Stobbe condensation with tetralone-1 and 1-keto-1,2,3,4-tetrahydrophenanthrene, has also been investigated. The γ-lactones and unsaturated acids thus produced were shown in three instances to be interconvertible in a true acid-catalyzed lacto–enoic tautomerism. In the polycyclic series the decarboxylation step proved to be faster than the tautomerism, making it possible to interrupt the process before equilibrium was reached, whereupon the lactone was always found in higher proportion than at equilibrium, suggesting that the lactone is the precursor of the unsaturated acid. This conclusion affords evidence in support of a hypothesis that paraconic acids are intermediates in the decarboxylation reaction.

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[CONTRIBUTION FROM THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH, DIVISION OF ORGANIC CHEMISTRY]

Streptomycin. II.[1] Reduction and Oxidation Products of Streptomycin and of Streptobiosamine[2]

BY J. FRIED AND O. WINTERSTEINER

Recent investigations on the structure of streptomycin have shown that it is composed of a base, streptidine, $C_8H_{18}O_4N_6$ (I),[1,3,4,5] linked glycosidically to streptobiosamine, a nitrogen-containing bisaccharide of the formula $C_{13}H_{21-23}O_9N$ (II).[3] The glycosidic linkage between these two moieties is easily cleaved by hydrogen chloride in methanol with the formation of streptidine hydrochloride and methyl streptobiosaminide dimethyl acetal.[3] The characterization and degradation of streptidine has been carried out independently in several laboratories[1,3,5,6] and has led to its formulation as one of the eight possible *meso*-forms of 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane.[5,6]

One of the outstanding characteristics of streptomycin is its reactivity toward carbonyl reagents. The isolation of an amorphous oxime of streptomycin[3] and the ultraviolet absorption characteristics of streptomycin thiosemicarbazone and phenylhydrazone[7] leave little doubt that a free carbonyl group must be present in the streptobiosamine portion of the molecule. It is evidently this group which accounts for two of the three methoxyl groups in methyl streptobiosaminide dimethyl acetal, while the third methoxyl is introduced by methanolysis of the glycosidic linkage to streptidine. Methyl streptobiosaminide dimethyl acetal forms a crystalline tetraacetate, in which three of the acetyl groups are attached to oxygen and the fourth to nitrogen.[3]

On hydrolysis with strong mineral acid methyl streptobiosaminide dimethyl acetal is cleaved into its components, one of which was identified as N-methyl-L-glucosamine (III).[8] For the other, as yet unidentified, component of streptobiosamine, which must have the composition $C_6H_{8-10}O_5$(IV), we propose the term streptonose.[9] Direct evidence for the presence of a six-carbon moiety in streptomycin other than N-methyl-L-glucosamine and streptidine has been adduced by Schenck and Spielman,[10] who showed that exposure of streptomycin to the action of dilute cold alkali results in the formation of the γ-pyrone maltol (V). The striking ease with which this reaction proceeds is not without analogy in sugar chemistry. Thus, tetraacetyl glucosone hydrate (VI), as well as the corresponding galactose derivative are transformed into diacetylkojic acid (VII) by pyridine and acetic anhydride at 0°.[11] While there is little doubt that maltol arises from the streptonose moiety, no conclusions as to the structure of this sugar can be drawn from this observation, in view of the known tendency of some dicarbonyl sugars to undergo rearrangement in alkaline media.[12] Thus the positions of the carbonyl groups, the points of attachment of streptidine and N-methyl-L-glucosamine, and the functions of the remaining oxygen atoms are still unknown.

It was felt that some information regarding the structure of streptonose could be gained by ascertaining the nature of the free carbonyl group in streptomycin. The present paper deals with derivatives of streptomycin and streptobiosa-

(1) Paper I of this series: J. Fried, G. A. Boyack and O. Wintersteiner, *J. Biol. Chem.*, **162**, 391 (1946).

(2) Most of the material in this paper was presented before the Division of Biological Chemistry of the American Chemical Society at the Chicago meeting in September, 1946.

(3) N. G. Brink, F. A. Kuehl, Jr., and K. Folkers, *Science*, **102**, 506 (1945).

(4) H. E. Carter, R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, J. S. Meek, P. S. Skell, W. A. Strong, J. T. Alberi, Q. R. Bartz, S. B. Binkley, H. M. Crooks, Jr., I. R. Hooper and M. C. Rebstock, *Science*, **103**, 53 (1946).

(5) R. L. Peck, C. E. Hoffhine, Jr., E. W. Peel, R. P. Graber, F. W. Holly, R. Mozingo and K. Folkers, THIS JOURNAL, **68**, 776 (1946).

(6) H. E. Carter, R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell and W. A. Strong, *Science*, **103**, 540 (1946).

(7) R. Donovick, G. Rake and J. Fried, *J. Biol. Chem.*, **164**, 173 (1946).

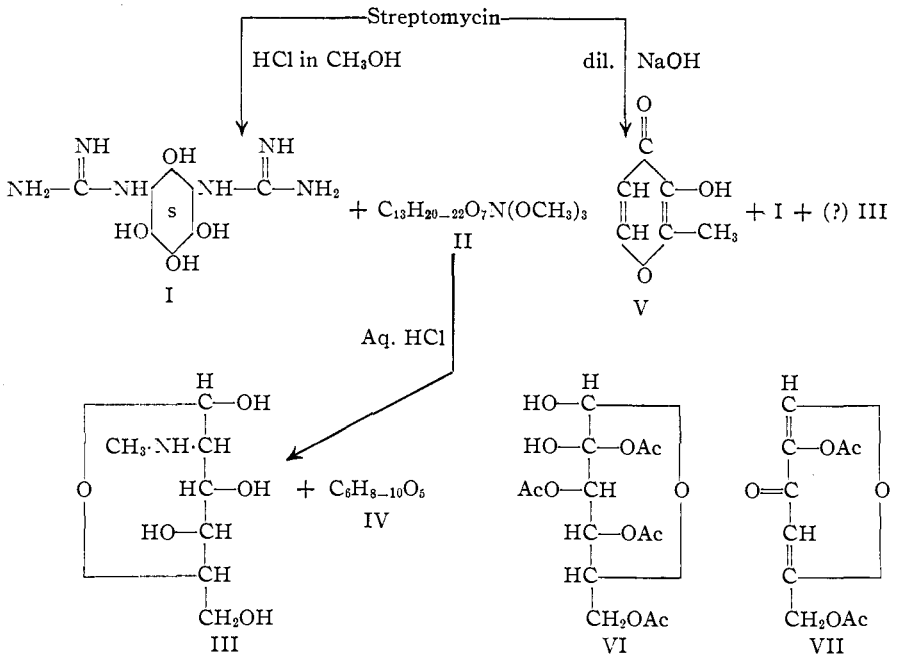
(8) F. A. Kuehl, Jr., E. H. Flynn, F. W. Holly, R. Mozingo and K. Folkers, THIS JOURNAL, **68**, 536 (1946).

(9) It will be clear from the following that streptonose contains an aldehyde group and at least one additional carbonyl group. This terminology is therefore in line with the customary designation of dicarbonyl sugars as onoses.

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

(11) K. Maurer, *Ber.*, **63**, 25, 2069 (1930).

(12) B. Helferich and N. M. Bigelow, *Z. physiol. Chem.*, **200**, 263 (1931).



mine in which this group has been modified by both reduction and oxidation.

When an aqueous solution of streptomycin trihydrochloride is shaken with hydrogen in the presence of platinum or palladium catalyst, approximately one mole of hydrogen is consumed. The resulting amorphous product, **dihydrostreptomycin** (VIII),[13] is biologically active. Its antibiotic potency toward the usual test organisms[14] and its toxicity[14] differ but slightly from those of streptomycin. In contradistinction to streptomycin, the dihydro compound is not inactivated by exposure to 1 *N* alkali at room temperature, in other words, the degradation of the molecule to streptidine,[14a] maltol and (presumably) N-methyl-L-glucosamine, which is brought about by the action of this reagent on streptomycin, is blocked as a consequence of the reductive change. The completeness of the reduction can therefore be conveniently judged by the failure of the reduced product to develop, on treatment with alkali, the ultraviolet band at 320 mμ characteristic for maltol. Dihydrostreptomycin yields crystalline salts with reinecke acid and helianthine, both of which are antibiotically active in proportion of their content of dihydrostreptomycin. On the other hand, dihydrostreptomycin trihydrochloride does not form a crystalline double salt with calcium chloride under conditions which in the case of streptomycin readily afford the calcium chloride double salt described by Peck, *et al.*[15] In the titration curve of dihydrostreptomycin only the weakly basic methylamino group ($pKa' = 7.70$) is in evidence, while the two guanido groups are completely ionized. Dihydrostreptomycin differs from streptomycin in that it fails to give rise to the typical thiosemicarbazone band at 270 mμ after treatment with thiosemicarbazide in neutral solution,[7] and that it is not inactivated by this and other carbonyl reagents. Furthermore, whereas streptomycin in consumes approximately two atoms of iodine per mole in the Willstätter–Schudel titration, dihydrostreptomycin is inert to hypoiodite. Obviously it is the free carbonyl group in streptomycin which acts as the hydrogen acceptor in the catalytic hydrogenation. Confirmatory evidence was adduced by degradation of dihydrostreptomycin to **methyl dihydrostreptobiosaminide** (IX) by means of methanolic hydrogen chloride. Methyl dihydrostreptobiosaminide contains only one methoxyl group, showing that it lacks the acetal-forming group present in the corresponding degradation product of streptomycin. Furthermore, on acetylation a crystalline **pentaacetate**[16] melting at 193° was obtained, which leaves little doubt that in the initial hydrogenation step a carbonyl group had been transformed into a carbinol group.

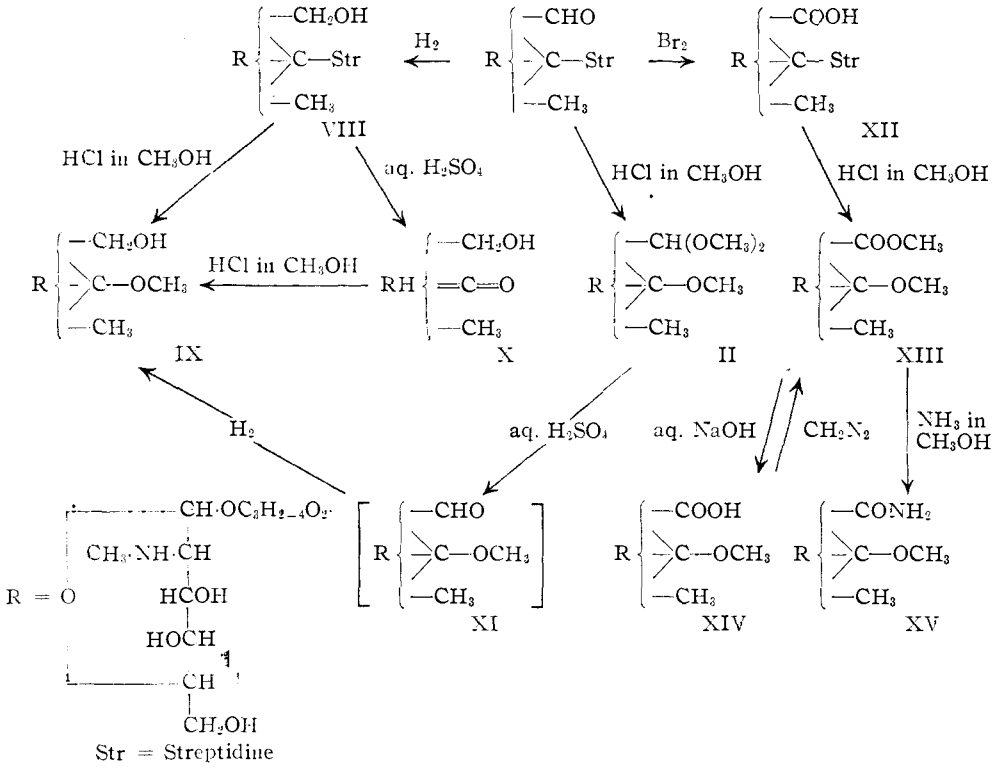
(13) After the completion of this work a communication appeared (Peck, Hoffhine and Folkers, THIS JOURNAL, **68**, 1390 (1946)), in which the preparation and properties of the trihydrochloride and trihelianthate of dihydrostreptomycin are described. Our own data are in substantial agreement with those presented by these authors. Another publication on this subject appeared while this paper was in press (Bartz, Controulis, Crooks, and Rebstock, *ibid.*, **68**, 2163 (1946)).

(14) R. Donovick and G. Rake, *J. Bact.*, in press (1947).

(14a) The formation of streptidine from streptomycin under the influence of alkali has not been previously reported. A solution of streptomycin trihydrochloride (310 mg.) in aqueous 0.2 *N* barium hydroxide (15 cc.) was allowed to stand at room temperature for eighteen hours and then made slightly acidic with sulfuric acid. After removal of the barium sulfate by filtration and of the maltol by extraction with chloroform, the solution was concentrated to a small volume. Addition of a concentrated aqueous solution of sodium sulfate resulted in the formation of a crystalline precipitate. On recrystallization from water 95 mg. of the typical prisms of streptidine sulfate monohydrate were obtained.

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

(16) It should be mentioned in this connection that in the acetyl determination according to Freudenberg, which tends to give low values with N-methyl-L-glucosamine pentaacetate, some of the figures obtained on the above acetate were one per cent. too high for a pentaacetate, but far too low for a hexaacetate. In contrast, the Kunz titration for O-acetal groups (Kunz and Hudson, THIS JOURNAL, **48**, 1982 (1926)) clearly indicated the presence of four such groups; prolongation of the saponification period from two to four hours did not alter this result. A fifth O-acetyl group, if present, would hardly be expected to resist saponification under these conditions. However, the possibility that the compound is a hexaacetate is not entirely excluded.



Hydrolysis of dihydrostreptomycin with aqueous sulfuric acid at 45° resulted in the formation of streptidine sulfate and of an amorphous product, which represents the expected **dihydrostreptobiosamine** (X), the hemiacetal corresponding to IX. Treatment of this product with methanolic hydrogen chloride followed by acetylation yielded the crystalline acetate previously obtained in the same manner directly from dihydrostreptomycin. This result shows that hydrolytic cleavage of the glycosidic linkage between streptidine and dihydrostreptobiosamine is not accompanied by any rearrangement in the sensitive streptonose moiety, unless the same rearrangement occurs also during methanolysis.

The crystalline pentaacetate of methyl dihydrostreptobiosaminide was also obtained in small amounts by selective hydrolysis of the acetal group in methyl streptobiosaminide dimethyl acetal followed by catalytic hydrogenation and acetylation.

The reactions described so far give little information about the nature of the free carbonyl group in streptomycin. There is no indication for the presence in the final product of a diastereoisomer of methyl pentaacetyl-dihydrostreptobiosaminide, as should be the case if a keto group had been reduced in the initial step. The stability of the acetal group in methyl streptobiosaminide dimethyl acetal also militates to a certain extent against the carbonyl group being ketonic. Proof that this group is aldehydic was brought about by oxidation of streptomycin with bromine water to an amorphous acid for which we propose the name **streptomycinic acid** (XII). The acidic nature of this product was demonstrated by electrometric titration, which revealed it to be a relatively strong acid (pK_1', 2.35; pK_2', 7.85). In the infrared[17] region the substance exhibited an absorption maximum at a wave length of 6.06 μ, which was partially displaced to 5.81 μ, when the spectrum was taken in the presence of D_2O and DCl. A shift of this type is usually found with zwitterions, and may therefore be taken as an indication of the expected zwitterion character of streptomycinic acid.

Streptomycinic acid is antibiotically inactive. It does not form maltol in alkaline medium, nor does it display any reactivity toward carbonyl reagents. More rigorous proof for the fact that streptomycinic acid differs from streptomycin merely by the replacement of an aldehyde by a carboxyl group was furnished by degradation of the acid with methanolic hydrogen chloride. This reaction afforded streptidine dihydrochloride and the amorphous hydrochloride of a product which contains two methoxyl groups and accordingly should be the methyl ester of the acid corresponding to methyl streptobiosaminide. In keeping with previous usage this substance will be referred to as **methyl streptobiosaminidic acid methyl ester** (XIII). The amorphous ester was acetylated, whereby it yielded a crystalline

(17) The infrared measurements were obtained through the courtesy of the Stamford Research Laboratories of the American Cyanamid Company.

tetraacetate, melting at 139°, the analytical composition of which was in good agreement with the above interpretation. The Kunz titration revealed four saponifiable groups in conformity with the above formulation, which demands three O-acetyl groups and one carbomethoxy group. When the unacetylated ester was treated with excess aqueous sodium hydroxide at room temperature for three days, one equivalent of alkali was consumed. The titration curve of the hydrolysis product, **methyl streptobiosaminidic acid** (XIV), showed two binding regions, with pK_1' 2.40 and pK_2' 8.20 (Fig. 1). It is to be noted that the methylamino group in methyl streptobiosaminidic acid methyl ester (pKa', 7.55) displays a markedly lower basicity than the same group in the free acid. This decrease in the pK' of the basic group is to be expected from theoretical considerations[18] and is generally observed with amino acids consequent to esterification.

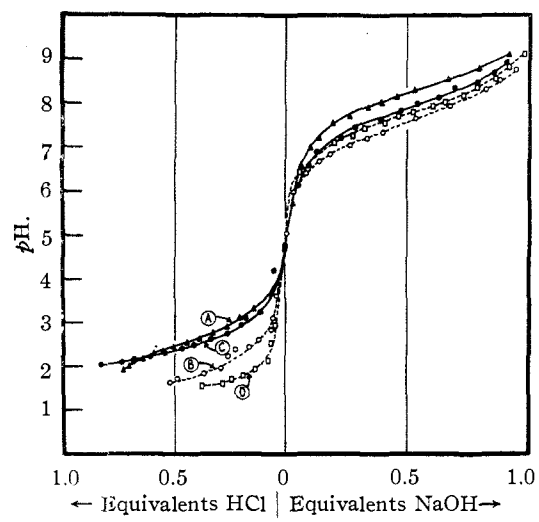


Fig. 1.—Titration curves: A, methyl streptobiosaminidic acid; **B**, methyl streptobiosaminidic acid methyl ester; C, streptomycinic acid; D, dihydrostreptomycin. Curves **B**, C and D were obtained by dissolving 0.1 millimole of substance in 1 ml. of 0.1 *N* hydrochloric acid and titrating the solutions with 0.1 *N* sodium hydroxide. To obtain curve A, 0.1 millimole of methyl streptobiosaminidic acid methyl ester was dissolved in 1 ml. of 1 *N* sodium hydroxide and allowed to stand at room temperature for sixty hours. 1 ml. of 1 *N* hydrochloric acid was then added and the mixture was titrated as above. All curves have been corrected for the water blank.

By the action of diazomethane on methyl streptobiosaminidic acid a product was obtained which, to judge from its methoxyl content, contained only a small proportion of the desired methyl ester. This is not surprising, as Kuhn and Brydówna[19] have shown that in the reaction of zwitterions with this agent betaine formation competes with esterification, sometimes to the exclusion of the latter. Nevertheless, a small amount of the crystalline tetraacetate could be obtained from the mixture upon acetylation.

Methyl streptobiosaminidic acid methyl ester upon treatment with alcoholic ammonia at room temperature yielded an amorphous product, the nitrogen and methoxyl content of which indicated that it consisted for the most part of the desired amide (XV). Acetylation of this material followed by chromatographic fractionation failed to yield any crystalline products. However, thorough analytical characterization of the main chromatographic fraction left little doubt that it represented the expected **methyl tetraacetylstreptobiosaminidic acid amide**.

The conclusion from the foregoing evidence as to the presence of a carboxyl group in the oxidized products discussed above receives further support from the infrared spectrum of the methyl ester XIII, which shows a strong band at 5.77 μ, the region characteristic for ester carbonyl.[17]

Though there is much presumptive evidence favoring the allocation of the aldehydic group to the streptonose moiety (for instance the abolition of the maltol reaction by reduction or oxidation of the aldehyde group of streptomycin), a more direct proof was desirable. This was accomplished by hydrolysis of methyl dihydrostreptobiosaminide with strong acid, which yielded N-methyl-L-glucosamine, identified as the crystalline pentaacetate[8] and as L-glucosazone. If the free carbonyl group in streptomycin were identical with the aldehyde group of N-methyl-L-glucosamine, it should have been reduced to carbinol in dihydrostreptomycin, and methyl dihydrostreptobiosaminide should have yielded N-methyl-L-glucosaminol instead of the N-methyl-L-glucosamine actually obtained. Streptomycin is therefore a N-methyl-L-glucosaminido-streptonoside, or a streptonosido-N-methyl-L-glucosaminide, of streptidine. The first-mentioned structure is preferred in view of the fact that in the hydrolysis (and methanolysis) of streptomycin and dihydrostreptomycin the glycosidic linkage to streptidine is cleaved in preference to that between the two components of streptobiosamine. This is in accord with the resistance shown by D-glucosaminides to similarly mild hydrolytic treatment.[20] Furthermore, the ready conversion of dihydrostreptobiosamine into its methyl glycoside is in agreement with a N-methyl-L-glucosaminidodihydrostreptonose structure for dihydrostreptobiosamine. The alternative structure would be expected to resist glycoside formation, in analogy to the behavior of D-glucosamine, which fails to yield methyl D-glucosaminide on treatment with methanolic hydrogen chloride.[20]

Streptobiosamine is a true *aldehydo*-sugar. This

(18) S. Glasstone and E. F. Hammel, Jr., THIS JOURNAL, **63**, 243 (1941).

(19) R. Kuhn and W. Brydówna, *Ber.*, **70**, 1333 (1937).

(20) R. C. G. Moggridge and A. Neuberger, *J. Chem. Soc.*, 745 (1938).

follows from its conversion into a dimethyl acetal under conditions which in the case of a hemiacetal structure would have produced a methyl glycoside. For streptomycin we likewise favor an open *aldehydo*-structure, in view of its lack of mutarotation and of its oxidation to an acid rather than to a lactone. The marked reactivity of streptomycin toward carbonyl reagents, which with thiosemicarbazide was found to surpass that of either glucose or fructose, is also in harmony with this concept.

Acknowledgment.—We wish to express our appreciation to Miss Doris E. Walz for her able assistance in the experimental work, to Mr. J. F. Alicino for the microanalyses, to Mr. E. A. Paredes for the purification of streptomycin and to Mr. F. Russo-Alesi for the numerous spectrophotometric streptomycin assays.

Experimental[21]

Purification of Streptomycin.—Streptomycin trihydrochloride of 300–400 units/mg. was chromatographed over acid-washed alumina from 80% methanol, essentially as described by Carter, *et al.*[22] Fractions having a potency of 500 units/mg.[23] or more were precipitated with methyl orange[24,25] (1.4 g. per gram of streptomycin trihydrochloride), and the crude helianthate recrystallized twice from 50% methanol. To insure efficient decomposition the pure, crystalline helianthate was finely ground and passed through a 60-mesh sieve. Twenty grams of the powder was suspended in 270 ml. of dry methanol and shaken in a closed vessel with three 25-ml. portions of 0.5 *N* hydrochloric acid in dry methanol. During this procedure, which was continued for twenty-five minutes, the temperature of the mixture was kept between 5 and 10°. The suspension was filtered, the filtercake washed with dry methanol and the combined filtrates decolorized with a small amount of charcoal. The colorless solution was diluted with 200 ml. of water and the methanol removed *in vacuo*. In some cases, where the *p*H of the concentrate was below 5.6 it was necessary to adjust the solution to that *p*H by shaking it with a small amount of silver oxide. This solution on freeze-drying yielded 8.5 g. of streptomycin trihydrochloride as a white amorphous powder, which contained approximately 5% moisture and assayed 750–800 units/mg.

Dihydrostreptomycin (VIII).—A solution containing 3.07 g. of streptomycin trihydrochloride in 60 ml. of water was shaken in an atmosphere of hydrogen with 300 mg. of platinum oxide at room temperature and atmospheric pressure.[26] At the end of twelve hours 143 ml. of hydrogen had been taken up, which corresponds to 1.3 moles per mole of streptomycin. The solution was filtered from the catalyst and lyophilized; 2.82 g. of a white, amorphous solid remained, which after drying *in vacuo* at 100° melted at 190–195° (dec.); $[\alpha]^{25}D$ −94.5° (*c*, 1.56 in water). It did not reduce Fehling's solution on boiling, and blackened Tollens reagent only slightly on standing. Its antibiotic activity against *K. pneumoniae* was 700 units/mg.

Anal. Calcd. for $C_{21}H_{41}O_{12}N_7 \cdot 3HCl$: C, 36.40; H, 6.40; N, 14.15; Cl, 15.37. Found: C, 35.19; H, 6.46; N, 13.72; Cl, 15.4.

Dihydrostreptomycin Trihelianthate.—A solution of 72 mg. of dihydrostreptomycin trihydrochloride in 1 ml. of water was mixed with a solution of 124 mg. of methyl orange in 5 ml. of 50% methanol. After standing for two hours at room temperature the crystals were filtered off and recrystallized twice from dilute methanol. After drying *in vacuo* at 100° they melted at 224–230° (dec.) and had a potency of 340 units/mg.

Anal. Calcd. for $C_{21}H_{41}O_{12}N_7 \cdot (C_{14}H_{15}N_3S)_3$: S, 6.40. Found: S, 6.36.

Dihydrostreptomycin Reineckate.—Forty-seven mg. of dihydrostreptomycin trihydrochloride was dissolved in 1 ml. of water and a solution of 71 mg. of reinecke salt in 4 ml. of water of 45° was added. On slow cooling to room temperature 62 mg. of well-shaped needles were obtained, which after one recrystallization from water sintered at 194° and melted at 204–206° (dec.) Their potency was 375 units/mg. An additional crop of crystals was obtained by chilling the mother liquors to 4°.

Anal. Calcd. for $C_{21}H_{41}O_{12}N_7 \cdot [HCr(SCN)_4(NH_3)_2]_2$: C, 28.57; H, 4.54; N, 21.78; S, 20.95; Cr, 8.51. Calcd. for $C_{21}H_{41}O_{12}N_7 \cdot [HCr(SCN)_4(NH_3)_2]_3$: C, 25.72; H, 4.06; N, 22.71; S, 24.92; Cr, 10.12. Found: C, 27.19; H, 4.52 N, 22.71; S, 23.59; Cr, 8.51.

Cleavage of Dihydrostreptomycin into Methyl Dihydrostreptobiosaminide (IX) and Streptidine.—Dihydrostreptomycin trihydrochloride (8.38 g.), which had been dried for two hours *in vacuo* over phosphorus pentoxide at 100° was dissolved in 173 ml. of 1 *N* hydrogen chloride in dry methyl alcohol. The solution was kept at room temperature for forty-eight hours. At the end of this period 360 ml. of dry ether was added with shaking. The resulting precipitate was filtered on a sintered glass funnel and dried (4.8 g.). It was identified as streptidine dihydrochloride through conversion into the difficultly soluble, crystalline sulfate. For this purpose 100 mg. of the material was dissolved in 2 ml. of water and a solution of 50 mg. of sodium sulfate in 3 ml. of water was added. After several hours in the refrigerator the crystals were filtered off and recrystallized from hot water. The water of crystallization was removed by drying *in vacuo* at 150°.

Anal. Calcd. for $C_8H_{18}O_4N_6 \cdot H_2SO_4$: C, 26.67; H, 5.60; N, 23.3; S, 8.88. Found: C, 26.63; H, 5.65; N, 23.3; S, 8.72.

The methanol ether filtrate was concentrated *in vacuo* to about 25 ml. and after removal of some solid material by centrifugation poured into 250 ml. of dry ether. The mixture was placed in the refrigerator for two hours. The resulting oily precipitate was separated from the methanol–ether solution by decantation, washed with two 50-ml. portions of dry ether and dried *in vacuo* over calcium chloride and potassium hydroxide. A hygroscopic solid (3.92 g.) remained, which represented essentially pure methyl dihydrostreptobiosaminide hydrochloride. It melted at 183–184° (dec.); $[\alpha]^{25}D$ −135° (*c*, 1.15 in methanol).

Anal. Calcd. for $C_{13}H_{22}O_8N(OCH_3) \cdot HCl$: C, 43.35; H, 6.76; N, 3.61; OCH_3, 7.99; N-methyl, 3.87; C-methyl 3.87; Cl, 9.14. Calcd. for $C_{13}H_{24}O_8N(OCH_3) \cdot HCl$: C, 43.13; H, 7.23; N, 3.59; OCH_3, 7.95; N-methyl, 3.85; C-methyl, 3.85; Cl. 9.10. Found: C 43.88; H, 8.00; N, 3.96; OCH_3, 6.68; N-methyl, 3.38; C-methyl, 4.45; Cl, 9.23.

For the preparation of its crystalline **pentaacetate** methyl dihydrostreptobiosaminide (3.92 g.) was dried at 100° for two hours and dissolved in 30 ml. of anhydrous pyridine and 30 ml. of acetic anhydride. After standing at room temperature for thirty hours, the mixture was poured onto crushed ice, and the aqueous solution was extracted with chloroform. The chloroform solution was

(21) All melting points were taken in capillary tubes and have been corrected for stem exposure.

(22) H. E. Carter, R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell and W. A. Strong, *J. Biol. Chem.*, **160**, 337 (1945).

(23) The progress of the purification was followed by means of a chemical method (Fried, Coy and Donovick, to be published), which involves the liberation of maltol[10] under standard conditions and the measurement of its ultraviolet absorption at 325 mμ in the Beckman spectrophotometer.

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

(25) F. A. Kuehl, Jr., R. L. Peck, C. E. Hoffhine, Jr., R. P. Graber and K. Folkers, THIS JOURNAL, **68**, 1460 (1946).

(26) Identical results were obtained with the use of palladium black.

mixed with crushed ice, and 5 *N* hydrochloric acid was added with shaking until the *p*H of the aqueous phase reached about 2. After successive washings with water, sodium bicarbonate and again water the chloroform layer was dried over sodium sulfate and freed from the solvent *in vacuo*. Addition of a small amount of absolute alcohol to the residue induced crystallization, which was complete after twenty-four hours in the refrigerator. Two recrystallizations from absolute alcohol yielded 600 mg. of stout prisms, which melted sharply at 193–194°, $[\alpha]^{25}D$ −120° (*c*, 1.01 in chloroform).

Anal. Calcd. for $C_{13}H_{17}O_8N(OCH_3)(CH_3CO)_5$: C, 51.33; H, 6.29; N, 2.49; OCH_3, 5.52; N-methyl, 2.66; total CH_3CO, 38.33; O-acetyl, 30.67; mol. wt., 561.5. Calcd. for $C_{13}H_{19}O_8N(OCH_3)(CH_3CO)_5$: C, 51.14; H, 6.62; N, 2.49; OCH_3, 5.50; N-methyl, 2.66; total CH_3CO, 38.19; mol. wt., 563.5. Calcd. for $C_{13}H_{18}O_8N(OCH_3)(CH_3CO)_6$: C, 51.56; H, 6.49; N, 2.31; OCH_3, 5.12; N-methyl, 2.48; total CH_3CO, 42.64; O-acetyl, 35.53; mol. wt., 605.6. Found: C, 51.23; H, 6.62; N, 2.43; OCH_3, 5.67; N-methyl, 2.03; total CH_3CO, 39.3, 39.6; O-acetyl, 29.3; mol. wt. (Rast), 531.

An additional amount of the substance was obtained by chromatographing the mother liquor material over aluminum oxide.[27] For this purpose the mother liquors were freed completely from alcohol and the residue taken up in 100 ml. of benzene. This solution was passed through a column containing 50 g. of alumina and the column washed with 250 ml. of benzene. Removal of the solvent from the combined effluent solutions yielded a residue from which 200 mg. of the crystalline acetate was obtained by crystallization from alcohol.

Cleavage of Dihydrostreptomycin into Dihydrostreptobiosamine (X) and Streptidine.—One gram of dihydrostreptomycin was dissolved in 8 ml. of 1 *N* sulfuric acid and the solution was allowed to stand at 45° for forty-five hours. The light yellow solution which was filled with crystals of streptidine sulfate was placed into the refrigerator for several hours to complete crystallization and then filtered. The dried crystals weighed 460 mg., which corresponds to 89% of the theoretical yield. The mother liquors were freed from sulfate ion by means of barium hydroxide and from excess chloride by treatment with silver carbonate to a *p*H of 6.5. Upon freeze-drying dihydrostreptobiosamine hydrochloride was obtained as a light brown hygroscopic powder, which readily reduced Fehling and Tollens reagents and consumed 1.3 moles of iodine in the Willstätter–Schudel titration. It melted at 110–130° (dec.). Although relatively stable in the solid state, the substance darkened appreciably on standing in aqueous solution at room temperature.

The product was used without further purification in the preparation of methyl pentaacetyl-dihydrostreptobiosaminide. Dihydrostreptobiosamine (360 mg.), which had been dried at room temperature over phosphorus pentoxide, was dissolved in 15 ml. of 1 *N* hydrochloric acid in anhydrous methanol and left at room temperature for forty-eight hours. After removal of the bulk of the hydrochloric acid by means of silver carbonate the methanol solution was concentrated to a small volume, and the methyl dihydrostreptobiosaminide was precipitated by the addition of dry ether. The product after drying *in vacuo* contained 5.7% methoxyl. Calcd. for $C_{13}H_{24}O_8N(OCH_3)\cdot HCl$: OCH_3, 7.95.

Acetylation of the crude product with pyridine and acetic anhydride yielded a crystalline substance which after two recrystallizations from absolute alcohol melted at 193° and did not depress the melting point of the preparation obtained by methanolysis of dihydrostreptomycin.

Methyl Pentaacetyl-dihydrostreptobiosaminide from methyl streptobiosaminide Dimethyl Acetal.[28]—A solution of 300 mg. of methyl streptobiosaminide dimethyl acetal (II)[3] in 4 ml. of 1 *N* sulfuric acid was allowed to stand at 45° for twenty-four hours. It was then neutralized with 0.5 *N* barium hydroxide and freeze-dried after removal of the barium sulfate by centrifugation. The dried product contained 7.32% OCH_3; calculated for methyl streptobiosaminide hydrochloride, $C_{13}H_{22}O_8N(OCH_3).HCl$: OCH_3, 7.99.

The above product was hydrogenated in aqueous solution in the presence of platinum oxide for four hours and the solution lyophilized after the removal of the catalyst. Acetylation of the resulting product with acetic anhydride in pyridine solution yielded the crystalline pentaacetate, m. p. 193°.

N-Methyl-L-glucosamine from Methyl Dihydrostreptobiosaminide.—A solution of 1.5 g. of methyl dihydrostreptobiosaminide in 15 ml. of 5 *N* hydrochloric acid was refluxed for ten hours. During this period a brown humin-like precipitate was formed (340 mg.), which was filtered off and washed with 20 ml. of hot water. The combined filtrates were decolorized with charcoal and evaporated to dryness *in vacuo*. The colorless residue was dried *in vacuo* over phosphorus pentoxide and potassium hydroxide and divided into two equal portions. The first portion was shaken for eighteen hours with 10 ml. of pyridine and 10 ml. of acetic anhydride and the resulting solution worked up as described above. The light brown residue from the dried chloroform extract was taken up in 20 ml. of benzene and chromatographed over 7.5 g. of aluminum oxide. The alumina column was washed with 100 ml. of benzene, and the combined effluent solutions were evaporated to dryness *in vacuo*. The colorless residue crystallized readily on rubbing with ether, yielding a product which after two recrystallizations from ethanol–ether melted at 157–158°, $[\alpha]^{25}D$ −101° (*c*, 0.396 in chloroform). Reported[8] for N-methyl-L-glucosamine, m. p. 160.5–161.5° (micro-block), $[\alpha]^{26}D$ −100° (*c*, 0.7 in chloroform).

Anal. Calcd. for $C_7H_{25}O_{10}N$: C, 50.62; H, 6.25; N, 3.76. Found: C, 50.71; H, 6.43; N, 3.38.

The remaining portion of the residue from the hydrolysis mixture was taken up in 6 ml. of water. The *p*H of the solution was adjusted to 7 by the addition of solid sodium acetate. After the addition of 0.7 ml. of phenylhydrazine and 0.35 ml. of glacial acetic acid, the mixture was refluxed for one and one-half hours. The yellow solution was centrifuged while hot to remove a small amount of brown gum, and kept in the refrigerator overnight. The resulting crystals were filtered off, dried *in vacuo* and recrystallized three times from absolute alcohol. They melted at 208° (dec.); $[\alpha]^{25}D$ +70° (*c*, 0.285 in 4 parts of pyridine + 6 parts of absolute alcohol). Levene and LaForge[29] report for D-glucosazone a melting point of 208° (dec.) and $[\alpha]^{25}D$ −62° (in same solvents).

Oxidation of Streptomycin with Bromine Water.—A solution of 5.2 g. of streptomycin trihydrochloride in 75 ml. of water to which 0.8 ml. of bromine had been added was allowed to stand at room temperature for five days in a dark place. After removal of excess bromine by vacuum distillation, the colorless solution was neutralized with silver carbonate to a *p*H of 6.7–6.9. The filtrate from the silver precipitate on freeze-drying yielded 4.8 g. of streptomycinic acid as a light pink-colored, amorphous product, which contained about 5% moisture. It melted at 231° (dec.) with previous sintering at 226°, $[\alpha]^{25}D$ −92° (*c*, 1.52 in water).

Cleavage of Streptomycinic Acid (XII) into Methyl Streptobiosaminidic Acid Methyl Ester (XIII) and Streptidine.—One and ninety-four hundredths grams of crude streptomycinic acid was dried at 100° *in vacuo* and dissolved in 38 ml. of 1 *N* hydrochloric acid in methanol. After standing at room temperature for sixty-five hours the streptidine dihydrochloride formed in the reaction was precipitated by the addition of 76 ml. of dry ether. The precipitate was filtered and washed with a mixture of 5 ml. of methanol and 10 ml. of ether. The filtrate was concen-

(27) The aluminum oxide used in this work was prepared by agitating Harshaw alumina with dilute acetic acid until a suspension of the alumina in water showed a *p*H of 6.5. It was activated by heating at 150° for thirty-six hours.

(28) We wish to thank Dr. H. E. Stavely of this Division for his participation in this experiment.

(29) P. A. Levene and F. B. LaForge, *J. Biol. Chem.*, **20**, 431 (1915).

trated to 10 ml., cleared by centrifugation and poured into 75 ml. of dry ether. The oily precipitate was washed with fresh portions of ether and dried *in vacuo* over calcium chloride and potassium hydroxide. The yield was 976 mg. (81%) of an amorphous hygroscopic solid, which started to sinter at 115° and melted at 170–180° (dec.), $[\alpha]^{25}D$ −133° (*c*, 1.06 in methanol).

Anal. Calcd. for $C_{12}H_{19}O_7N(OCH_3)COOCH_3 \cdot HCl$: C, 43.33; H, 6.30; N, 3.37; OCH_3, 14.92; Cl, 8.53. Calcd. for $C_{12}H_{21}O_7N(OCH_3)COOCH_3$: C, 43.12; H, 6.76; N, 3.35; OCH_3, 14.85; Cl, 8.48. Found: C, 41.33; H, 7.01; N, 3.87; OCH_3, 14.94; Cl, 9.22.

The acetylation of the crude, vacuum-dried methyl ester was carried out as described for methyl dihydrostreptobiosaminide. The chloroform residue was taken up in 25 ml. of benzene and chromatographed on 25 g. of alumina. After removing a small amount of amorphous material by washing with benzene, the column was eluted with 300 ml. of a mixture of equal volumes of benzene and ether. The effluent solution was evaporated to dryness *in vacuo* and the residue moistened with absolute alcohol. Crystallization rapidly ensued, yielding an almost pure product. By two recrystallizations from a small volume of absolute alcohol well-shaped prisms melting sharply at 139–140° were obtained, $[\alpha]^{25}D$ −134° (*c*, 1.18 in chloroform).

Anal. Calcd. for $C_{12}H_{15}O_7N(OCH_3)(CH_3CO)_4COOCH_3$: C, 50.45; H, 6.08; N, 2.56; OCH_3, 11.33; N-methyl, 2.74; CH_3CO, 31.42; mol. wt., 547.5. Calcd. for $C_{12}H_{17}O_7N(OCH_3)(CH_3CO)_4COOCH_3$: C, 50.26; H, 6.42; N, 2.55; OCH_3, 11.29; N-methyl, 2.73; CH_3CO, 31.30; mol. wt., 549.5. Found: C, 50.37; H, 6.31; N, 2.68; OCH_3, 11.27; N-methyl, 2.50; CH_3CO, 31.4; mol. wt. (Rast), 494.

In the titration according to Kunz and Hudson 45.15 mg. consumed 3.34 ml. of 0.1 *N* sodium hydroxide. Calcd. for $C_{12}H_{17}O_7N(OCH_3)(CH_3CO)_4COOCH_3$: 3 O-acetyl + 1 $COOCH_3$, 3.29 ml.

An additional amount of this material was obtained by a subsequent elution of the column with 100 ml. of a mixture of two parts of ether and one part of acetone.

Saponification of Methyl Streptobiosaminidic Acid Methyl Ester.—A solution of 594 mg. of methyl streptobiosaminidic acid methyl ester hydrochloride in 6 ml. of 1 *N* alkali was titrated electrometrically with 1 *N* hydrochloric acid after it had been allowed to stand for sixty-seven hours at room temperature. Evaluation of the titration curve showed that 1.33 ml. of alkali had been consumed during the saponification. Calculated: 1.42 ml. The neutralized solution, which had a *p*H of 5.6, was evaporated to dryness and the sodium chloride removed by repeatedly dissolving the residue in dry methanol, centrifuging and concentrating the supernatant solution. The final concentrate yielded methyl streptobiosaminidic acid (XIV) as an amorphous solid, which contained 9.01% methoxyl. Calculated for $C_{12}H_{21}O_7N(OCH_3)COOH$: OCH_3, 8.44.

This product was remethylated by dissolving 300 mg. in 10 ml. of methanol and adding an ethereal solution of diazomethane until the mixture remained permanently yellow. After removal of the solvents *in vacuo* the residue was acetylated with acetic anhydride in pyridine at room temperature. The crude acetylation product was chromatographed on an alumina column, from which a small amount of crystalline product was obtained by elution with a mixture of equal volumes of benzene and ether. The crystals melted at 142–143°. When mixed with a sample of methyl streptobiosaminidic acid methyl ester tetraacetate the melting point was found to be 140–141°.

When the same preparation of methyl streptobiosaminidic acid was acetylated directly and the crude product was worked up in exactly the same manner, no crystals of methyl tetraacetyl-streptobiosaminidic acid methyl ester could be obtained from the chromatogram.

Methyl Streptobiosaminidic Acid Amide (XV).—Methyl streptobiosaminidic acid methyl ester (400 mg.) was dissolved in 5 ml. of methanol which had previously been saturated with ammonia at 0°. After standing in a tightly stoppered vessel for four days at room temperature, the solvent was removed *in vacuo* and the resulting amorphous solid was dried *in vacuo* at 80° over phosphorus pentoxide.

The crude amide from 350 mg. of methyl streptobiosaminidic acid methyl ester was acetylated with acetic anhydride and pyridine at room temperature. The crude acetylation product was dissolved in 50 ml. of a mixture of equal volumes of benzene and ether and chromatographed over 10 g. of alumina. A small amount of ether-soluble material was removed by washing the column with 200 ml. of a mixture of one part of acetone and four parts of ether. The desired product was eluted from the column with 500 ml. of a mixture of one part of acetone and two parts of ether. The residue obtained by removal of the solvents *in vacuo* was dissolved in a minimum of warm ether. Concentration of the ether solution to about one-half of the original volume and cooling in the refrigerator produced a semisolid deposit, which was purified by a second precipitation from ether. The amorphous product melted at 93–103° and had $[\alpha]^{25}D$ −70° (*c*, 1.00 in chloroform). For analysis it was dried over phosphorus pentoxide at room temperature.

Anal. Calcd. for $C_{12}H_{15}O_7N(OCH_3)(CH_3CO)_4CONH_2$: C, 49.58; H, 6.06; N, 5.26; OCH_3, 5.82; N-methyl, 2.82; CH_3CO, 32.30; mol. wt., 532.5. Calcd. for $C_{12}H_{17}O_7N(OCH_3)(CH_3CO)_4CONH_2$: C, 49.39; H, 6.41; N, 5.24; OCH_3, 5.80; N-methyl; 2.81; CH_3CO, 32.18; mol. wt., 534.5. Found: C, 48.70; H, 6.58; N, 4.96; OCH_3, 5.68; N-methyl, 2.17; CH_3CO, 28.2; mol. wt. (Rast), 525.

Summary

Streptomycin possesses an aldehyde group, which resides in the maltol-forming moiety $C_6H_{8-10}O_5$. The term streptonose is proposed for this entity.

Catalytic hydrogenation of streptomycin yields dihydrostreptomycin, $C_{21}H_{39-41}O_{12}N_7$, in which the aldehydic group is reduced to a carbinol group. The helianthate and reineckate of this substance were obtained in crystalline form.

Dihydrostreptomycin trihydrochloride possesses an antibiotic potency of about 700 units/mg. against *K. pneumoniae.*

Dihydrostreptomycin is degraded by methanolic hydrogen chloride to streptidine and methyl dihydrostreptobiosaminide, $C_{13}H_{22-24}NO_8(OCH_3)$. The latter compound forms a crystalline pentaacetate. Methyl dihydrostreptobiosaminide is also obtained by hydrolysis of dihydrostreptomycin with aqueous acid and conversion of the resulting dihydrostreptobiosamine into the methyl glycoside. A third procedure involves the hydrolysis of methyl streptobiosaminide dimethyl acetal to methyl streptobiosaminide and catalytic hydrogenation of the latter.

Streptomycin is oxidized by bromine water to the corresponding acid, streptomycinic acid, which is antibiotically inactive.

Streptomycinic acid in the presence of methanolic hydrogen chloride is degraded to streptidine and methyl steptobiosaminidic acid methyl ester, $C_{12}H_{19-21}N(OCH_3)COOCH_3$, which forms a crystalline tetraacetate. Methyl streptobiosaminidic acid methyl ester can be saponified with dilute cold alkali to the corresponding acid, which is partially remethylated by the action

of diazomethane. Methyl streptobiosaminidic acid has been further characterized by the preparation of the amide and its tetraacetate.

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[CONTRIBUTION FROM THE RESEARCH LABORATORY OF GALAT CHEMICAL DEVELOPMENT, INC.]

An Improved Synthesis of *dl*-Lysine

BY ALEXANDER GALAT

The von Braun synthesis of *dl*-lysine,[1] which has been improved recently by Eck and Marvel,[2] presents one of the most satisfactory ways of preparing this amino-acid. The key intermediate in this synthesis is ε-aminocaproic acid. This is benzoylated, the benzoyl derivative converted with phosphorus and bromine to the α-bromo acid and the latter ammonolyzed and finally hydrolyzed to *dl*-lysine.

We have simplified this method by using sulfuryl chloride in the halogenation of ε-benzoylaminocaproic acid and proceeding *via* the α-chloro acid. In contrast to phosphorus and bromine, which produce a violent exothermic reaction and form mixtures of such a consistency that efficient stirring is impossible, sulfuryl chloride reacts at moderate temperatures, mildly, non-exothermically and forms a homogeneous solution. Moreover, the yields with phosphorus and bromine are erratic and vary between 60 and 90%, whereas sulfuryl chloride consistently gives yields of 96–98%. Finally, the use of sulfuryl chloride is by far more economical than that of bromine of which considerable amounts are required in the von Braun synthesis.

These improvements, together with the fact that ε-caprolactam and ε-aminocaproic acid have recently become commercially available, render the preparation of large amounts of *dl*-lysine convenient and economical.[3]

Experimental

ε-Benzoylamino-caproic Acid.—Sixty-seven and eight-tenths g. (0.6 mole) of ε-caprolactam, 48 g. (1.2 mole) of sodium hydroxide and 300 ml. of water were refluxed for thirty minutes.[4] The mixture was cooled in an ice-bath and 86.5 g. (0.62 mole) of benzoyl chloride was added dropwise with stirring and keeping the temperature at 20°. When the addition was finished, the mixture was stirred for an additional fifteen minutes. It was then diluted to 1500 ml. with water and made acid by the addition of an excess of hydrochloric acid (1:1). The acid must be added slowly at the beginning, otherwise the material may precipitate as an oil or in large lumps. Seeding and cooling helps to obtain it in fine crystals. The mixture is allowed to stand in an ice-bath for two and one-half hours whereupon the crystals were filtered off, washed Cl^- free and dried at 40–50°; yield, 132 g. (93.5%), m. p. 75–78°.

ε-Benzoylamino-α-chlorocaproic Acid.—Thirty-five and three-tenths grams (0.15 mole) of ε-benzoylaminocaproic acid and 0.5 g. of finely powdered iodine were dissolved in 70 ml. of sulfuryl chloride and heated under reflux in a water-bath at 60–65°. There was a copious evolution of gas which slowed down after one to one and one-half hours. During the next one and one-half hours the bath was gradually brought to the boiling point and the reaction was complete. The excess sulfuryl chloride was removed by distillation and the residue shaken with 120 ml. of water, decanted and washed with two portions of hot water, 120 ml. each. The crude chloro-acid is a pale yellow solid which was dried *in vacuo* and weighed 38.7–39.32 g. (96–97.5%), m. p. 115–125°. It was used directly, without further purification, in the next step. A small additional amount of material crystallized out from the mother liquors used in the washing of the crude acid.

Pure ε-benzoylamino-α-chlorocaproic acid obtained by recrystallization from a very dilute aqueous solution melted at 145–147° and had a neutral equivalent of 268–269 (theory, 269.5).

Anal. Calcd. for $C_{13}H_{16}O_3NCl$: C, 57.9; H, 5.94; N, 5.2; Cl, 13.17. Found: C, 57.5; H, 6.0; N, 5.3; Cl, 12.9.

***dl*-ε-Benzoyllysine.**—Two and two-tenths grams of crude chloro-acid was dissolved in 40 ml. of concentrated ammonia and heated in a pressure-bottle for seven and one-half hours at 85–90°. The mixture was then evaporated *in vacuo* to dryness, the residue taken up in a small amount of water, filtered, washed with water followed by methanol and dried; yield 1.30 g. (60% of ε-benzoylaminocaproic acid), m. p. 265–270° (dec.).

dl-ε-Benzoyllysine was converted to *dl*-lysine hydrochlorides according to the procedures described in the literature.[1,2]

Summary

An economical and convenient procedure for the preparation of *dl*-lysine is described. It consists in converting ε-caprolactam or ε-aminocaproic acid to ε-benzoylaminocaproic acid, chlorinating the latter with sulfuryl chloride in the presence of iodine, ammonolyzing the resulting α-chloro acid and hydrolyzing to *dl*-lysine.

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(1) von Braun, *Ber.*, **42**, 839 (1909).

(2) Eck and Marvel, *J. Biol. Chem.*, **106**, 387 (1934); "Organic Syntheses," Coll. Vol. 2, John Wiley and Sons, New York N. Y., 1943.

(3) ε-Caprolactam and ε-aminocaproic acid are available from E. I. du Pont de Nemours & Co., Inc.; sulfuryl chloride from Hooker Electrochemical Company.

(4) When an equivalent amount of ε-aminocaproic acid is used, this step, of course, is not necessary.