316. *The Streptothricin Group of Antibiotics. Part I. The General Structural Pattern.*

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An antibiotic mixture produced by *Streptomyces lavendulae* has been shown to consist of six compounds, one of which is streptothricin, and another streptothricin containing an additional β-lysine unit. Hydrolyses and periodate oxidations have indicated how the main fragments of these antibiotics are linked. Apart from the precise nature of the group which causes the evolution of carbon dioxide and ammonia after very mild hydrolysis, a complete structure of streptothricin is suggested, as well as its general relation to the other members of this group of antibiotics.

STREPTOTHRICIN, first isolated by Waksman and Woodruff in 1942,[1] is a basic water-soluble antibiotic produced by *Streptomyces lavendulae*. Since its discovery, a number of related antibiotics have been isolated, including geomycin,[2] mycothricin,[3] pleocidin,[4] streptin,[5] streptolin,[6] racemomycin,[7] racemomycin O,[8] and roseothricin.[9] All are active against a wide spectrum of Gram positive and Gram negative bacteria, fungi, and viruses, but are also highly nephrotoxic. Streptothricin was isolated by adsorption and solvent extraction, and, although it has not been obtained crystalline, crystalline derivatives have been reported, *e.g.*, the reineckate [10] and the helianthate.[11] Streptothricin picrate [12] and sulphate [13] have been used in other isolation procedures. The antibiotic is stated to contain three basic groups (pK_a 7·1, 8·2, and 10·1) and to have a molecular formula $C_{20}H_{34}N_8O_9$ (amended to $C_{19}H_{34}N_8O_8$ in the present paper). Colour tests suggested a peptide structure; *O*-, *C*-, and *N*-methyl groups were absent and there was only end absorption in the ultraviolet spectrum. Hydrolyses and other published degradation reactions of streptothricin are discussed below.

[*Added, December 11th*, 1961.] Since this paper was submitted a series of notes have appeared by Carter, and van Tamelen and their collaborators (*J. Amer. Chem. Soc.*, 1961, **83**, 4287, 4295, 4296) in which they reach a similar conclusion as to the structure of streptothricin with the exception of the relative positions of the amino- and hydroxy-groups in the streptolidine side-chain. Our component D is evidently equivalent to their streptolin in containing two β-lysine units.

[1] Waksman and Woodruff, *Proc. Soc. Exp. Biol. Med.*, 1942, **49**, 207.
[2] Brockmann and Musso, *Chem. Ber.*, 1954, **87**, 1779.
[3] Schaffner, Rangaswami, and Waksman, 3rd Intern. Congr. Biochem., Brussels, 1955; Résumés communs., p. 95.
[4] Charney, Roberts, and Fischer, *Antibiotics and Chemotherapy*, 1952, **2**, 307.
[5] Woodruff and Foster, *J. Bact.*, 1946, **52**, 502.
[6] Rivett and Peterson, *J. Amer. Chem. Soc.*, 1947, **69**, 3006.
[7] Taniyama and Takemura, *J. Pharm. Soc. Japan*, 1957, **77**, 1210, 1215; 1958, **78**, 742.
[8] Taniyama and Takemura, *Chem. and Pharm. Bull. (Japan)*, 1960, **8**, 150.
[9] Nakanishi, Ito, and Hirata, *J. Amer. Chem. Soc.*, 1954, **76**, 2845.
[10] Fried and Wintersteiner, *Science*, 1945, **101**, 613.
[11] Kuehl, Peck, Walti, and Folkers, *Science*, 1945, **102**, 34.
[12] Peck, Walti, Graber, Flynn, Hoffhine, Allfrey, and Folkers, *J. Amer. Chem. Soc.*, 1946, **68**, 772.
[13] Carter, Clark, Kohn, Rothrock, Taylor, West, Whitfield, and Jackson, *J. Amer. Chem. Soc.*, 1954, **76**, 566.

Meanwhile the development of more efficient chromatographic methods revealed that several of the antibiotics of the streptothricin group, formerly believed to be single substances, were in fact mixtures, *e.g.*, the separation of streptolins A and B,[14] and the isolation of three components from racemomycin [7] and from roseothricin [15] by chromatography and paper electrophoresis, and by ion-exchange chromatography.[16] Possibly the best chromatographic technique for assessing the purity of compounds of the streptothricin group is that developed by Horowitz and Schaffner,[17] using circular paper chromatography and a solvent system developed from that of Brockmann and Musso [2] in the separation of the geomycins.

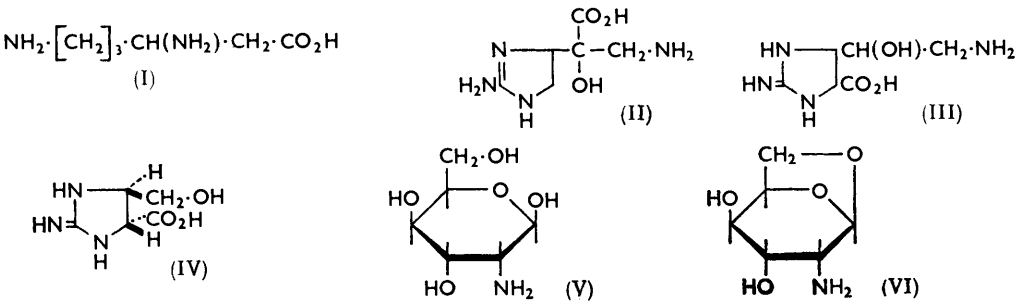
Through the courtesy of Dr. M. Lumb and his colleagues of Boots Pure Drug Company, we have been able to examine a preparation, A 8265, which has proved to be a mixture of antibiotics of the streptothricin group. Preparation A 8265 was isolated from the broth filtrate by carbon adsorption, elution with methanolic hydrogen chloride, neutralisation, and evaporation.[17a] Further purification was achieved through the picrate and hydrochloride. The colour reactions of the product corresponded to those of streptothricin, and negative ferric, Brady, Fehling's, and Schiff's reactions demonstrated the absence of free phenolic or aldehydic groupings. Circular paper chromatography, with the solvent system of Horowitz and Schaffner,[17] showed the presence of six ninhydrin-positive spots which were named A to F in the order of ascending R_F values, component D being present in the largest amount. Samples of streptothricin (Upjohn), pleocidin (Merck), streptolin (Professor E. van Tamelen), and geomycin (Professor Brockmann) were run parallel and gave results similar to those of Horowitz and Schaffner. Streptothricin appeared to correspond to our component F and, apart from geomycin, all the antibiotics examined contained appreciable amounts of it; moreover with the exception of streptothricin they all contained also a high proportion of our component D. Components D and F were each separated from the mixture by chromatography of the antibiotic sulphate on a column of powdered cellulose, n-propanol–pyridine–acetic acid–water (15 : 10 : 3 : 12) being used as developing solvent. Each component was tested for antibacterial activity against a number of organisms, and component D was more active than component F which had an antibacterial spectrum almost identical with that of streptothricin.

Component F and component D have been examined in detail from a structural viewpoint. They were converted into the hydrochlorides by way of the picrates and it was found that the infrared spectrum of component F hydrochloride ($[\alpha]_D = -47°$) was superimposable on that of streptothricin hydrochloride ($[\alpha]_D = -50{\cdot}1°$). When an aqueous solution of component F (streptothricin) hydrochloride (pH *ca.* 2·5) was kept for about one week at room temperature, a degradation product was observed whose R_F value was identical with that of component D hydrochloride; after about two weeks the conversion was complete. However, this compound was biologically inactive and therefore differed from component D; it was provisionally named compound X. Similar treatment of component D hydrochloride gave a compound with an R_F value corresponding to component B, but this biologically inactive reaction product has not been examined in detail. Component D, isolated by chromatography of the original antibiotic complex, always contained some of the deactivated component F, but they could be separated by chromatography using phenol–water systems, and quantitatively by countercurrent distribution between phenol and water.

Most of the knowledge of the structure of streptothricin has been derived from studies of its hydrolysis. Under mild conditions,[13] it yielded carbon dioxide and then ammonia. More vigorous hydrolysis produced three ninhydrin-positive compounds which could

[14] Larson, Sternberg, and Peterson, *J. Amer. Chem. Soc.*, 1953, **75**, 2036.
[15] Hosoya, Soeda, and Arai, *J. Antibiotics*, 1951, **4**, 313.
[16] Goto, Hirata, Hosoya, and Komatsu, *Bull. Chem. Soc. Japan*, 1957, **30**, 304, 729.
[17] Horowitz and Schaffner, *Analyt. Chem.*, 1958, **30**, 1616.
[17a] Lumb, Chamberlain, Cross, Macey, Spyvee, Uprichard, and Wright, paper in preparation.

be separated by chromatography on paper or a column of cellulose.[13] With t-butyl-alcohol–acetic acid–water (2 : 1 : 1) as solvent, the first to be eluted was an amino-acid, the second was a cyclic guanidine derivative, and the third, a weakly reducing product referred to as compound C [13] or compound B [16] (compound Y in the present paper), proved to be a condensation product of the cyclic guanidine with an amino-sugar.



The Amino-acid.—This was a diaminohexanoic acid ($C_6H_{14}N_2O_2$) which did not react with periodate and was not an α-amino-acid. The structure, suggested independently by Carter [18] and van Tamelen,[19] was 3,6-diaminohexanoic acid (I) but it is generally named β-lysine, and has been synthesised.[20] The same β-amino-acid has also been isolated from hydrolyses of viomycin,[21] and from other members of the streptothricin group, *e.g.*, streptolin,[22] racemomycin O,[23] geomycin,[2,24] roseothricin,[9,16] and mycothricin.[3]

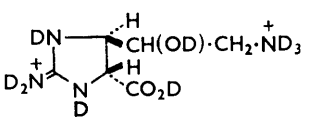
The Cyclic Guanidine Derivative.—This also has been isolated from several antibiotics of the group, and has been variously named streptolidine [13] (from streptolin and streptothricin), geamine [24] (from geomycin), and roseonine [9] (from roseothricin and racemomycin). Analyses indicated a molecular formula $C_6H_{12}N_4O_3$ and, from its physical and chemical properties, Nakanishi [9] suggested the 2-aminoimidazoline structure (II, or the 5-substituted imidazoline). Others, *e.g.*, Brockmann and Musso,[24] felt that alternative arrangements of the functional groups of the side-chain had not been eliminated satisfactorily, although in later papers [25,26] Nakanishi has reported further studies, especially on infrared spectra, which were claimed to support structure (II). However, Carter and McNary [27] have recently produced evidence which necessitates that structure (II) for streptolidine should be modified to the imidazolidine (III). Oxidation of streptolidine with 1 mole of periodate, followed by reduction, gave *threo*-5-hydroxymethyl-2-iminoimidazolidine-4-carboxylic acid (IV), which was synthesised and identified by paper-chromatographic comparisons. The remaining ambiguity in structure (III) for streptolidine on this basis is the relative positions of the hydroxyl and amino-groups in the side-chain, and our studies on this are described below.

The Amino-sugar.—The isolation of a ninhydrin-positive reducing fraction from the hydrolysates of both streptothricin and streptolin was reported in 1956 [28] (see also ref. 16). Two related compounds were isolated which were identified as 2-amino-2-deoxy-α-D-gulose (V) (D-2-gulosamine) and its 1,6-anhydro-derivative (VI). Degradation of the former by means of ninhydrin gave D-xylose, and this, with certain other properties, established

[18] Carter, Hearn, Lansford, Page, Salzman, Shapiro, and Taylor, *J. Amer. Chem. Soc.*, 1952, **74**, 3704.
[19] van Tamelen and Smissman, *J. Amer. Chem. Soc.*, 1952, **74**, 3713.
[20] van Tamelen and Smissman, *J. Amer. Chem. Soc.*, 1953, **75**, 2031.
[21] Haskell, Fusari, Frohardt, and Bartz, *J. Amer. Chem. Soc.*, 1952, **74**, 599.
[22] Smissman, Sharpe, Aycock, van Tamelen, and Peterson, *J. Amer. Chem. Soc.*, 1953, **75**, 2029.
[23] Takemura, *Chem. and Pharm. Bull.* (*Japan*), 1960, **8**, 154.
[24] Brockmann and Musso, *Chem. Ber.*, 1955, **88**, 648.
[25] Goto, Nakanishi, and Ohashi, *Bull. Chem. Soc. Japan*, 1957, **30**, 723.
[26] Nakanishi and Ohashi, *Bull. Chem. Soc. Japan*, 1957, **30**, 725.
[27] Carter and McNary, *Diss. Abs.*, 1960, **20**, 4525.
[28] van Tamelen, Dyer, Carter, Pierce, and Daniels, *J. Amer. Chem. Soc.*, 1956, **78**, 4817.

the degradation product as the first naturally occurring derivative of D-gulose. The structures (V) and (VI) have now been established by synthesis.[29] Racemomycin O [23] is unique in this group of antibiotics in that it is claimed to contain D-2-glucosamine rather than D-2-gulosamine.

Vigorous hydrolysis of preparation A 8265 showed that three ninhydrin-positive compounds were produced which corresponded to β-lysine, streptolidine, and combined streptolidine–D-2-gulosamine (compound Y corresponding to compound C of Carter *et al.*[13]). The hydrolysis mixture was separated on a column of Amberlite IRC-50 ion-exchange resin, and both β-lysine and streptolidine were obtained as their picrates. Free D-2-gulosamine was destroyed under these conditions but by milder hydrolysis it was obtained by chromatography on Dowex 50 × 8 resin. After further purification on a charcoal–Celite column (van Tamelen, private communication), the crystalline amino-sugar hydrochloride was obtained. Ninhydrin degradation [30] gave xylose as observed previously.[28] Of the three major hydrolysis products of streptothricin, the structures of both β-lysine and D-2-gulosamine are considered to be correct. Although we have observed 1,6-anhydro-D-2-gulosamine on chromatograms of streptothricin hydrolysates, it is believed to be an artefact and it is readily formed by heating the amino-sugar with 6N-hydrochloric acid. In an attempt to identify the side-chain of streptolidine, we had the nuclear magnetic resonance spectrum determined by Dr. R. J. Abraham of the National Physical Laboratory, Teddington, and we are grateful to him for his observations. The dihydrochloride was dissolved in trifluoroacetic deuteroacid which exchanged protons for deuterons on the nitrogen and oxygen but not carbon atoms, *i.e.*, indicating structure (VII) or (VIII). If the former were correct, the lowest field peak would be a complex signal from the single proton on the carbon adjacent to the hydroxyl group, whereas the latter should give a



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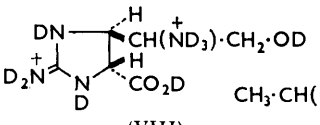

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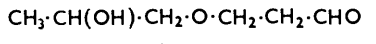


(IX)

low-field doublet due to the methylene group. The presence of a low field doublet (τ 6·05) in the spectrum favoured structure (VIII).

Components F (streptothricin) and D were hydrolysed with 6N-hydrochloric acid at 100° and the two hydrolysates were examined separately. Measurement of the intensities (with a self-recording optical densitometer) of the absorption of the ninhydrin colours produced by the respective hydrolysates indicated that the β-lysine : streptolidine ratio was 1 : 1 in streptothricin but 2 : 1 in component D. Thus, increase in this ratio decreases the R_F value of the antibiotic, which accords with the observations on the geomycin [31] complex, one of the components of which contains a tetra-β-lysine peptide and has a very low R_F value.

The next problem was to discover how the various components, β-lysine, streptolidine, and D-2-gulosamine, were linked to each other, and a study of the partial hydrolysis of streptothricin was undertaken following the methods described by Goto *et al.*[16] The slow conversion of an aqueous solution of streptothricin hydrochloride (pH *ca.* 2·5) at room temperature into compound X has already been mentioned. Hydrolysis of streptothricin hydrochloride with 3N-hydrochloric acid under reflux caused the rapid (complete after *ca.* 10 min.) evolution of 1 mole of carbon dioxide (based on a C_{19} formulation of streptothricin). At this stage the major product was compound X, but further hydrolysis produced free β-lysine, streptolidine, and D-2-gulosamine, as well as a second partial

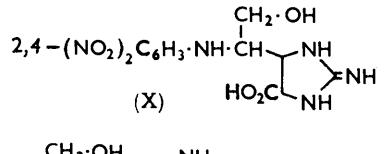
[29] Tarasiejska and Jeanloz, *J. Amer. Chem. Soc.*, 1957, **79**, 4215; Kuhn and Bister, *Annalen*, 1958, **617**, 92.
[30] Stoffyn and Jeanloz, *Arch. Biochem. Biophys.*, 1954, **52**, 373.
[31] Brockmann and Cölln, *Chem. Ber.*, 1959, **92**, 114.

hydrolysis product, compound Y. More vigorous conditions caused the break-down of compound Y and the decomposition of D-2-gulosamine. No ether-soluble material corresponding to racemonic aldehyde [23] (IX), a hydrolysis product of racemomycin O, was observed at any stage. A mild-acid hydrolysate of streptothricin was fractionated on a column of Dowex-50 and the elution with 2·5N-hydrochloric acid carried out at −5° to preclude further hydrolysis of the products. After D-2-gulosamine hydrochloride, ammonium chloride was obtained (0·8 mole of ammonia per mole of streptothricin on a C_{19} formulation), and then a β-lysine fraction followed by a mixture of β-lysine and streptolidine. Finally compound Y was obtained as a discrete band. Compound X was obtained by subsequent elution with 4N-hydrochloric acid.

Compound Y, obtained as a crystalline hydrochloride, was hydrolysed with 6N-hydrochloric acid under reflux and gave streptolidine and a trace of D-2-gulosamine, which is known to decompose under these conditions. Reaction of compound Y with 1-fluoro-2,4-dinitrobenzene [16] and hydrolysis of the 2,4-dinitrophenyl derivative gave a mono-(2,4-dinitrophenyl)streptolidine (X), in which the side-chain amino-group had been arylated, and some *N*-(2,4-dinitrophenyl)-α-D-gulosamine. Thus the side-chain amino-group of the streptolidine fragment and the amino-group of the gulosamine fragment were free in compound Y, which had no reducing properties and consequently contained a glycosidic linkage involving $C_{(1)}$ of the sugar fragment. The remaining structural problem is therefore the position of linkage of the streptolidine in compound Y, and it was first established that it did not involve the carboxyl or the hydroxyl group. Addition of excess of trimethylamine to the hydrochloride of compound Y caused a band at 1736 cm.$^{-1}$ (free carboxyl) in the infrared spectrum to be replaced by one at 1600 cm.$^{-1}$ (carboxylate ion). This indicates that the carboxyl group is not involved in the linkage with gulosamine and is free in compound Y. Structure (XI), an *O*-guloside (cf. the partial structure for geomycin [31]), for compound Y was eliminated because it should result in an initial uptake of 2 moles of periodate, whereas a compound with the favoured structure (XII) would be expected to consume 3 moles, which was found to be the case, although a fourth mole was consumed after about two days and probably represents the slow oxidation of the streptolidine ring. Periodate oxidation of streptolidine itself caused the rapid consumption of the first mole of reagent and a slow consumption of a second mole.

Consequently compound Y contained the streptolidine fragment linked through one of the three guanidine nitrogen atoms. From Nakanishi's [25] rules for the derivation of the degree of substitution of guanidines from the number of bands in the infrared spectrum around 1650 cm.$^{-1}$, the single band at 1665 cm.$^{-1}$ in the spectrum of compound Y is interpreted as favouring a symmetrical trisubstituted guanidine, *i.e.*, structure (XII). On the other hand Carter and McNary [27] favour an *N*-guloside structure (XIII) for their compound C, which resembles our compound Y although the reported infrared bands in the 1650 cm.$^{-1}$ region do not correspond exactly. The corresponding product from roseothricin A [16] is believed to contain gulosamine and to be a dimer (XIV) formed by an ether linkage between the $C_{(6)}$ carbon atoms of the two hexosamines, although the evidence for this is tenuous.

On the basis of structure (XII) for compound Y, it is now possible to consider the larger fragment, compound X, from streptothricin. This substance, which has the same R_F value as component D of the A 8265 complex in the solvent system propanol–pyridine–acetic acid–water (15 : 10 : 3 : 12), is produced by very mild hydrolysis of streptothricin. It is best purified from streptothricin hydrolysates by chromatography on Dowex 50 as it is the only compound not eluted by 2·5N-hydrochloric acid and the acid must be 4N before compound X is obtained. Hydrolysis of compound X gives β-lysine, streptolidine, and a small amount of gulosamine (largely decomposed under the hydrolysis conditions). Like Y, compound X has a peak in the infrared spectrum near 1736 cm.$^{-1}$ which is absent after reaction with trimethylamine, and is therefore associated with a free carboxyl group. Reaction of compound X with 1-fluoro-2,4-dinitrobenzene and subsequent hydrolysis gave



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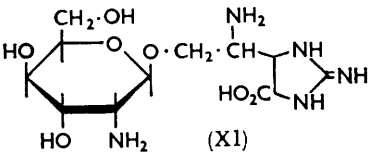
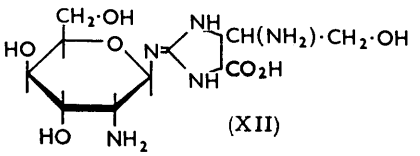
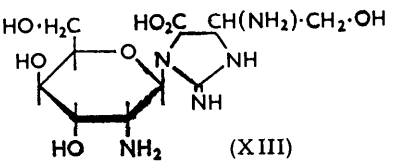


(XI)

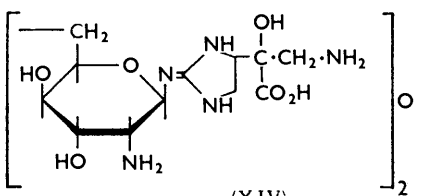


(XII)

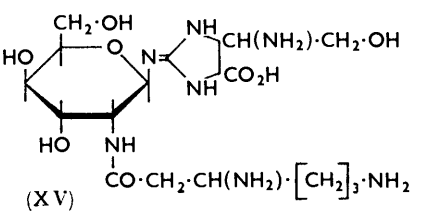


(XIII)

mono-(2,4-dinitrophenyl)streptolidine (X), *NN'*-bis-(2,4-dinitrophenyl)-β-lysine, and free gulosamine. These results indicate that compound X (XV) is formed by the condensation



(XIV)



(XV)

of β-lysine with compound Y through an amide-linkage involving the carboxyl group of β-lysine and the amino-group of the gulosamine fragment. Periodate oxidation of compound X, followed by hydrolysis, gave only β-lysine as a recognisable fragment; and, as expected from structure (XV), neither streptolidine nor gulosamine was observed.

Compound X is formed from streptothricin by very mild hydrolysis during which 1 mole of carbon dioxide and one of ammonia are eliminated. The precise nature of the grouping which is involved in this reaction has not yet been established but a urethane structure presumably as a substituent on either the 3- or 4-hydroxyl group of the amino-sugar such as is found in novobiocin [32] is an obvious possibility. However, hydrolytic fission of the grouping in streptothricin occurs more readily than that of the novobiocin urethane grouping although this may be caused by steric factors. A peak at 1712 cm.$^{-1}$ in the infrared spectrum is unaffected by brief treatment of the antibiotic with trimethylamine and from this and a potentiometric titration it appears that a free carboxyl group is not present. Reaction of streptothricin with 1-fluoro-2,4-dinitrobenzene followed by hydrolysis gives *NN'*-bis-(2,4-dinitrophenyl)-β-lysine together with free streptolidine and D-2-gulosamine, and hence the side-chain amino-group of streptolidine is also substituted in streptothricin. This is supported by the lack of any rapid reaction of streptothricin with periodate.

Complete hydrolysis of component D of the A 8265 complex has been referred to already, and the ratio of streptolidine to β-lysine has been shown to be 1 : 2. Partial hydrolysis of component D with 3N-hydrochloric acid under reflux gave carbon dioxide, ammonia, β-lysine, streptolidine, D-2-gulosamine, compounds X and Y, and a compound Z with an R_F value, in the usual solvent system, just less than that of streptolidine. Finally, there was obtained another compound of very low R_F value. These products were separated by chromatography on Dowex-50; gulosamine and ammonium chloride were eluted first with 2·5N-hydrochloric acid, and were followed successively by β-lysine, a mixture of β-lysine and streptolidine, compound Y, and finally compound Z as a separate band. This product, which had chromatographic properties similar to the partial

[32] Hoeksema, Caron, and Hinman, *J. Amer. Chem. Soc.*, 1956, **78**, 2019.

hydrolysis product S_2 of geomycin,[31] gave only β-lysine on hydrolysis and consequently the probable structure is an N^{ω}-β-lysyl-β-lysine.

The slow-moving component observed in the partial hydrolysis of component D is probably compound X (XV) but containing two β-lysine units instead of one and component D would bear the same structural relation to this as streptothricin does to compound X.

The structural pattern thus obtained for these antibiotics of the streptothricin group is therefore the linked streptolidine–D-2-gulosamine fragment, to which is attached the small grouping giving rise to carbon dioxide and ammonia on hydrolysis, as well as a varying number of β-lysine units: one in streptothricin (identical with our component F and probably roseothricin A), two in component D, and up to four in geomycin. Another variant in the molecules will be the nature of the amino-sugar if the Japanese claim [23] to have isolated glucosamine from racemomycin O is upheld. Experiments designed to establish the complete structure of streptothricin are in progress.

Experimental

Isolation of Crude Antibiotic A 8265.—The brew (300 gallons) from a strain of Streptomyces, A8265, was clarified by filtration through kieselguhr at pH 5. Biologically active material was absorbed from the filtrate on active carbon and eluted with methanolic hydrogen chloride at pH 3. After filtration, neutralisation, and evaporation, water was added and the solution freeze-dried to give a crude solid (750 g.), which was active against *Micrococcus pyogenes* var. *aureus* at a dilution of 1 in 10^4.

Partial Purification of the Crude Antibiotic as its Salts.—The crude solid (100 g.) was treated with water (120 ml.) and stirred for 1 hr.; the solid was filtered off and washed with water (*ca.* 50 ml.), and the combined filtrate and washings were treated with saturated picric acid solution (2 l.). After being kept overnight, the mixture deposited a gum which was separated by decantation and dissolved in acetone (80 ml.). Saturated aqueous picric acid (75 ml.) was added to cause incipient turbidity, followed by more picric acid (10 ml.), and the solution, decanted from the precipitated gum, was poured into saturated picric acid (2 l.) and water (200 ml.) and kept at 0° overnight. The resulting gum was collected and reprecipitated by the same procedure three more times, to yield a yellow sticky solid. This was triturated with ethanol and the solid formed (1·2 g.) was washed with ethanol and ether. It could not be crystallised.

The picrate (10 g.) was dissolved in acetone (50 ml.) and treated with concentrated hydrochloric acid (5 ml.) which caused immediate precipitation of a pale brown hydrochloride. This was separated, dissolved in water (10 ml.), and reprecipitated with acetone. The hydrochloride was again removed and the operation repeated until all of the picric acid had been removed and the aqueous solution was almost colourless. It was then freeze-dried, giving a hygroscopic solid (5·3 g.), $[\alpha]_D^{20}$ −27°. It was further purified by chromatography of a methanolic solution of this product (5 g.) on acid-washed alumina (100 g.), the elution being effected with methanol. The biologically active hydrochloride (1·5 g.) had $[\alpha]_D^{20}$ −40° and gave positive colour tests with the ninhydrin, Elson–Morgan, biuret, Fehling, van Slyke, and Weber reagents, an atypical reaction with the Pauly reagent, and negative colour tests with ferric chloride, Brady, Sakaguchi, Molisch, Schiff, and Ehrlich reagents. This behaviour was exactly the same as that of streptothricin hydrochloride (kindly provided by Merck and Co.).

Acid Hydrolysis of Antibiotic A 8265 *Hydrochloride. β-Lysine and Streptolidine Dipicrates.*—The hydrochloride (10 g.) of antibiotic A 8265 was dissolved in 6N-hydrochloric acid (50 ml.), and the solution heated under reflux for 24 hr. The hydrolysate was distilled under reduced pressure and the residue dried (NaOH) *in vacuo* for 16 hr. The hydrolysate was dissolved in water (40 ml.), heated under reflux (charcoal) for 5 min., and filtered. The pale yellow filtrate was shaken with Amberlite IR-45 (OH^- form) for 1 hr. during which time the pH of the solution increased from 2 to 8. The resin was separated and the filtrate distilled *in vacuo* to give a pale yellow solid (8·3 g.).

An aqueous solution (25 ml.) of the hydrolysate was brought on to a column (60 × 2 cm.) of

Amberlite IR5-50 (Na^+ form), and eluted with water at the rate of 1 ml./min. Fractions (12 ml.) were collected and tested for amino-compounds with saturated phosphotungstic acid solution. Fractions 2—13 contained amino-compounds but fraction 14 did not and the solvent was changed to 0·1N-hydrochloric acid. The next 47 fractions (500 ml. in all) all gave positive phosphotungstic acid reactions, but after fraction 60 amino-compounds were not detected, even with N-hydrochloric acid as eluent. Each of the fractions containing amino-compounds was tested on a descending paper chromatogram using t-butyl alcohol–acetic acid–water (2 : 1 : 1) as the solvent and ninhydrin to locate the products. Fractions 1—13 (after acidification with hydrochloric acid) had R_F 0·43; 14—50, R_F 0·43; 51—53, R_F 0·32 and 0·43, and 54—59, R_F 0·32 with a faint spot at R_F 0·20. Authentic β-lysine dihydrochloride and streptolidine (= roseonine) dihydrochloride, run on the same chromatograms, had R_F 0·43 and 0·32, respectively.

Fractions 1—50 (600 ml.) were bulked and adjusted to pH 7 with 10% sodium hydroxide solution. The solution was evaporated to dryness and the residue extracted with methanol (100 ml.) at room temperature. The extract was filtered and evaporated *in vacuo* to a pale yellow syrup (4·82 g.). This was dissolved in water (50 ml.), and the solution heated to boiling, whereupon picric acid was added until the hot solution was saturated. On cooling yellow needles of β-lysine dipicrate crystallised and after two recrystallisations from water the picrate (3·51 g.) had m. p. 204°, not depressed on admixture with an authentic specimen (kindly provided by Professor Nakanishi). The infrared spectra of the two specimens were identical [Found: C, 36·0; H, 3·25; N, 18·7. Calc. for $C_6H_{14}O_2N_2,(C_6H_3O_7N_3)_2$: C, 35·75; H, 3·35; N, 18·55%]. Fractions 54—60 (90 ml.) were bulked, adjusted to pH 7, and evaporated to dryness. The residue was extracted with methanol and treated with picric acid as for fractions 1—50. The picrate was treated with hot water and most of it dissolved leaving a brown gum which was removed. Orange prisms (1·5 g.), m. p. 238°, were obtained from the filtrate [Found: C, 33·3; H, 3·1; N, 21·8. Calc. for $C_6H_{12}O_3N_4,(C_6H_3O_7N_3)_2$: C, 33·45; H, 2·8; N, 22·1%]. A mixed melting point with authentic roseonine dipicrate (from Professor Nakanishi) showed no depression, and the infrared spectra of the two samples were superimposable. Professor van Tamelen has confirmed that the specimen is identical with streptolidine dipicrate.

NN′-*Bis*-(2,4-*dinitrophenyl*)-β-*lysine*.—β-Lysine dihydrochloride (20 mg.) and sodium hydrogen carbonate (60 mg.) were dissolved in 50% ethanol (5 ml.) and shaken with a 5% ethanolic solution of 1-fluoro-2,4-dinitrobenzene (1 ml.) for 24 hr. at room temperature. The solution was acidified (HCl) and extracted with ethyl acetate. The ethyl acetate layer was then washed with water and extracted with 5% aqueous potassium carbonate. The aqueous layer was acidified with hydrochloric acid; the precipitate, washed and crystallised from methanol–acetone, formed yellow needles, m. p. 196° (lit.,[16] 195—196°) (Found: C, 44·9; H, 3·65. Calc for $C_{18}H_{18}N_6O_{11}$: C, 45·2; H, 3·8%).

Streptolidine Dihydrochloride.—Streptolidine dipicrate (1 g.) was suspended in water (20 ml.) and treated with concentrated hydrochloric acid (1 ml.). Ether (20 ml.) was added and the mixture shaken until all the picrate had dissolved. The aqueous layer was extracted with ether (2 × 20 ml.). The solution of the dihydrochloride was concentrated (to 5 ml.) and acetone added to the warm solution until precipitation commenced. Streptolidine dihydrochloride (255 mg.) was obtained, m. p. 220° (lit.[9] 215°), $[\alpha]_D^{20}$ +50° (in water) (Found: C, 27·7; H, 5·65; Cl, 26·7; N, 21·5. Calc. for $C_6H_{12}N_4O_3,2HCl$: C, 27·65; H, 5·4; Cl, 27·15; N, 21·45%). The infrared spectrum had principal bands at 1740, 1689, and 1582 $cm.^{-1}$. Potentiometric titration showed pK_a values of 2·5, 9·0, and 11·5. The hydrochloride gave positive tests with ninhydrin and the Weber reagent but negative tests with the Sakaguchi, Elson–Morgan, biuret, and Schiff reagents.

In oxidation of an aqueous solution of the dihydrochloride with sodium metaperiodate, 0·95 mol. of reagent was consumed after 15 min., 1·04 mol. after 30 min., 1·57 mol. after 12 hr., and 1·89 mol. after 24 hr. During repetition of the oxidation for 30 min. 0·88 mole of ammonia was evolved per mole of streptolidine dihydrochloride and in a similar oxidation 1·01 mol. of formaldehyde (estimated as the dimedone derivative, m. p. 190°) was also evolved. The main product from the periodate oxidation of streptolidine dihydrochloride was an amorphous solid which could not be further purified.

N-(2,4-*Dinitrophenyl*)*streptolidine*.—Streptolidine dihydrochloride (20 mg.) and sodium hydrogen carbonate (45 mg.) were dissolved in 50% ethanol (5 ml.) and treated with a 5%

ethanolic solution of 1-fluoro-2,4-dinitrobenzene (0·8 ml.). The mixture was shaken for 24 hr. at room temperature, added to water (5 ml.), acidified with hydrochloric acid, and extracted with ethyl acetate. The aqueous layer was concentrated (to 2 ml.). The pH was adjusted to *ca.* 5 with sodium acetate and *N*-(2,4-dinitrophenyl)streptolidine slowly formed yellow needles, m. p. 230° (decomp.) (lit.,[16] 226—229°).

D-2-*Amino-2-deoxygulose* (*Gulosamine*) *Hydrochloride.*—In preliminary small-scale experiments, antibiotic A 8265 hydrochloride was hydrolysed under mild conditions and the products examined by chromatography on paper by the descending technique, t-butyl alcohol–acetic acid–water (2 : 1 : 1) being used as solvent. Duplicate chromatograms were developed with ninhydrin (amino-compounds) and a 5% aqueous solution of triphenyltetrazolium chloride followed by N-sodium hydroxide (for amino-sugars), and exposure of the paper to steam. Under the most favourable conditions for the production of the amino-sugar, hydrolysis with 3N-hydrochloric acid at 100° for ½ hr., two products which reacted with both reagents were obtained: R_F 0·39 (D-2-amino-2-deoxygulose) and 0·20 (compound Y; see below), and three products which reacted only with ninhydrin: R_F 0·15 (compound X; see below), 0·32 (streptolidine dihydrochloride), and 0·43 (β-lysine dihydrochloride). Of the two amino-sugar spots, that with high mobility (R_F 0·39) always appeared before that with R_F 0·20 when using the triphenyltetrazolium chloride reagent.

The antibiotic A 8265 hydrochloride (10 g.) was dissolved in 3N-hydrochloric acid and heated under reflux for 30 min. during which time the colour of the solution changed from pale yellow to brown. The solvent was removed by distillation and the residue kept overnight *in vacuo* over sodium hydroxide.

Dowex-50 × 8 (200—400 mesh) was slurried into a column (60 × 2 cm.) with 0·6N-hydrochloric acid and the column washed for 24 hr. with 0·6N-acid. The dried hydrolysate, dissolved in water (30 ml.), was applied to the column which was eluted with 0·6N-hydrochloric acid at the rate of 1 ml./min. Fractions (25 ml.) were collected and tested with ninhydrin, alkaline triphenyltetrazolium chloride, and Nessler's reagent. Ninhydrin-positive fractions were spotted on paper chromatograms and the paper developed with t-butyl alcohol–acetic acid–water. Fractions 11—33 gave positive reactions with ninhydrin and the triphenyltetrazolium chloride reagent but a negative reaction with Nessler's reagent, and in the chromatogram appeared as a single spot, R_F 0·39. Fractions 51—70 gave a positive Nessler reaction but failed to react with the other reagents. Fractions 51—70 contained ammonium chloride (1·06 g.). Fractions 11—33 were distilled *in vacuo*, giving a pale yellow syrup (803 mg.). This (*ca.* 150 mg.), dissolved in water (5 ml.), was applied to a column (40 × 1·2 cm.) of charcoal–Celite-545 (1 : 1), and eluted with water. The flow rate was 3—4 ml./hr. The effluent gave positive reactions with ninhydrin and with the triphenyltetrazolium chloride reagent after approximately 250 ml. had been collected. All the ninhydrin-positive material was eluted in a small fraction which was taken to dryness and yielded crystals (35 mg.), m. p. 158—160° (decomp.) (from methanol–ethanol) (lit.,[28] m. p. of D-2-amino-2-deoxygulose hydrochloride, 152—162°). The remainder of the syrup (600 mg.) was crystallised from methanol–ethanol by seeding the solution with crystals obtained from the charcoal–Celite chromatography. The crystalline hydrochloride (120 mg.) had $[\alpha]_D^{20}$ −19·2° (water) (lit.,[28] −18·7°) (Found: C, 32·9; H, 6·7; N, 6·75. Calc. for $C_6H_{13}NO_5$,HCl: C, 33·4; H, 6·55; N, 6·5%).

Ninhydrin Degradation of the Hexosamine (cf. ref. 30).—The hexosamine (5 mg.) was dissolved in water (0·1 ml.) and an excess of a 2% solution of ninhydrin in water containing 4% of pyridine was added. The mixture was sealed into a capillary and heated in boiling water for ½ hr. It was examined on a paper chromatogram, with the product from similar treatment of glucosamine hydrochloride, and samples of arabinose, ribose, lyxose, and xylose. The paper was developed by descending flow of n-butyl alcohol–ethanol–water (4 : 1 : 1), dried, and treated with alkaline silver nitrate solution (as modified by Trevelyan *et al.*[33]). The R_F values were: arabinose, 0·20; xylose, 0·23; lyxose, 0·25; ribose, 0·27; glucosamine degradation product, 0·20; hexosamine degradation product, 0·23.

Separation of Antibiotic A 8265 into Individual Components.—Chromatography on paper. Circular paper chromatograms (Whatman No. 1; 24 cm. diam.) were developed from a central wick of rolled filter paper with n-propanol–pyridine–acetic acid–water[17] (15 : 10 : 3 : 12). After about 3 hr. the papers were dried, sprayed with a 0·2% solution of ninhydrin in acetone

[33] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.

and heated for 10 min. at 100°. Antibiotic A 8265 hydrochloride and sulphate were both chromatographed in this manner and the components were named from A to F in the order of ascending R_F values. The intensity of the ninhydrin bands was measured by an optical densitometer. The figures quoted for each component are the R_F values from the chromatography of the antibiotic hydrochloride and sulphate respectively: A (7%) 0·20, 0·17; B (18%) 0·24, 0·20; C (18%) 0·29, 0·27; D (30%) 0·35, 0·32; E (9%) 0·40, 0·36; F (18%) 0·48, 0·43. A number of known antibiotics of the streptothricin group were examined as their hydrochlorides in this solvent system and all consisted of one or more of the components of antibiotic A 8265 (identical R_F values).

TABLE.

Composition of some antibiotics of the streptothricin group.

	A	B	C	D	E	F
	Percentage optical density of bands at R_F:					
Antibiotic and source	0·20	0·24	0·29	0·35	0·40	0·48
Streptothricin 8804 (Upjohn) ...	0	0	0	0	0	100
Pleocidin L532748 (Merck)	0	0	0	32	32	36
Pleocidin 272 (Merck)	0	10	10	40	30	20
Streptolin (van Tamelen)	0	10	0	40	0	50
Geomycin (Brockmann)	10	40	20	30	0	0

Cellulose-column Chromatography of Antibiotic A 8265 *Sulphate.*—Standard Whatman ashless cellulose (3800 g.) was dry-packed in a column (diam. 10 cm.), and tamped down after each addition (10 g.). The final length of the column was 86 cm. Antibiotic A 8265 sulphate (8 g.) was dissolved in n-propanol–pyridine–acetic acid–water (15 : 10 : 3 : 12) (20 ml.) and applied to the column. After 16 hr., solvent started to flow from the column at the rate of 90 ml./hr., and fractions (45 ml.) were collected. The first 30 were all dark brown but from 31 onwards were colourless. Each was extracted with ether (50 ml.) and the aqueous phase (5 ml.) separated and freeze-dried. The solid so obtained was dissolved in water (0·5 ml.) and chromatographed on paper. A solution of antibiotic A8265 sulphate (50 mg./ml.) was used as a control and the chromatogram was developed for 16 hr. by descending chromatography, the same solvent being used. From these chromatograms, fractions giving identical ninhydrin-positive spots were bulked and again freeze-dried. After 300 hr. the eluent no longer contained ninhydrin-positive compounds and the elution was stopped. All the bulked solids were dissolved in water (50 mg./ml.) and applied to circular paper chromatograms.

The following bulked fractions were obtained: 1, fractions 1—30, R_F 0·43, component F (383 mg.); 2, fractions 31—147, R_F 0·43, component F (655 mg.); 3, fractions 148—190, R_F 0·43, 0·36, components E + F (354 mg.); 4, fractions 191—215, R_F 0·36, component E (527 mg.); 5, fractions 220—256, R_F 0·32, component D (793 mg.); 6, fractions 257—310, R_F 0·32, 0·27, components C + D (1407 mg.); 7, fractions 311—330, R_F 0·27, component C (520 mg.); 8, fractions 331—371, R_F 0·27, 0·20, components B + C (635 mg.); 9, fractions 372—419, R_F 0·20, component B (273 mg.); 10, fractions 420—462, R_F 0·20, 0·17, components A + B (199 mg.); 11, fractions 463—570, R_F 0·17, component A (180 mg.).

Purification of Hydrochlorides of Components D and F.—Bulk solid 2 (655 mg.) was dissolved in water (*ca.* 2 ml.), and concentrated aqueous picric acid (50 ml.) was added. A gum which was deposited after about 4 hr. was separated by decantation of the mother liquor, and dissolved in acetone (5 ml.). This solution was treated with concentrated hydrochloric acid (4 drops), and a gum separated during the night at 0°. It was separated by decantation and dissolved in methanol (5 ml.). Addition of an excess of acetone precipitated an amorphous white solid which was recovered by centrifugation and decantation. This was repeated until the solution of the hydrochloride in methanol was colourless. The white solid (450 mg.) had $[\alpha]_D^{20}$ −47°. Potentiometric titration gave pK_a values of 7·6, 8·7, and 10·2. The procedure was repeated with bulk solid 5 (component D). The reineckate of component F crystallised from warm (45°) water as fine needles [Found: C, 25·65; H, 4·5; Cr, 10·55; N, 23·75. Calc. for $C_{20}H_{34}N_8O_9,(C_4H_7N_6S_4Cr)_3$: C, 25·2; H, 3·95; Cr, 10·4; N, 23·75%].

Stability of Aqueous Solutions of the Hydrochlorides of Components F and D.—Aqueous solutions of the two hydrochlorides (pH 2·5) when kept at room temperature decompose to some extent to give new ninhydrin-positive products. The solutions were tested at intervals

on circular paper chromatograms, n-propanol–pyridine–acetic acid–water (15 : 10 : 3 : 12) being used, and on descending chromatograms, phenol–water (4 : 1) being used with a 1% aqueous solution of sodium cyanide in a beaker in the chromatography tank. Thus, a solution of component F initially had R_F 0·48 and 0·2 respectively, in the two solvent systems, but after 1 week it had R_F 0·48, 0·35, and 0·20, 0·03. Similarly, component D hydrochloride initially had R_F 0·35, and 0·18, 0·03, respectively, but after 1 week it had R_F 0·35, 0·24, and 0·18, 0·10, 0·03. This demonstration, by chromatography in phenol–water, that component D was contaminated with compound X (R_F 0·18 and 0·03) led to the use of this solvent system for separation by countercurrent distribution (following experiment).

Countercurrent Distribution of Crude Component D.—Liquid phenol (850 ml.) was equilibrated with 0·1% aqueousammonium formate (2550 ml.), and the top phase had a pH of 5·2. Equal volumes of bottom phase (25 ml.) were placed in the first 21 tubes of a countercurrent machine. Top phase (25 ml.) was put into the first 5 tubes. A solution of the hydrochloride (500 mg.) of component D was converted into the free base by treatment with Amberlite IR-45 (OH^- form), taken to dryness, and dissolved in the solution in the second tube. After 20 transfers, portions (0·2 ml.) of top and bottom phases were treated with the ninhydrin solution (1 ml.) described by Moore and Stein,[34] heated for ½ hr. in boiling water; the absorption at 575 mμ showed two distinct maxima at tubes 2 and 18. Tubes 1—6 were treated with an excess of ether and the aqueous phase separated, extracted twice with ether, and freeze-dried to give a pale brown solid (80 mg.). Tubes 14—20 gave a colourless solid (310 mg.). On chromatography in phenol–water (4 : 1), the first solid (tubes 1—6) had R_F 0·20 (component D) and the second (tubes 14—20) had R_F 0·03 (compound X, see below).

Total Hydrolysis of Components D and F.—Each component (5 mg.), dissolved n 6N-hydrochloric acid (0·5 ml.), was sealed in a capillary tube and heated in boiling water for 30 hr. The solutions were evaporated to dryness *in vacuo* over sodium hydroxide. They were then chromatographed alongside solutions of mixtures of β-lysine and streptolidine dihydrochlorides (1 : 1, 2 : 1, 3 : 1; molar). After descending development the chromatogram was dried and sprayed with a 0·2% solution of ninhydrin in butanol containing 10% of pyridine, and then heated for 10 min. at 110°. This produced five pairs of purple spots, one of each pair due to streptolidine (R_F 0·32) and the other due to β-lysine (R_F 0·43). Their intensities were measured with a densitometer; the chromatogram was scanned at right angles to the direction of the moving solvent and the intensity of absorption was plotted against the position on the chromatogram in each case. The curves had twin peaks due to the pairs of spots and the area under the curve corresponding to each peak was measured. The ratio of the two areas was calculated in each case and it was seen that the component F hydrolysate was identical with a 1 : 1 molar ratio of β-lysine : streptolidine, and component D hydrolysate was identical with a 2 : 1 molar ratio of β-lysine : streptolidine.

Partial Hydrolysis of Component F.—Component F hydrochloride (100 mg.) was dissolved in 3N-hydrochloric acid (10 ml.). A stream of carbon dioxide-free nitrogen was passed through the solution and then through saturated barium hydroxide solution. The hydrolysis solution was heated under reflux and, immediately heating commenced, barium carbonate was precipitated, but after the first 10 min. no more carbon dioxide was evolved. The precipitated barium carbonate (27·7 mg.) corresponded to 1·01 mole of carbon dioxide per mole of $C_{19}H_{34}N_8O_8$. The products of several hydrolyses were located on descending chromatograms, t-butyl alcohol–acetic acid–water (2 : 1 : 1) being used: component F hydrochloride, R_F 0·25; hydrolyses with 3N-hydrochloric acid for (*a*) 24 hr. at 23°, R_F 0·15; (*b*) 10 min. at 100°, R_F 0·15, 0·43; (*c*) 2 hr. at 100°, R_F 0·15, 0·20, 0·32, 0·39, 0·43; hydrolyses with 6N-hydrochloric acid for (*a*) 8 hr. at 100°, R_F 0·20, 0·32 and 0·43; (*b*) 40 hr. at 100°, R_F 0·32, 0·43; β-lysine dihydrochloride, R_F 0·43; streptolidine dihydrochloride, R_F 0·32; gulosamine hydrochloride, R_F 0·39. The partial hydrolysis product of R_F 0·15 has been named compound X, and the product of R_F 0·20 has been named compound Y.

Separation of the Products from the Partial Hydrolysis of Component F.—Component F hydrochloride (400 mg.) was dissolved in 3N-hydrochloric acid (10 ml.) and refluxed for 2 hr. under nitrogen. Paper chromatography showed the presence of β-lysine, streptolidine, gulosamine, compound Y, and compound X. The hydrolysate was evaporated to dryness and kept over sodium hydroxide *in vacuo* for 24 hr.

[34] Moore and Stein, *J. Biol. Chem.*, 1948, **176**, 367.

Dowex-50 × 8 (*ca.* 200 g.; 200—400 mesh) was treated with water, 2N-sodium hydroxide, water, and 2N-hydrochloric acid. The resin was slurried into a column (60 × 2 cm.) and washed with 2·5N-hydrochloric acid for 2 days. The hydrolysate was dissolved in water (4 ml.) and applied to the column which was kept at −5°. It was eluted with 2·5N-hydrochloric acid (25 ml./hr.). Fractions were collected for ½ hr. at a time and each was tested with ninhydrin, Weber's reagent, Nessler's reagent, and alkaline triphenyltetrazolium chloride. Alternate fractions were reduced to dryness and tested on paper chromatograms, t-butyl alchol–acetic acid–water (2 : 1 : 1) being used. Fractions 18—21 (25 mg.), gulosamine hydrochloride, gave positive reactions with ninhydrin and triphenyltetrazolium chloride, and had R_F 0·39. Fractions 24—27 (30 mg.), ammonium chloride, 0·8 mol. of ammonia per mol. of $C_{19}H_{34}N_8O_8$. Fractions 89—95 (43 mg.), β-lysine dihydrochloride, gave a positive reaction with ninhydrin and had R_F 0·43. Fractions 96—110 (168 mg.), a mixture of β-lysine dihydrochloride and streptolidine dihydrochloride, gave positive reactions with ninhydrin and the Weber reagent and had R_F 0·43, 0·32. Fractions 114—125 (111 mg.), compound Y, gave positive reactions with ninhydrin, triphenyltetrazolium chloride, and the Weber reagent, and had R_F 0·20. Fractions 151—170 (41 mg.), compound X, was eluted with 4N-hydrochloric acid. It gave positive reactions with ninhydrin and the Weber reagent and had R_F 0·15.

Compound Y.—Fractions 114—125 from the above separation were bulked and taken to dryness. The pale brown residue (111 mg.) was dissolved in methanol (2 ml.), the solution filtered, and acetone added to the hot methanolic solution to cause incipient precipitation. Crystals of compound Y hydrochloride, m. p. 240° (decomp.), were slowly obtained. The infrared spectrum showed main bands at 1404m, 1504m, 1616s, 1665s, 1736m, and a broad band at 3310—2920s cm.$^{-1}$ (Found: N, 14·9. Calc. for $C_{12}H_{22}N_5O_7$,3HCl: N, 15·3%).

Hydrolysis of Compound Y.—Compound Y hydrochloride (5 mg.) was dissolved in 6N-hydrochloric acid (0·5 ml.), sealed in a capillary tube, and heated in boiling water for 3 hr. The hydrolysate was evaporated to dryness *in vacuo* over sodium hydroxide. The residue, dissolved in water (0·1 ml.), was applied to two chromatograms alongside authentic β-lysine dihydrochloride, streptolidine dihydrochloride, and gulosamine hydrochloride. The chromatograms were developed by the descending technique, t-butyl alcohol–acetic acid–water (2 : 1 : 1) being used as solvent. They were dried, one was sprayed with 0·2% solution of ninhydrin in butanol and the other with a 5% solution of triphenyltetrazolium chloride followed by N-sodium hydroxide. The R_F values were: β-lysine dihydrochloride, 0·43; streptolidine dihydrochloride, 0·32; gulosamine hydrochloride, *0·39*; compound Y hydrochloride, 0·20; compound Y hydrolysate, *0·20*, 0·32, and *0·39*. All the spots gave a positive ninhydrin reaction, and those whose R_F values are in italics also gave a positive reaction with alkaline triphenyltetrazolium chloride. The spot with R_F value 0·39 in compound Y hydrolysate was faint.

Hydrolysis of the 2,4-Dinitrophenyl Derivative of Compound Y.—Compound Y hydrochloride (20 mg.) and sodium hydrogen carbonate (50 mg.) were dissolved in 50% ethanol (10 ml.), and shaken with ethanolic 1-fluoro-2,4-dinitrobenzene in ethanol (5%; 0·6 ml.) for 2 days at 23°. A yellow precipitate of the 2,4-dinitrophenyl derivative of compound Y was produced. The solution was acidified with hydrochloric acid and the precipitate washed with water, ethanol, and ether. The 2,4-dinitrophenyl derivative (10 mg.) was treated with 6N-hydrochloric acid (1 ml.), sealed in a capillary tube, and heated in boiling water for 24 hr. The hydrolysate was dried *in vacuo* over sodium hydroxide, and a solution of the residue examined on a paper chromatogram alongside *N*-(2,4-dinitrophenyl)streptolidine and *N*-(2,4-dinitrophenyl)gulosamine (prepared from D-2-gulosamine by the method given for the streptolidine derivative). The chromatogram was developed by descending flow of butanol–acetic acid–water (4 : 1 : 5). The hydrolysate gave two spots with R_F values identical with *N*-(2,4-dinitrophenyl)streptolidine (0·80) and *N*-(2,4-dinitrophenyl)gulosamine (0·75), the latter being of low intensity. The chromatogram showed no colour with ninhydrin.

Periodate Oxidation of Compound Y Hydrochloride.—Compound Y hydrochloride was oxidised with periodate as described for the oxidation of streptolidine dihydrochloride. 2·93 Mol. of reagent were consumed after ½ hr., 2·98 mol. after 1 hr., 3·41 mol. after 24 hr., and 3·85 mol. after 48 hr.

Compound X.—Fractions 157—170 from the ion-exchange resolution of component F hydrolysate (see above) were bulked and taken to dryness. The resulting solid (41 mg.) was dissolved in methanol (1 ml.), filtered, and precipitated by the addition of an excess of acetone (5 ml.). Compound X hydrochloride was a white amorphous powder whose R_F value was

identical with that of component D hydrochloride (0·35) on circular chromatograms developed with n-propanol–pyridine–acetic acid–water (15 : 10 : 3 : 12). It gave positive ninhydrin, Elson–Morgan, Weber, and biuret reactions, but negative reactions with the triphenyltetrazolium and Schiff's reagent.

Hydrolysis of Compound X.—Compound X hydrochloride (5 mg.) was dissolved in 6N-hydrochloric acid (0·5 ml.), sealed in a capillary tube and heated in boiling water for 3 hr. The hydrolysate was evaporated to dryness *in vacuo* over sodium hydroxide. The residue was dissolved in water (0·1 ml.) and introduced on to two chromatograms alongside β-lysine dihydrochloride, streptolidine dihydrochloride, gulosamine hydrochloride, and compound Y hydrochloride. The chromatogram was developed and sprayed as for the hydrolysis product from compound Y hydrochloride, and the R_F values were: β-lysine dihydrochloride, 0·43; streptolidine dihydrochloride, 0·32; gulosamine hydrochloride, *0·39*; compound Y hydrochloride, *0·20*; compound X hydrochloride, 0·15; compound X hydrolysate, 0·15, *0·20*, 0·32, *0·39*, 0·43. The values in italics are those which gave positive reactions with both ninhydrin and alkaline triphenyltetrazolium chloride. The spot corresponding to gulosamine (R_F = 0·39) in compound X hydrolysate was faint.

Hydrolysis of the 2,4-Dinitrophenyl Derivative of Compound X.—The 2,4-dinitrophenyl derivative of compound X was prepared and hydrolysed in the same way as that of compound Y. Paper chromatography with butanol–acetic acid–water (4 : 1 : 5) showed that the hydrolysate consisted of two compounds having R_F values identical with those of *N*-(2,4-dinitrophenyl)-streptolidine (0·80) and *NN'*-bis-(2,4-dinitrophenyl)-β-lysine (0·85). The chromatogram was sprayed with ninhydrin and heated; a faint spot appeared with an R_F value (0·10) identical with that of gulosamine hydrochloride in this system.

Hydrolysis of the 2,4-Dinitrophenyl Derivative of Component F.—Component F hydrochloride (100 mg.) and sodium hydrogen carbonate (300 mg.) were dissolved in 50% ethanol (40 ml.), and shaken with ethanolic 1-fluoro-2,4-dinitrobenzene (5%; 6 ml.) for 2 days at 23°. A yellow precipitate which formed was washed with water, ethanol, and ether, dissolved in tetrahydrofuran, and re-precipitated with ether. The 2,4-dinitrophenyl derivative of component F so formed was hydrolysed with 6N-hydrochloric acid for 3 hr. at 100°. Paper chromatography of the hydrolysate showed the presence of *NN'*-bis-(2,4-dinitrophenyl)-β-lysine, free streptolidine, and a small amount of D-2-gulosamine.

Periodate Oxidation of Component F Hydrochloride.—Component F hydrochloride was subjected to periodate oxidation under the same conditions as those used for the oxidation of streptolidine (above); 0·03 mol. of reagent was consumed after ½ hr., 0·10 mol. after 1 hr., 1·02 mol. after 24 hr., and 1·63 mol. after 48 hr.

Partial Hydrolysis of Component D.—Component D hydrochloride (100 mg.), which had been freed from compound X by countercurrent distribution of crude component D (above), was dissolved in 3N-hydrochloric acid (10 ml.) and heated under reflux for 2 hr. in a stream of nitrogen. The effluent gas was bubbled through barium hydroxide, and barium carbonate was precipitated during the first 10 min. of hydrolysis, as in that of component F. The hydrolysate was evaporated to dryness and kept over sodium hydroxide *in vacuo* for 24 hr. Descending paper chromatograms of the hydrolysate in t-butyl alcohol–acetic acid–water (2 : 1 : 1) showed β-lysine, streptolidine, gulosamine, compound Y, compound X, a new partial hydrolysis product (compound Z) with an R_F value (0·28) just below that of streptolidine, and also a partial-hydrolysis product with a very low R_F (0·05).

The hydrolysate was resolved by ion-exchange chromatography on a column (60 × 2 cm.) of Dowex 50 × 8, which was eluted with 2·5N-hydrochloric acid at a rate of 25 ml./hr. The following fractions (½ hr. elution each) were collected and identified by colour reactions and paper chromatography: fraction 21 (5 mg.), gulosamine hydrochloride; fractions 27—28 (6 mg.), ammonium chloride; fractions 91—97 (10 mg.), β-lysine dihydrochloride; fractions 98—105 (35 mg.), β-lysine dihydrochloride + streptolidine dihydrochloride; fractions 116—120 (30 mg.), compound Y; fractions 122—123 (5 mg.), compound Z, R_F 0·28. Compound Z gave a positive ninhydrin reaction.

Hydrolysis of Compound Z.—A solution of compound Z (5 mg.) in 6N-hydrochloric acid (0·5 ml.) was heated in a capillary tube in boiling water for 3 hr. The hydrolysate was evaporated to dryness *in vacuo* over sodium hydroxide. The residue was dissolved in water (0·1 ml.) and examined on chromatograms alongside β-lysine dihydrochloride, streptolidine dihydrochloride, gulosamine hydrochloride, compound Y, and compound X. The R_F values

were: β-lysine dihydrochloride, 0·43; streptolidine dihydrochloride, 0·32; gulosamine hydrochloride, *0·39*; compound X hydrochloride, 0·15; compound Y hydrochloride, *0·20*; compound Z hydrochloride, 0·28; compound Z hydrolysate, 0·28, 0·43. Only the spots with R_F values in italics gave positive reactions with alkaline triphenyltetrazolium chloride.

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