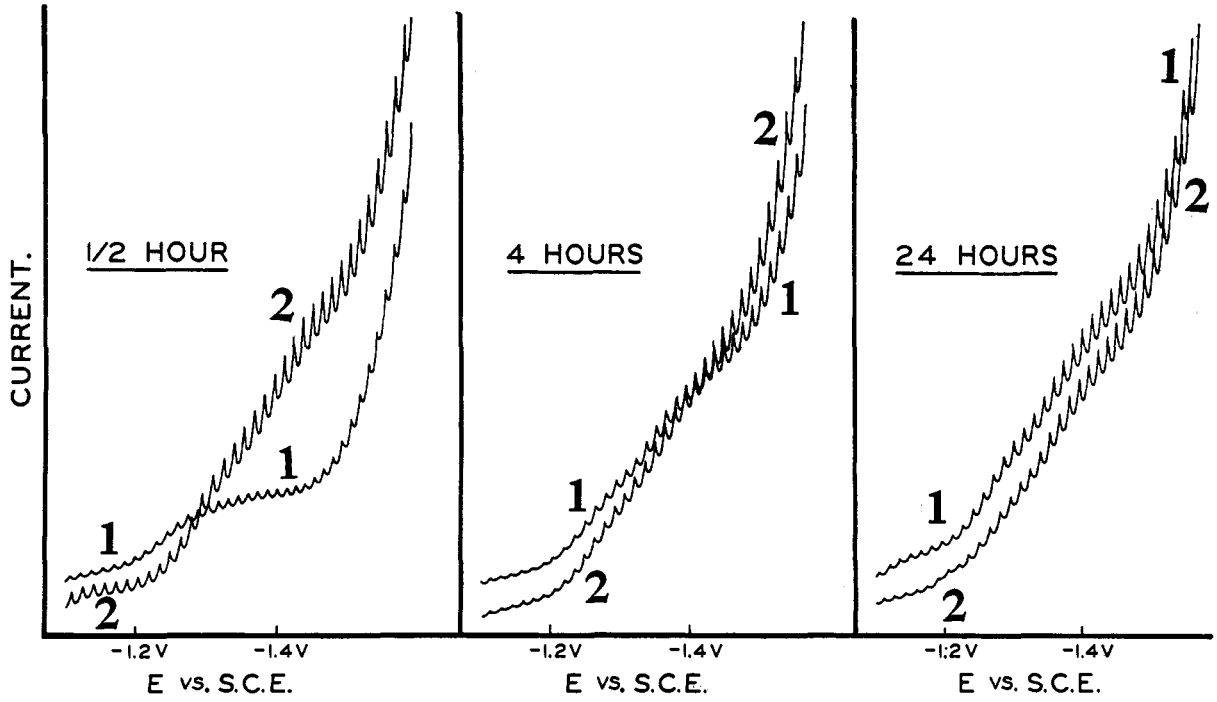

Fig. 4.—Polarograms of crystalline streptomycin trihydrochloride (1) and amorphous streptomycin trihydrochloride (2).

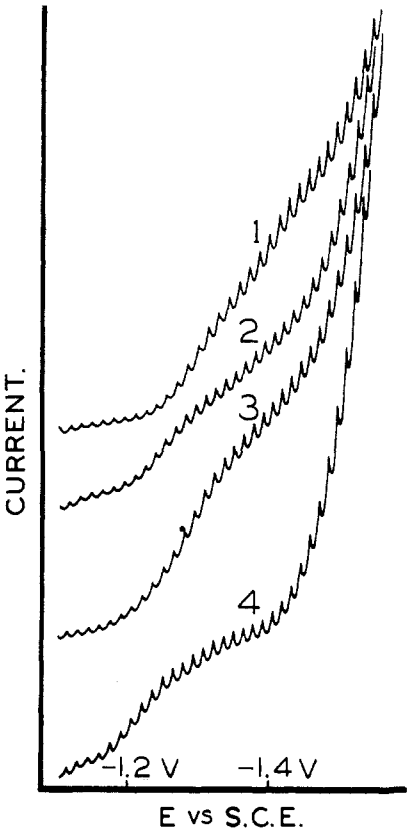

Fig. 5.—Streptomycin tergitate polarograms: 1, freshly precipitated; 2, after 3 days at 5°; 3, after 11 days at 5°; 4, crystalline tergitate.

crystalline form. Furthermore, the yield of crystalline streptomycin sulfate increased with aging time to a maximum of 60–70% at eight days (Fig. 2) and remained constant at that level, whereas the crystalline tergitate was found to give a quantitative yield of crystalline streptomycin sulfate.

These tautomeric transformations are attributed to changes in the nature of the aldehyde group of the streptomycin molecule since they could not be demonstrated with dihydrostreptomycin salts. Bricker and Vail[12] found that streptomycin exhibits a single-wave and a double-wave polarogram depending upon the *p*H of the solution being recorded. They consider the first wave, which occurs alone at *p*H 12.5 or higher, to be due to the reduction of a free aldehyde group and the second wave to the reduction of an aldehyde group bound by one or both of the protonated guanidino groups.

Accepting this interpretation in view of the similarity of the polarograms obtained, we conclude that of the tautomeric streptomycins, only that one having a free aldehyde group may be crystallized as the salt of an inorganic acid. The ready conversion of the crystalline modification to the non-crystalline form explains the abnormalities encountered in the crystallization and recrystallization of these salts.

Acknowledgment.—We wish to express our appreciation to Mr. J. Alicino for the analyses, Dr. N. Coy for the polarograms, Mr. F. Russo-Alesi and Mr. D. Lapedes for the chemical and biological assays, and Mr. C. F. Anderson for his assistance in the experimental details.

(12) G. E. Bricker and W. A. Vail, THIS JOURNAL, 72, 585 (1951).

Experimental

Preparation of Streptomycin Tergitate.—To 10 liters of a solution of streptomycin hydrochloride at a concentration of 3000 μg./ml., 230 ml. of Tergitol-7 was added slowly with agitation maintaining a *p*H of 7.0–7.5 by the occasional addition of dilute hydrochloric acid. The mixture was allowed to stand overnight at 5° and was then filtered. After drying under vacuum, 96.5 g. of streptomycin tergitate was obtained with an activity of 305 μg./mg. (spectrophotometric determination)[9] representing a yield of 98%.

Although the end-point of the precipitation may be determined rapidly by testing the filtrate with additional precipitating agent, surface tension and conductivity measurements can also serve to indicate the end-point as shown in Table II.

TABLE II

RELATION BETWEEN SURFACE TENSION, SPECIFIC CONDUCTIVITY AND STREPTOMYCIN CONCENTRATION DURING TERGITOL PRECIPITATION

Tergitol-7 added, ml.	Activity of mother liquor, μg./ml.	Surface tension, dynes/sq. cm.	Specific conductivity × 10^4
0	396	60.0	2.27
2	213	59.3	2.70
6	34	49.9	3.69
7	30	40.3	3.92
8	<12.3	31.8	4.08

Fifteen grams of crude tergitate was dissolved in 100 ml. of methanol, filtered and 10 ml. of water added to cloudiness. Crystals gradually formed and were filtered and dried; yield 8.1 g., m.p. (dec.) 141–142°. Analysis after drying in high vacuum at room temperature over phosphorus pentoxide gave: rotation $[\alpha]^{25}D$ −25° (*c* 1% in methanol).

Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3HC_{17}H_{35}SO_4\cdot 6H_2O$: C, 50.89; H, 9.43; N, 5.77; S, 5.66; MeO, 0.0. Found: C, 41.48; H, 9.42; N, 6.03; S, 5.82; MeO, 0.0.

Recovery of Amorphous Streptomycin from Streptomycin Tergitate.—Twenty grams of crude streptomycin tergitate activity was dissolved in 100 ml. of methanol and the solution was filtered. The rich solvent was added to a mixture of 300 ml. of water and 60 g. of neutral IR-4B[13] resin at a temperature of 50–55°. Maintaining this temperature, 5.4 ml. of concentrated hydrochloric acid was added with agitation at such a rate as to maintain a *p*H of 3.0–3.5. After the addition, the *p*H was allowed to rise to 6.5 and the resin filtered off and washed with 50 ml. of water. The methanol was removed under vacuum and the resulting solution was freeze dried. The yield of streptomycin hydrochloride having an activity of 725 μg./mg. was 6.5 g.

Preparation of Crystalline Streptomycin Hydrochloride.—Twenty grams of crude streptomycin tergitate was dissolved in 100 ml. of methanol and filtered. To the rich solvent was added a solution of 70 ml. of 10% calcium chloride in methanol containing 0.7 ml. of concentrated hydrochloric acid. Crystallization took place rapidly and the mixture was allowed to stand overnight at 5°. After filtration and drying, 4.5 g. of the crystalline hydrochloride was obtained having an activity of 860 μg./mg. An additional 2.4 g. of amorphous hydrochloride was precipitated from the mother liquor with acetone having an activity of 620 μg./mg.

The crystalline fraction, recrystallized from methanol, gave the following analysis after drying at 100° in high vacuum over phosphorus pentoxide.

Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3HCl$: C, 36.50; H, 6.13; N, 14.19; Cl, 15.40. Found: (1) C, 36.27; H, 6.14; N, 14.29; Cl, 15.59. (2) C, 36.43; H, 6.38; N, 13.67; Cl, 15.80. $[\alpha]^{26}D$ −86.1° (*c* 1% in water). Activity *vs. K. Pneumoniae* = 891 μg./mg.

Preparation of Crystalline Streptomycin Hydrobromide.—Twenty grams of crystalline streptomycin tergitate was dissolved in 100 ml. of methanol and 125 ml. of 10% methanolic calcium bromide was added. After standing overnight at

(13) Manufactured by the Resinous Products Division of the Rohm and Haas Co., Washington Square, Philadelphia 5, Pa.

5°, 3.8 g. of crystalline hydrobromide was obtained. Recrystallization from methanol gave crystals with the following analysis after drying at 100° in high vacuum over phosphorus pentoxide.

Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3HBr$: C, 30.59; H, 5.14; N, 10.68; Br, 29.08. Found: C, 30.97; H, 5.53; N, 11.07; Br, 28.70. $[\alpha]^{26}D$ −72.2 (*c* 1% in water). Activity *vs. K. Pneumoniae* = 797 μg./mg.

Preparation of Crystalline Streptomycin Nitrate.—Ten grams of crystalline streptomycin tergitate was dissolved in 50 ml. of methanol. Thirty-five ml. of 10% methanolic calcium nitrate was added followed by 0.3 ml. of concd. HNO_3. The cloudy solution was allowed to stand in the cold room 4 hours and filtered. Recrystallization from methanol gave crystals with the following analysis (dried in high vacuum over phosphorus pentoxide at 56°):

Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3HNO_3$: C, 32.73; H, 5.49; N, 18.18. Found: C, 33.11; H, 5.53; N, 17.91. $[\alpha]^{26}D$ −82.3° (*c* 1% in water). Activity *vs. K. Pneumoniae* = 733 μg./mg.

Preparation of Crystalline Streptomycin Sulfate.—Twenty grams of crude streptomycin tergitate was dissolved in 100 ml. of butanol and filtered. The rich solvent was added slowly with agitation to 100 ml. of 5% aqueous guanidine sulfate solution which had been adjusted to *p*H 3.0 with dilute sulfuric acid. The crystals were allowed to remain overnight at 5° and then filtered and dried. The yield was 5.3 g. Recrystallization from warm 5% aqueous guanidine sulfate solution yielded crystals with the following analysis after drying in high vacuum phosphorus pentoxide at 100°.

Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3/2H_2SO_4$: C, 34.61; H, 5.81; N, 13.45; S, 6.60. Found: (1) C, 34.85; H, 5.77; N, 14.43; S, 6.65. (2) C, 34.83; H, 5.84; N, 13.46; S, 6.82. $[\alpha]^{26}D$ −83.2 (*c* 1% in water). Activity *vs. K. Pneumoniae* = 843 μg./mg.

Preparation of Crystalline Streptomycin Phosphate.—Five grams of crystalline streptomycin hydrochloride was dissolved in 10 ml. of water and 40 ml. of 5% aqueous guanidine phosphate solution, acidified to *p*H 2.5 with phosphoric acid, was added. Methanol was added until the solution became turbid and the solution allowed to stand overnight at 5°. After filtration and drying, 0.81 g. crystalline streptomycin phosphate was obtained. Recrystallization from 2.5% phosphoric acid in methanol solution gave crystals with the following analysis after drying in high vacuum phosphorus pentoxide at 100°.

Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3/2H_3PO_4$: C, 34.62; H, 6.02; N, 13.45; P, 6.39. Found: C, 34.31; H, 6.30; N, 13.35; P, 6.58. $[\alpha]^{26}D$ −77.2° (*c* 1% in water). Activity *vs. K. Pneumoniae* = 770 μg./mg.

NEW BRUNSWICK, N. J.

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

Terramycin.[1] IX. The Synthesis of Indanone Degradation Products of Terramycin[2]

BY L. H. CONOVER

RECEIVED APRIL 25, 1953

Decarboxyterracinoic acid and isodecarboxyterracinoic acid, degradation products of Terramycin, have been synthesized confirming the structures assigned these compounds. The synthesis of decarboxyterracinoic acid provides additional proof of structure of terracinoic acid since the relationship between these two products has already been established. Terracinoic acid has also been converted to 5-methoxy-2,3-dimethylindanone which has been synthesized.

Included among the alkaline degradation products of Terramycin are terracinoic acid (I) and isodecarboxyterracinoic acid (II). The structure of terracinoic acid has been shown[3] to be 4-carboxy-5-hydroxy-3-methylindanone-2-acetic acid. Isodecarboxyterracinoic acid, the "bicarbonate soluble fraction"[4] which was obtained from the alkaline degradation of Terramycin, has been identified[5] as 7-hydroxy-3-methylindanone-2-acetic acid.[6] The present work was undertaken to provide synthetic confirmation of the structures of these important Terramycin degradation products.

Proof of the structure of decarboxyterracinoic acid (III) through synthesis would also constitute confirmation of the structure of terracinoic acid. This follows from previous studies which have established the position of the aromatic carboxyl function which is eliminated when terracinoic acid is decarboxylated.[3]

The preparation of decarboxyterracinoic acid (III) was accomplished through introduction of an acetic acid side chain at the 2-position of 5-hydroxy-3-methylindanone (IV) by a modification of the method employed by Knott[7] for the conversion of substituted acetophenones, acetonaphthones, etc., to the corresponding aroylpropionitriles and propionic acids. This useful procedure consists of the formation of an N,N-dialkyl ketonic Mannich base, followed by treatment of the Mannich base with aqueous alkali cyanide to form the nitrile, which may be hydrolyzed to the corresponding carboxylic acid.[8] Knott concluded that the method failed in the case of cyclic ketones and propiophenones; however, in the present case 5-methoxy-3-methylindanone (V) was converted to 5-methoxy-3-methylindanone-2-acetonitrile (VI), which on hydrolysis and demethylation yielded 5-hydroxy-3-methylindanone-2-acetic acid (III). The latter was shown to be identical with decarboxyterracinoic acid obtained by degradation of Terramycin.

The indanone (IV) was prepared in two ways. The preferred method involved treatment of phenyl α-bromobutyrate with aluminum chloride at 145°. This synthesis was suggested by the previously reported[9] conversion of phenyl α-bro-

(1) Terramycin is the registered trademark of Chas. Pfizer & Co., Inc. for oxytetracycline.

(2) Part of this work was presented before the Division of Organic Chemistry at the 121st National Meeting of the American Chemical Society, Buffalo, N. Y., March 25, 1952.

(3) Paper III of this series, R. Pasternack, L. H. Conover, A. Bavley, F. A. Hochstein, G. B. Hess and K. J. Brunings, THIS JOURNAL, **74**, 1928 (1952).

(4) Paper II of this series, R. Pasternack, A. Bavley, R. L. Wagner, F. A. Hochstein, P. P. Regna and K. J. Brunings, *ibid.*, **74**, 1926 (1952).

(5) Paper VII of this series, F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, K. J. Brunings and R. B. Woodward, *ibid.*, **74**, 3708 (1952).

(6) The structure proof of isodecarboxyterracinoic acid is to be published by F. A. Hochstein, *et al.*

(7) E. B. Knott, *J. Chem. Soc.*, 1190 (1947).

(8) An analogous reaction has been used to convert other types of Mannich bases to substituted acetic acids. *Cf.* H. R. Snyder and F. J. Pilgrim, THIS JOURNAL, **70**, 3770 (1948), and E. L. Eliel, *ibid.*, **73**, 43 (1951).

(9) K. v. Auwers and E. Hilliger, *Ber.*, **49**, 2410 (1916).