oily product (89 mg.) and recrystallization from methanol gave 19 mg. (22%) of impure material, m. p. 80–82.5°. Further crops were oily and difficult to purify even through the derivatives.

Reduction of β-17-Equilenone (probably *trans*). (a) **Clemmensen Reduction.**—Reduction of 298 mg. of β-17-equilenone (m. p. 190–192° vac., prepared by the procedure of Bachmann and Wilds)[3] was carried out as described for 16-equilenone. After evaporative distillation at 160–185° (0.1 mm.) the product crystallized only partially and contained a small amount of high-melting material (probably the result of incomplete reduction). This was removed by dissolving the product in petroleum ether (b. p. 30–60°) at room temperature and filtering. The petroleum ether soluble portion was converted to the picrate and recrystallized to give 30 mg., m. p. 104–107.5°. The hydrocarbon obtained by decomposing the picrate melted at 76–83°. Further recrystallizations afforded a few mg. of the hydrocarbon, m. p. 85.5–86.5°. A mixture with the hydrocarbon from 16-equilenone melted at 85.5–88°.

(b) **Wolff-Kishner Reduction.**—The **semicarbazone of β-17-equilenone** was prepared in 94% yield as described for 16-equilenone, except with refluxing for three hours. The analytical sample was recrystallized from pyridine–alcohol as fine colorless needles, m. p. 256.5–257.5° (dec.).

Anal. Calcd. for $C_{19}H_{21}ON_3$: C, 74.2; H, 6.9. Found: C, 74.2; H, 6.9.

Reduction of 160 mg. of the semicarbazone was carried out as described for the 16-equilenone derivative, except the time of heating was twenty-one hours. Crystallization of the oily product (119 mg.) from methanol gave 65 mg. (53%) of colorless crystals, m. p. 73–80°. The purest sample of β-equilenane from β-17-equilenone, obtained by repeated recrystallization and purification through the picrate, melted at 86–86.5°. A mixture with the hydrocarbon obtained from 16-equilenone (m. p. 87.5–89.5°) melted at 86.5–88.5°.

The picrate, m. p. 107.5–108.5°, and the trinitrobenzene complex, m. p. 120–121.5°, gave no depression in melting point when mixed with the corresponding derivative of the hydrocarbon from 16-equilenone.

Reduction of α-17-Equilenone (probably *cis*).—α-17-Equilenone was prepared by the procedures of Bachmann and Wilds,[3] with essentially the same results reported. The product of the Arndt–Eistert reaction, the dimethyl ester of the α-isomer of 2-methyl-2-carboxy-1,2,3,4-tetrahydrophenanthrenepropionic acid, however, was obtained as a lower melting polymorphic modification, m. p. 71–73.5° (reported,[3] m. p. 98–99°). Upon cyclization and decarboxylation this afforded α-17-equilenone, m. p. 100–102° (reported,[3] 100–101°). The semicarbazone was prepared in alcohol–pyridine solution and recrystallized from *n*-butyl alcohol, m. p. 264–266.5° (dec.).

Anal. Calcd. for $C_{19}H_{21}ON_3$: C, 74.2; H, 6.9. Found: C, 74.5; H, 7.1.

Clemmensen reduction of 709 mg. of α-17-equilenone, as described for 16-equilenone, afforded 660 mg. of colorless oil after evaporative distillation at 160–180° (0.1 mm.) This was converted to the trinitrobenzene derivative in absolute alcohol, giving 830 mg., m. p. 110–112.5°, and 200 mg., m. p. 106–109°, for a total yield of 81%. Regeneration of the hydrocarbon from a pure sample of the derivative (m. p. 113.5–115°) by passing a benzene solution through a column of alumina, gave a colorless viscous oil which failed to crystallize. Adsorption of the hydrocarbon on alumina and fractional elution also failed to result in a crystalline sample of α-equilenane.

The **picrate of α-equilenane** was formed in methanol solution using an excess of picric acid. Purification was difficult due to the tendency of the complex to dissociate. The analytical sample was obtained as orange blades from methanol, m. p. 100.5–101°. A mixture with the picrate of β-equilenane (from 16-equilenone) gave a depression in m. p. to 93–100°.

Anal. Calcd. for $C_{18}H_{20}\cdot C_6H_3O_7N_3$: C, 61.9; H, 5.0. Found: C, 62.3; H, 5.1.

The **trinitrobenzene complex of α-equilenane** crystallized well from methanol as fine yellow needles, m. p. 113.5–115°. A mixture with the derivative from β-equilenane was depressed to 104–109°.

Anal. Calcd. for $C_{18}H_{20}\cdot C_6H_3O_6N_3$: C, 64.1; H, 5.2. Found: C, 64.2; H, 5.1.

Summary

The *cis* and *trans* forms of *dl*-equilenane have been prepared by reduction of the two forms of 17-equilenone. By comparison with the hydrocarbon obtained from 16-equilenone the latter ketone has been shown to belong to the β-series, which probably has the same C:D ring configuration as equilenin.

MADISON 6, WISCONSIN RECEIVED JUNE 12, 1946

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS & CO.]

Dihydrostreptomycin[1]

BY QUENTIN R. BARTZ, JOHN CONTROULIS, HARRY M. CROOKS, JR., AND MILDRED C. REBSTOCK

Streptomycin has been found to be a strong base with an empirical formula $C_{21}H_{37-9}N_7O_{12}$.[2] Of its component parts streptidine, 1,3-biguanido-2,4,5,6-tetrahydroxycyclohexane[3a,3b,4,5] and N-methyl *l*-glucosamine[6] have been characterized and identified. A third degradation product, maltol, has been isolated[7] and presumably is derived from the remaining six-carbon portion of the molecule. This missing portion combined with the N-methyl *l*-glucosamine constitutes the degradation product designated "streptobiosamine."[8]

The presence of a carbonyl function has been demonstrated in streptomycin[8] and its location assigned to the streptobiosamine portion of the molecule. Since the formation of maltol occurs in alkaline solution a base-catalyzed cleavage of a carbonyl compound appears likely and the carbonyl function may tentatively be assigned to

(1) Presented before the Division of Medicinal Chemistry at Chicago, Illinois, September, 1946.

(2) Peck, Brink, Kuehl, Flynn, Walti and Folkers, THIS JOURNAL, **67**, 1866–1867 (1945).

(3) (a) Carter, Clark, Dickman, Loo, Meek, Skell, Strong, Alberi, Bartz, Binkley, Crooks, Hooper and Rebstock, *Science*, **103**, 53, 4 (1946); (b) Carter, Clark, Dickman, Loo, Skell and Strong, *ibid.*, **103**, 540 (1946).

(4) Fried, Boyack and Wintersteiner, *J. Biol. Chem.*, **162**, 391–392 (1946).

(5) Peck, Hoffhine, Peel, Graber, Holly, Mozingo and Folkers, THIS JOURNAL, **68**, 776–781 (1946).

(6) Kuehl, Flynn, Holly, Mozingo and Folkers, *ibid.*, **68**, 536 (1946).

(7) Schenk and Spielman, *ibid.*, **67**, 2276–2277 (1945).

(8) Brink, Kuehl and Folkers, *Science*, **102**, 506–507 (1945).

the maltol-precursor portion of the molecule. Streptobiosamine, *per se*, is relatively unstable under a number of reaction conditions and it was felt that the stability might be increased by reduction of the carbonyl grouping.

Reduction of streptomycin in the presence of platinum (from Adams platinic oxide catalyst) was found to result in the addition of one molecular equivalent of hydrogen per molecular equivalent of streptomycin at pressures of from one to four atmospheres. The resulting dihydrostreptomycin has been found to have not only increased stability, particularly toward alkaline reagents but to have biological activity qualitatively and quantitatively comparable to streptomycin itself. In addition, dihydrostreptomycin is not inactivated by cysteine[9] nor by carbonyl reagents.[10] The relative stability of the two compounds at various hydrogen ion concentrations are presented in Tables I and II and toward various carbonyl reagents in Table IV. In addition to this no maltol is formed from dihydrostreptomycin under conditions which give an essentially quantitative yield from streptomycin.

An experiment in further support of the thesis that it is a carbonyl group which has undergone reduction was suggested by Dr. M. A. Spielman of Abbott Laboratories based upon the ultraviolet absorption of thiosemicarbazide and thiosemicarbazones.[11,12] The data, Fig. 1, indicate a definite carbonyl derivative formation with thiosemicarbazide and streptomycin while no such

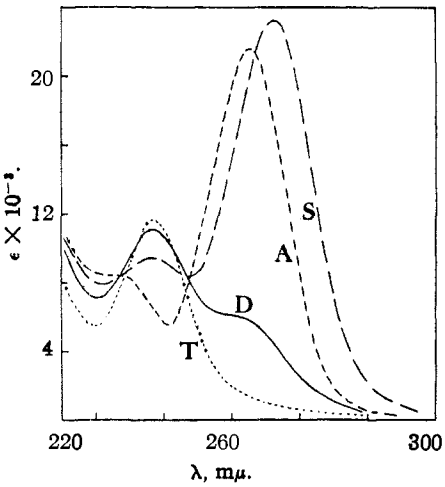

Fig. 1.—Ultraviolet absorption curves of the compounds in the presence of thiosemicarbazide (solvent, 50% methanol): D, dihydrostreptomycin; S, streptomycin; A, acetone; T, thiosemicarbazide.

(9) Denkelwater, Cook and Tishler, *Science*, **102**, 12 (1945).

(10) Geiger, Green and Waksman, *Proc. Soc. Exptl. Biol. Med.*, **61**, 187 (1946).

(11) Evans and Gillam, *J. Chem. Soc.*, 565–567 (1943).

(12) Added in proof, October 14.—After the submission of this paper the publication of Donovick, Rake and Fried, *J. Biol. Chem.*, **164**, 173–177 (1946), appeared in which essentially these same data were given.

compound is indicated when dihydrostreptomycin is treated in an identical manner.[13]

Streptomycin, when inactivated by hydrogen chloride in dry methanol, yields streptidine dihydrochloride and methyl streptobiosaminide dimethyl acetal hydrochloride. This latter product may in turn be acetylated to give a crystalline tetraacetyl derivative.[8] In contrast to this, dihydrostreptomycin, inactivated in the same manner, forms streptidine dihydrochloride and methyl dihydrostreptobiosaminide hydrochloride which in turn forms a crystalline hexa-acetyl derivative.[14] The same hexa-acetyl compound is obtained if the original methanolic hydrogen chloride solution of methyl streptobiosaminide dimethyl acetal hydrochloride from streptomycin is evaporated to dryness *in vacuo* and then hydrogenated (0.6–1.0 molecular equivalent uptake of hydrogen) and the product acetylated. The evaporation process apparently results in hydrolysis of the acetal grouping. The relation of these products to the complete structure of streptomycin will be discussed in a subsequent paper.

We wish to acknowledge our indebtedness to Dr. Arne Wick of the Upjohn Company and Dr. John Leighty of the Lilly Laboratories for supplies of crude streptomycin. Crystalline streptomycin hydrochloride calcium chloride double salt was supplied by Messrs. J. T. Alberi and R. L. Harris of this Laboratory. For the bacteriological data we are indebted to Dr. John Ehrlich and Mr. D. A. Joslyn of this Laboratory and to Dr. Guy P. Youmans of Northwestern University for the test on the tubercle bacillus. The helpful advice of Dr. H. E. Carter in the course of this work is gratefully acknowledged.

Experimental

Preparation of Dihydrostreptomycin Salts.—When hydrogenation of streptomycin trihydrochloride calcium chloride double salt was carried out in a quantitative micro-hydrogenation apparatus, a rapid uptake of one mole of hydrogen per 0.5 mole of compound was observed. In one experiment 0.07905 millimole of pure crystalline $(\text{streptomycin}\cdot 3HCl)_2\cdot CaCl_2$ dissolved in 20 ml. of distilled water when reduced at 760.1 mm. pressure in the presence of 32.3 mg. of Adams platinum oxide catalyst absorbed 90% of the calculated amount of hydrogen in one hour and 95% in two hours (basis 2 moles hydrogen per mole streptomycin double salt). The catalyst had been saturated with hydrogen before the streptomycin was added.

Dihydrostreptomycin·3HCl was prepared in quantity by hydrogenation according to the following directions.

(13) Ultraviolet data by Dr. John M. Vandenbelt of this Laboratory.

(14) Added in proof, October 14.—The assay method used in obtaining these high values was that of L. P. Clark utilizing alcoholic potassium hydroxide. After discussion of this point with Drs. Fried and Wintersteiner at Chicago, September 9, 1946, since their data indicate five total acetyls rather than six, determinations were carried out by the method of Kunz and sharp values of four O-acetyl groups per molecule were obtained. The formation of additional volatile acid in the Clarke method must presumably be due to degradation of the reduced maltol precursor fragment.

Three grams of streptomycin·3HCl (660 units/mg.)[15] was dissolved in 150 ml. of distilled water and hydrogenated in the presence of 1 g. of Adams platinum oxide catalyst which had previously been saturated with hydrogen. The reaction was continued for four hours at 22 lb./sq. in. The catalyst was then removed by filtration and washed with distilled water. Filtrate and washings were combined and concentrated to dryness at 40° (reduced pressure). The residue was dissolved in 70 ml. of methanol and four volumes of acetone added. After one hour the precipitated solid was separated by centrifugation and stirred with 80 ml. of anhydrous acetone. The product was again centrifuged, the supernatant acetone decanted, and the solid dried to a pure white powder in a vacuum desiccator under reduced pressure. A yield of 2.75 g. (642 units/mg.) was obtained.

If the calcium chloride double salt of streptomycin was hydrogenated and processed in this same manner an amorphous white solid was obtained which contained calcium but actual molecular complex formation has not been conclusively demonstrated.

Acid Degradation of Dihydrostreptomycin.—Both $(\text{streptomycin·3HCl})_2\text{·CaCl}_2$ and the dihydro derivative were completely inactivated by 1.0 *N* methanolic hydrochloric acid in eighteen hours at room temperature. Aqueous 1.0 *N* sulfuric acid effected similar inactivation at 37°

TABLE I

ACID INACTIVATION OF DIHYDROSTREPTOMYCIN

Compound	Acid 1 *N*	0 hr. units/ml.	24 hr. units/ml.	48 hr. units/ml.
Streptomycin	CH_3OH—HCl	5,400	0	..
Dihydrostreptomycin	CH_3OH—HCl	6,000	0	..
Streptomycin	H_2SO_4	11,600	1850	440
Dihydrostreptomycin	H_2SO_4	12,000	3030	1180

One gram of hydrogenated $(\text{streptomycin·3HCl})_2\text{·CaCl}_2$ was degraded with 61 ml. of 1.0 *N* methanolic hydrochloric acid for seventy-two hours. Two volumes of anhydrous ether were then added and the mixture placed in the refrigerator overnight. The precipitate was then centrifuged and identified as streptidine dihydrochloride by conversion to the picrate. This fraction represented 490 mg. of material. The supernatant solution was neutralized with 10% methanolic sodium hydroxide, the sodium chloride removed, and the solution evaporated to dryness at 40° under reduced pressure. This fraction consisting of 475 mg. of amorphous material was acetylated to form a crystalline hexaacetyl derivative, m. p. 198–199°, further described in detail below.

Streptidine sulfate was similarly isolated from a sulfuric acid inactivated dihydrostreptomycin solution and identified by conversion to the picrate.

Methyl Hexaacetyldihydrostreptobiosaminide.[14]—Methyl dihydrostreptobiosaminide hydrochloride from the degradation of dihydrostreptomycin with 1.0 *N* methanolic hydrochloric acid was treated with pyridine and acetic anhydride at 0° for four to six days. The solvents were then removed at 40° (reduced pressure). The residue was extracted with acetone, the acetone evaporated, and the crystalline solid recrystallized from absolute ethanol or chloroform–ether.[16] The product melted at 198–199° (micro block); $[\alpha]^{23.5}D$ −125.7° (*c*, 0.98% in $CHCl_3$). *Anal.* Calcd. for $C_{13}H_{18}NO_8(CH_3CO)_6(OCH_3)$: C, 51.56; H, 6.49; N, 2.31; acetyl, 42.6; OCH_3, 5.12. Found: C, 51.23; H, 6.42; N, 2.40; acetyl, 42.6; OCH_3, 4.45.[17]

(15) The assay method used to report potencies through Table V was based on the "plate-disc" method of Loo, *et al.*, *J. Bact.*, **50**, 701 (1945), employing ATCC strain #6633 of *B. subtilis* and the F.D.A. streptomycin sulfate working standard.

(16) Microanalyses by A. W. Spang of this Laboratory.

(17) Calcd. for $C_{13}H_{18}NO_8(CH_3CO)_6(OCH_3)$: O-acetyl, 30.6. Found: O-acetyl, 30.9 (Kunz method).

The methanolic hydrogen chloride solution containing methyl streptobiosaminide dimethyl acetal hydrochloride from inactivation of 1.0 g. of streptomycin[3a] was concentrated *in vacuo* at 40° without neutralization of the acid. When 62.3 mg. of the residue (equivalent to 0.1412 millimole of streptomycin) was hydrogenated 0.0878 millimole of hydrogen was absorbed and the product, when acetylated by the above technique, yielded the same hexaacetyl derivative. No appreciable hydrogen absorption occurred if the methanolic hydrogen chloride solution was neutralized before evaporation.

Alkaline Stability of Dihydrostreptomycin.—In contrast to streptomycin itself, the dihydro derivative was found to exhibit marked stability in the presence of alkali. The results of these studies are summarized in the following table.

TABLE II

STABILITY OF DIHYDROSTREPTOMYCIN IN ALKALINE SOLUTION (ROOM TEMPERATURE)

Compound	Solvent, NaOH	0 hr. units/ml.	20 hr. units/ml.	44 hr. units/ml.	56 hr. units/ml.
Streptomycin	0.1 *N* NaOH	8610	0	..	..
Dihydrostreptomycin	0.1 *N* NaOH	5830	5610	..	5140
Dihydrostreptomycin	0.78 *N* NaOH	7910	7600	7280	..

When dihydrostreptomycin was treated with 0.1 *N* sodium hydroxide for ten minutes at 100°, conditions which result in the quantitative formation of maltol from streptomycin,[7] ultraviolet absorption studies showed the presence of no significant amount of this degradation product.[12]

TABLE III

ULTRAVIOLET ABSORPTION FOR MALTOL FORMATION

Conditions	λ	Streptomycin $\epsilon \times 10^{-4}$	Dihydrostreptomycin $\epsilon \times 10^{-4}$
0.1 *N* HCl after treatment with 0.1 *N* NaOH, 10 min., 100°	324	9.34	0.21
	275	11.00	0.25
0.1 *N* NaOH, 10 min., 100°	324	17.5	0.38

Stability in the Presence of Carbonyl Reagents.—In contrast to streptomycin which is inactivated by a variety of carbonyl reagents,[10] similar systems substituting the dihydro derivative were stable. Solutions of the calcium chloride salt of streptomycin·3HCl and the hydrogenated material were prepared. The carbonyl reagent was then added and a sample of the mixture immediately diluted for assay. After four to four and one-half hours a second sample was taken. The results of these experiments are given in the Table IV.

Although thiosemicarbazide did not inactivate streptomycin under the above conditions, ultraviolet absorption studies gave evidence of compound formation when a mixture of 50 mg. of streptomycin and 7.14 mg. of thiosemicarbazide in 2 ml. of 50% methanol was warmed at 40–50° for two to three minutes. The dihydro derivative when treated similarly did not show this reaction, Fig. 1. The "hump" at 265 mμ is not clearly understood. That it cannot result from unreduced streptomycin is indicated by two observations: (1) It disappears on acidification of the solution whereas streptomycin thiosemicarbazone similarly treated loses about 1/3 intensity with no shift of wave length. (2) A portion of this dihydro material when treated with alkali for maltol formation showed that less than 2% streptomycin was present, an amount too small to be responsible for the height of the "hump."

Stability in the Presence of Cysteine.—Samples of $(\text{streptomycin·3HCl})_2\text{·CaCl}_2$ and its hydrogenation product were dissolved in 1.0 *M* phosphate buffer each and cysteine added to a concentration of 1 milligram per milliliter. Samples of solution were removed and diluted for assay at intervals.

TABLE IV

STABILITY OF DIHYDROSTREPTOMYCIN IN THE PRESENCE OF CARBONYL REAGENTS

Compound	Carbonyl reagent[a]	pH	0 hr. units/ml.	4–4.5 hr. units/ml.
Streptomycin	0.002 *M* $NH_2NHCONH_2 \cdot HCl$	4.6	28	<7
Dihydrostreptomycin	.002 *M* $NaC_2H_3O_2$	4.7	30	29
Streptomycin	.002 *M* $NH_2OH \cdot HCl$	5.3	32–15	<5
Dihydrostreptomycin	.002 *M* $NaC_2H_3O_2$	5.4	40	41
Streptomycin	.002 *M* $C_6H_5NHNH_2$	6.8	27–13	<4.8
Dihydrostreptomycin		6.8	25	29
Streptomycin	.002 *M* $NH_2NHCSNH_2$	6.4	22	22
Dihydrostreptomycin		6.2	29	29
Streptomycin	Distilled water	6.6	27	29
Dihydrostreptomycin		6.7	34	33
Streptomycin	.002 *M* $NaC_2H_3O_2$	6.7	37	36
Dihydrostreptomycin		6.8	33	33

[a] All carbonyl reagents showed 0 activity.

TABLE V

Compound	0 hr. units/ml.	2 hr. units/ml.	24 hr. units/ml.
Streptomycin	100–42	0	..
Dihydrostreptomycin	95	91	85

TABLE VI

COMPARISON OF ANTIBACTERIAL ACTION OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN

Organism	Streptomycin u./ml.	Dihydrostreptomycin u./ml.
Escherichia coli[a]	0.3	0.3
Bacillus subtilis[a]	1.0	0.3
Serratia marcescens[a]	1.0	3.0
Bacillus mycoides[a]	>3.0	>3.0
Escherichia coli[b]	10	6
Salmonella schottmülleri[b]	8	8
Shigella paradysenteriae (Sonne)[b]	15	8
Klebsiella pneumoniae[b]	6	4
Staphylococcus aureus[b]	7	5
Streptococcus (hemolytic)[b]	4	2
Streptococcus (non-hemolytic)[b]	12	8

[a] FDA streptomycin identity test method, complete inhibition. [b] Turbidimetric method which determines concentration necessary to give 50% density of control culture.

Dihydrostreptomycin has been found to be equal to streptomycin in its effect on the H37Rv strain of M. Tuberculosis var. hominis *in vitro* by Dr. Guy P. Youmans of Northwestern University Medical School. Dr. Youmans has been kind enough to permit us to make this report.

Summary[18]

1. Streptomycin salts have been reduced to form the corresponding dihydrostreptomycin salts.

2. Dihydrostreptomycin is more stable in alkaline solution than streptomycin and does not form maltol when heated with alkali.

3. Dihydrostreptomycin has antibiotic activity of the order of streptomycin and in contrast to streptomycin does not lose this activity in the presence of cysteine or carbonyl reagents.

4. Dihydrostreptomycin has been cleaved with methanolic hydrogen chloride to give methyl dihydrostreptobiosaminide hydrochloride which yields a crystalline hexa-acetyl derivative.[14]

DETROIT, MICH. RECEIVED JULY 15, 1946

(18) Since submission of this manuscript a communication on dihydrostreptomycin (Peck, Hoffhine and Folkers, THIS JOURNAL, **68**, 1390 (July 1946)) has appeared in which some of these same conclusions were reported: added July 30, 1946.