REVIEW ARTICLE

THE CHEMISTRY AND BIOCHEMISTRY OF STREPTOMYCIN AND RELATED COMPOUNDS

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Streptomycin

The discovery of penicillin was due to a chance observation by Sir Alexander Fleming of the inhibition and lysis of staphylococcal colonies on an agar plate by a mould contaminant. Streptomycin, on the other hand, was discovered as the result of a deliberate search begun in 1939 by Waksman and his associates. They undertook the examination and testing of a large number of micro-organisms, and looked particularly for evidence of activity against Gram-negative bacteria. The first potentially useful antibiotic resulting from this investigation was streptothricin, but this was found to be too toxic for general use. The discovery of streptomycin soon followed and was first published[1] in 1944. The amount of work since undertaken on all aspects of streptomycin testifies to the importance of this antibiotic in chemotherapy.

The experience gained in the production of penicillin and the large scale plant available for conducting submerged mould fermentations permitted rapid progress in the building up of stocks of streptomycin which were necessary for adequate clinical trials and the assessment of its value in medicine.

Organism. Two active strains of the actinomycete producing streptomycin, namely *Streptomyces griseus*, were isolated from heavily manured soil and from a chicken's throat respectively[1,2]. The organism was somewhat unstable and gave rise to inactive variants although it was later found that by suitable treatment these strains could be made to revert to an active form[3]. This difficulty may arise in large scale production of streptomycin where the maintenance of the activity of a high yielding strain is naturally of extreme importance. The fact that streptomycin inhibits the growth of inactive but not of active strains can be made use of in the isolation of active variants. Methods similar to those used for the production of strains of *Penicillium notatum* giving high yields of penicillin, e.g. X-ray or ultra-violet irradiation can be applied to the development of strains of *Streptomyces griseus* of high activity. The existence of a phage capable of lysing cells of *S. griseus* was reported by Saudek and Colingsworth[4] and has since been confirmed by other workers. This phenomenon can be a source of considerable anxiety in streptomycin production. However, active strains resistant to the phage can be developed.

Production of streptomycin. Although streptomycin can be produced by means of surface cultures, and some was in fact so produced in the

earlier stages of its development, the surface culture method has now been abandoned in favour of deep fermentation as in the case of penicillin. The best method of keeping the spores is in soil. To produce an active inoculum the spores are sown on an agar medium and when sufficient growth has occurred the spores are washed off, often with the use of a wetting agent, e.g. "calsolene." The spore suspension is used to inoculate a rich liquid medium containing casein hydrolysate and meat extract. The heavy mycelial growth which develops in 1 to 2 days is transferred to a larger seed tank or to the fermentation tank, which usually contains a cheaper medium. During all these operations asepsis is maintained by steam sterilisation of media and all parts of the apparatus from which infection might arise. Sterile filtered air is introduced into the tanks during fermentation. When fermentation is complete the mycelium is separated from the liquor by means of rotary drum vacuum filters with the addition of kieselguhr to aid filtration. The streptomycin is removed from the fermentation liquor by adsorption on charcoal which is washed with neutral alcohol to remove impurities and then the streptomycin is eluted with acid alcohol. Evaporation of the acid alcohol leaves an aqueous concentrate used for preparation of a crystalline complex. Whilst the free base does not crystallise it forms crystalline double salts. The complex with calcium chloride was first employed by the Merck workers[5] and has proved highly successful in practice. The crystalline product is difficult to obtain sterile, it is therefore dissolved in water, contaminating organisms and pyrogens are removed by passage through a biological filter and the product is freeze-dried, giving a sterile powder. It is filled aseptically into vials which are then sealed and packed.

The above is merely a broad general outline of the process, much detailed information as to the exact method used by the individual producers has not been divulged. In some cases purification is accomplished by means of base exchange reagents, e.g. permutit. Certain dyestuffs have also been proposed as adsorbents, e.g. naphthol black and orange II. The latter is said to be used on a commercial scale in America.

Unit and assay. The first unit officially recognised for streptomycin was defined as that quantity which will inhibit a given strain of *E. coli* in 1 ml. of nutrient broth or agar. When streptomycin was obtained in a pure state the potency of a concentrate came to be expressed in terms of its equivalent in weight of pure streptomycin base. By chance 1 μg. of the base is equal to 1 unit in the former nomenclature.

For the biological assay of streptomycin the turbidimetric method is frequently employed in America with *Klebsiella pneumoniæ* as test organism. The cylinder plate or cup plate method is commonly used in Britain with *B. coli* or *B. subtilis.*

Various methods of chemical assay have been proposed. Boxer, Jelinek and Leghorn[6] used a colorimetric method for streptomycin assay in clinical preparations, urine and broth. This was based on the formation of maltol by the action of alkali and its subsequent determination colorimetrically by means of the phenol reagent of Folin and Ciocalteu[7]

or by means of ferric ammonium sulphate. This method is specific for the streptose portion of the streptomycin molecule which must contain an intact carbonyl group and must be linked glycosidically to another group. In the assay in urine or broth the maltol is separated by chloroform extraction from the bulk of interfering substances.

Other methods depend on the formation of derivatives of the carbonyl group. Boxer and Jelinek[8] used a fluorimetric method for the assay of streptomycin in blood and cerebrospinal fluid. The method is based on the formation of a hydrazone of streptomycin with the fluorescent 9-hydrazinoacridine hydrochloride. The excess reagent together with the hydrazones of acidic neutral and weakly basic compounds is separated from the strongly basic streptomycin hydrazone by extraction from acid solution with benzyl alcohol. This method was later applied to urine[9]. Marshall, Blanchard and Buhle[10] used a similar method depending on condensation with the coloured 4-[4-(*p*-chlorophenylazo)-1-naphthyl]-semicarbazide to give a semicarbazone which is estimated colorimetrically.

Chemistry of Streptomycin

Properties. Streptomycin is water-soluble, and is a relatively strong base since its hydrochloride gives an almost neutral solution in water. It is quite stable between the *p*H limits of 3 and 7 and relatively so between *p*H 1 and *p*H 10. It gives no reaction for primary amino nitrogen but gives a Sakaguchi reaction indicating the presence of a guanidine group. It also reacts with carbonyl reagents and is thereby inactivated. Several crystalline salts and complexes of streptomycin have been prepared. Streptomycin reineckate, plates, m.pt. 164° to 165°C. (decomp.), helianthate, m.pt. 220° to 226°C. (decomp.), and the double salt of the trihydrochloride with calcium chloride, decomp. 200° to 230°C. are crystalline. The hydrochloride is a white amorphous powder, $[\alpha]_D$ −84°. More recently the crystalline naphthalene β-sulphonates of streptomycin and dihydrostreptomycin have been described[11]. Analysis of the salts led to the acceptance of the formula $C_{21}H_{37(39)}O_{12}N_7$ for streptomycin. The actual number of hydrogen atoms present was only settled by analysis of certain degradation products which indicated that the H_{39} formula was correct.

Derivatives

(a) *Dihydrostreptomycin.* Hydrogenation of streptomycin trihydrochloride in presence of platinum or palladium catalyst in aqueous solution affords amorphous dihydrostreptomycin trihydrochloride, m.pt. 190° to 195°C. (decomp.), $[\alpha]_D^{25}$ −94·5° (in water). The trihelianthate of dihydrostreptomycin is crystalline, m.pt. 224° to 230°C. (decomp.). Analysis of these compounds indicates that dihydrostreptomycin has the formula $C_{21}H_{41}O_{12}N_7$ agreeing with the uptake of 2 atoms of hydrogen as observed in the hydrogenation.

(b) *Streptomycinic acid.* Oxidation of streptomycin trihydrochloride with bromine water gave an amorphous product, m.pt. 231°C. (decomp.)

$[\alpha]_D^{25}$ $-92°$. It had the formula $C_{21}H_{39}O_{13}N_7$, 2HCl, i.e., it contained 1 atom of oxygen more than streptomycin and revealed on electrometric titration an acidic group of *p*K 2·35 as well as a basic group of *p*K 7·85. Since neither dihydrostreptomycin nor streptomycinic acid gives reactions for the carbonyl group which is present in streptomycin, these conversions indicate the presence of an aldehydo group in streptomycin. Whilst dihydrostreptomycin has a biological activity similar to that of streptomycin, streptomycinic acid shows no such activity.

DEGRADATION

The brilliant chemical investigations which led to the elucidation of the main outline of the structure of streptomycin were undertaken largely by three groups of workers, that headed by Folkers at the Research Laboratories of Merck and Co. Inc., New Jersey, the team of Wintersteiner at the Squibb Institute for Medical Research, and Carter and his colleagues at the University of Illinois. With such a large molecule as that of streptomycin it was obviously desirable to degrade it into smaller fragments which could be investigated separately. This fission was achieved by dilute acid. On acid hydrolysis the molecule is cleaved hydrolytically giving rise to two new bases, streptidine and streptobiosamine as follows:—

$$\underset{\text{Streptomycin}}{C_{21}H_{39}O_{12}N_7} + H_2O \rightarrow \underset{\text{Streptidine}}{C_8H_{18}O_4N_6} + \underset{\text{Streptobiosamine}}{C_{13}H_{23}O_9N}$$

Streptidine. For the isolation of streptidine various methods of acid hydrolysis of streptomycin have been used.

1. Streptomycin hydrochloride was hydrolysed with 10 per cent. aqueous hydrochloric acid for 2 hours at 120°C. The dry residue after evaporation was extracted with ethanol. After evaporation to dryness the residue was dissolved in water and the crystalline streptidine dipicrate was precipitated by addition of picric acid[12].

2. Streptomycin hydrochloride was dissolved in methanol containing 3 per cent. (by volume) of concentrated sulphuric acid. After several days at 25°C. streptidine sulphate crystallised out[12].

3. Streptomycin sulphate was hydrolysed with 4 parts of N sulphuric acid for 48 hours at 45°C. Streptidine sulphate separated in crystalline form[13].

4. Streptomycin hydrochloride was hydrolysed by N methanolic hydrochloric acid for 24 hours at room temperature and the streptidine hydrochloride was precipitated by addition of 2 volumes of ether and converted into the sulphate or picrate[14].

Streptidine sulphate crystallises from methanol or water with one mol. of solvent of crystallisation, $C_8H_{18}O_4N_6,H_2SO_4,CH_3OH$ and $C_8H_{18}O_4N_6,H_2SO_4,H_2O$. Streptidine also forms a methanolic dihydrochloride, $C_8H_{18}O_4N_6$, 2HCl, CH_3OH, m.pt. 170° to 210°C. Streptidine is thus a di-acidic base. It is optically inactive and gives no reaction for primary amino, carbonyl or carboxyl groups, but it contains OH and

NH groups as indicated by the infra-red spectrum and its formation of an octa-acetyl derivative[12].

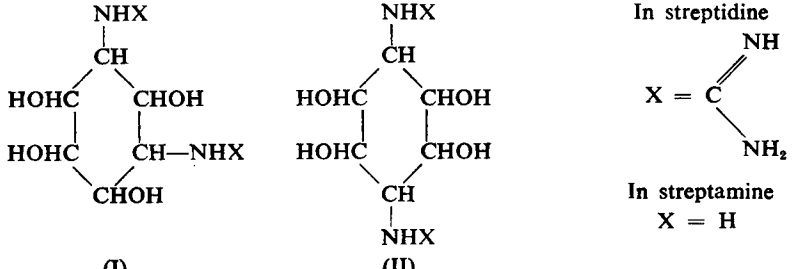
Alkaline hydrolysis of streptidine (6 N alkali) affords ammonia (4 mol.) and a new base named streptamine[14]. The base forms a slightly soluble sulphate by means of which it may be isolated. The hydrolysis occurs as shown:

$$\underset{\text{Streptidine}}{C_8H_{18}O_4N_6} + 4H_2O = \underset{\text{Streptamine}}{C_6H_{14}O_4N_2} + 4NH_3 + 2CO_2$$

The inference from this reaction was that the six nitrogen atoms of streptidine were present as two mono-substituted guanido groups which were degraded to primary amino groups in streptamine. This was confirmed by alkaline hydrolysis of streptidine under milder conditions. Two mol. of ammonia and the corresponding diurea $C_6H_{10}O_4(NHCONH_2)_2$ were obtained[13,15]. Direct evidence of the presence of the guanido groups in streptidine was given by permanganate oxidation which afforded 1·3 mols. of guanidine (isolated as picrate) per mol. of streptidine.

Streptamine gave a hexabenzoyl derivative and a hexa-acetyl derivative by the usual methods. The hexabenzoyl streptamine was converted into a *NN'*-dibenzoyl derivative by heating with 0·5N methanolic sodium hydroxide. This evidence indicates that the four oxygen atoms are present as hydroxyl groups.

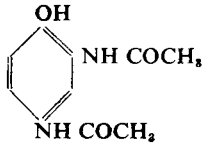
Further evidence as to the structure of streptamine was obtained by a study of its reaction with periodate. Streptamine reduced 6 mols. of periodate whereas dibenzoyl streptamine and streptidine reduced 2 mols. The utilisation of 6 mols. of periodate and the absence of formaldehyde as a reaction product indicated that the streptamine molecule had a cyclic structure with a hydroxy or amino group attached to each carbon. The resulting diaminotetrahydroxycyclohexane structure must have the amino groups arranged in the 1:3 or 1:4 positions (I or II), since the 1:2 position would involve reaction of 3 mols. of periodate (not 2) with streptidine and dibenzoyl streptamine.



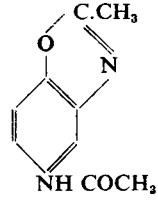
Streptidine and Streptamine

A definite choice in favour of (I) was made by Carter *et al.*[16] when they found that a periodate oxidation product of *NN'*-dibenzoyl streptamine further oxidised by bromine water gave a dibenzoylamino hydroxyglutaric acid. A compound of formula (II) should give rise on oxidation with periodate to two similar 3-carbon fragments. Further proof of structure I was advanced by Peck *et al.*[15], who obtained

2:4-diacetamidophenol (III) and 5-acetamido-2-methyl benzoxazole (IV) by heating hexa-acetyl streptamine in a sealed tube at 350°C. for one hour.

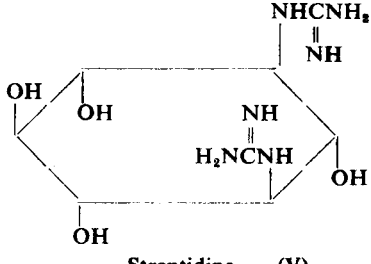


(III)



(IV)

The configuration of streptidine was still undecided, although it was expected to be a meso-form since it was optically inactive. Wintersteiner and Klingsberg[17] by degrading *O*-tetramethyl streptamine to DL-dimethoxysuccinic acid showed that the 5-hydroxyl group was orientated *trans* with respect to the 4- and 6-hydroxyl groups. Complete evidence of configuration was obtained when Wolfrom and Olin[18] synthesised streptamine starting from D-glucosamine. In the final stage of a series of reactions one of the two crystalline products was hexa-acetyl streptamine. Further Wolfrom and Polglase[19] were able to convert hexa-acetyl streptamine to streptidine sulphate monohydrate. From the method of synthesis and the knowledge that streptidine was *meso* it followed that streptidine had the all *trans*-configuration (V).

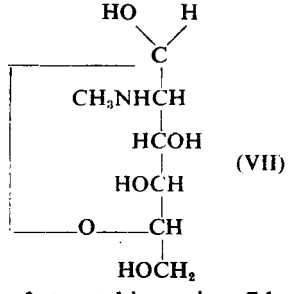


Streptidine (V)

Streptobiosamine. The structure of the larger fragment of the streptomycin molecule, streptobiosamine, remains to be considered. On fission of streptomycin by methanolic hydrogen chloride the products consist of streptidine hydrochloride and the amorphous hydrochloride of a base known as methyl streptobiosaminide dimethyl acetal (VI), $C_{13}H_{22}O_7N(OCH_3)_3$, HCl which on acetylation gave a crystalline tetra-acetyl derivative $C_{13}H_{18}O_7N\ (CH_3CO)_4\ (OCH_3)_3$ with three *O*-acetyl and one *N*-acetyl group. Since (VI) contained no primary amino group and afforded methylamine on alkaline hydrolysis, it appeared that the nitrogen atom in streptobiosamine was present as a methylamino group.

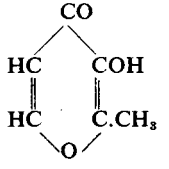
Acid hydrolysis of (VI) and acetylation of the product[20] gave a penta-acetyl hexosamine $C_{17}H_{25}O_{10}N$, m.pt. 160° to 163°C. By hydrolysis with hydrochloric acid this was converted to the hydrochloride of a hexosamine $C_7H_{15}O_5N$, which could be oxidised with mercuric oxide to an acid of m.pt. 230° to 232°C. This m.pt. agreed with the m.pt. of the known *N*-methyl-D-glucosamic acid. The optical rotations of the two

acids were identical in magnitude but of opposite sign. The inference was that the degradation product was *N*-methyl-L-glucosamic acid and a synthesis of the latter from L-arabinose, methylamine and hydrocyanic acid showed that such was indeed the case. Thus the configurations about the C_3, C_4, and C_5 atoms of the hexosamine were the same as those about the C_2, C_3, and C_4 of L-arabinose or about C_3, C_4 and C_5 of L-glucose. The configuration about the C_2 in the hexosamine must be the opposite of that of D-glucose, therefore that of L-glucose. One acid hydrolysis fragment of methyl streptobiosaminide dimethyl acetal was therefore *N*-methyl-L-glucosamine (VII).



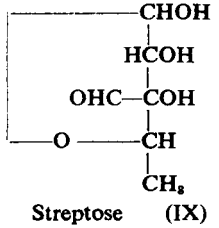
(VII)

Of the 13 carbon atoms of streptobiosamine, 7 had now been identified, being present in the *N*-methyl-L-glucosamine fragment. There still remained a six-carbon fragment which was named streptose to be identified. A certain amount of information as to the nature of this fragment can be gleaned from the evidence already presented. Streptomycin contains a free aldehydo group. This must be present in the six-carbon fragment. Further the γ-pyrone maltol (VIII)[21] is a product of alkaline hydrolysis of streptomycin, but not of dihydrostreptomycin or streptomycinic acid.



(VIII)

It was assumed (correctly) that the six carbon atoms of maltol were derived from streptose, although the actual formula of maltol was misleading as a guide to the structure of stretose since it transpired that the conversion involved a carbon-carbon rearrangement. As a result of a considerable amount of further work the structure of streptose was finally shown to be (IX).

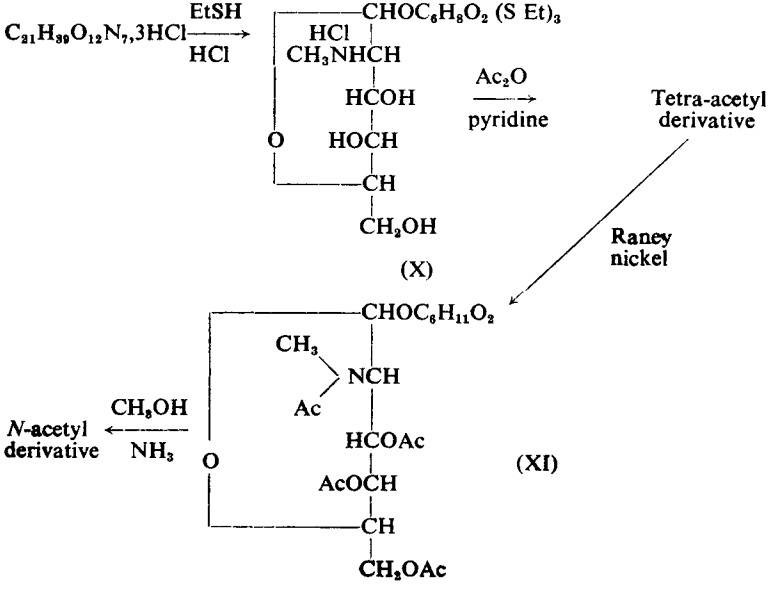


Streptose (IX)

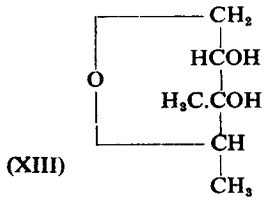
Considerable light was thrown on the constitution of streptose and its manner of linkage by degrading streptomycin with ethyl mercaptan[22]. With ethyl mercaptan and hydrogen chloride the products were streptidine and ethyl thiostreptobiosaminide diethyl mercaptal hydrochloride (X):

$$C_{21}H_{39}O_{12}N_7,\ 3HCl + 3C_2H_5SH \rightarrow$$
$$C_8H_{18}O_4N_6,\ 2HCl + C_{13}H_{22}O_7N\ (SC_2H_5)_3,\ HCl + H_2O.$$

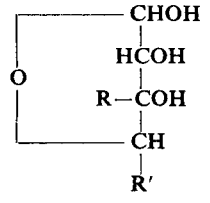
(X) was acetylated with acetic anhydride in pyridine to a crystalline tetra-acetyl compound which on hydrogenolysis by refluxing in 70 per cent. ethanol with Raney nickel afforded a sulphur-free product $C_{13}H_{21}O_7N(CH_3CO)_4$. It appeared that three ethylmercapto groups had being replaced by hydrogen atoms, the product being named tetra-acetyl-bisdeoxystreptobiosamine (XI). Partial deacetylation removed three acetyl groups to give the *N*-acetyl compound, which did not reduce Fehling's solution. It thus appeared that the linkage between the two hexose portions of streptobiosamine involved carbon atom one of *N*-methyl-L-glucosamine the reactions being formulated in accordance with the scheme below.



Tetra-acetylbisdeoxystreptobiosamine (XI) on acid hydroylsis afforded *N*-methyl-L-glucosamine and bisdeoxystreptose (XII) $C_6H_2O_3$, m.pt. 90° to 91°C. which contained 2 C-methyl groups, and formed a bis-*p*-nitrobenzoate. Oxidation with 1 mol. of periodic acid, acid hydrolysis and treatment of the product with substituted hydrazines afforded osazones of biacetyl. (XII) was therefore formulated as 3:4-dihydroxy-2:3-dimethyltetrahydrofuran (XIII). The hydroxy groups are assumed to have the *cis* configuration since the substance forms an acidic complex with boric acid.



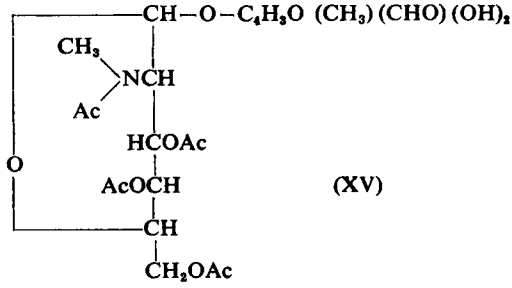
(XIII)



(XIV)

Streptose should then have the configuration (XIV) where R=CHO and R′=CH_3 or R=CH_3 and R′=CHO.

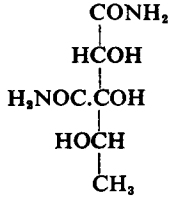
A final solution of the structures of streptose and streptobiosamine was obtained[22] by further degradation of (X). Reaction with mercuric chloride in presence of cadmium or strontium carbonate buffer afforded tetra-acetyl streptobiosamine (XV).



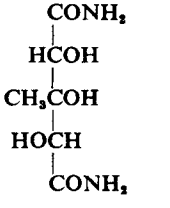
(XV)

This on oxidation with bromine yielded an acid which on reacetylation was obtained as a penta-acetyl derivative (XVI), $C_{13}H_{21}O_{10}N(CH_3CO)_5$, which was named penta-acetylstreptobiosamic acid monolactone.

After hydrolysis of (XVI) with hydrochloric acid and extraction of the dried mixture with acetone a crystalline compound derived from the streptose portion of the molecule was obtained. This compound $C_6H_8O_6$ was named streptosonic acid monolactone (XVII) in accordance with its properties. It contained one C-methyl group, and formed a diamide (XVIII) which afforded acetaldehyde on oxidation with 2 mol. of periodate. On the basis of the alternative formulæ for streptose indicated in (XIV) there are two possible structures for (XVIII) as shown below:



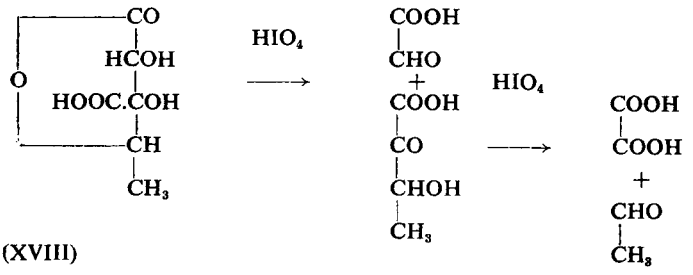
(XVIII a)



(XVIII b)

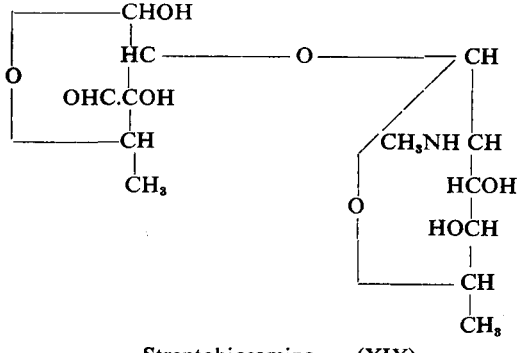
Only one of these (XVIII a) could give rise to acetaldehyde on periodate oxidation, (XVIII b) is therefore eliminated. Streptose is therefore to be represented by structure (IX). Confirmatory evidence of the correctness of this structure was obtained by oxidising (XVII) with 2 mol.

of periodic acid. This oxidation afforded glyoxylic acid and oxalic acid as follows:



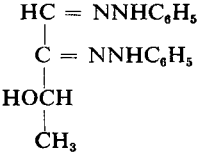
(XVIII)

Since the streptobiosamine derivatives had been shown to contain a free tertiary hydroxy group resistant to acetylation the linkage of the two moieties must involve the hydroxy group attached to carbon atom 2 of streptose. This indicated that streptobiosamine should be formulated as (XIX)



Streptobiosamine (XIX)

The configuration of streptose was decided by the action of phenylhydrazine on streptobiosamine. By this means Fried, Walz and Wintersteiner[23] obtained a crystalline osazone which was identified as 4-deoxy-L-erythrose (threose) phenyl osazone (XX)



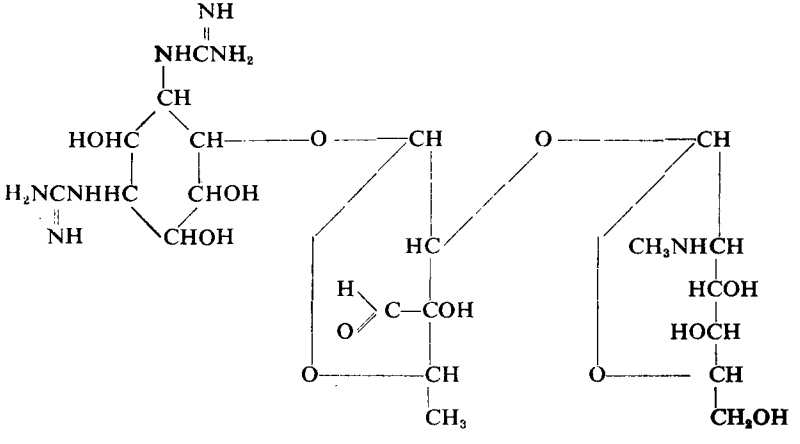
(XX)

The asymmetric carbon atom of this compound was obviously the 4-carbon of streptose and since it had the L-configuration streptose was an L-sugar.

By bromine oxidation and hydrolysis of penta-acetyldihydrostreptobiosamine Kuehl *et al.*[24] obtained dihydrostreptosonic acid lactone and from it the corresponding hydrazide. By applying Hudson's rule to the hydrazide and to streptosonic acid diamide it became evident that the hydroxyl group at C_2 of streptose was oriented to the right according

to the conventional representation. Since the hydroxy groups at C_2 and C_3 have been shown to be *cis*, the configuration of L-streptose is correctly represented as (IX). By applying the Hudson rules of isorotation to various derivatives of streptomycin the glycosidic linkage between streptose and *N*-methyl-L-glucosamine was found to be α-L.

It had been assumed and was later confirmed that the streptidine portion of the streptomycin molecule was linked through one of its hydroxyl groups to the rest of the molecule. Evidence for the participation of the hydroxy group in position 4 was obtained by Kuehl *et al.*[25] They benzoylated streptomycin and hydrolysed the product to yield streptidine heptabenzoate, which was finally converted by a series of reactions to *NN'*-dibenzoyldeoxystreptamine. This product and *NN'*-dibenzoyl streptamine react with 1 mol. and 2 mols. respectively of periodate. These facts are consistent with the derivation of deoxystreptamine from streptamine by elimination of the hydroxy group at C_4. Further conclusive evidence on this point was later advanced by Kuehl, Peck, Hoffhine and Folkers[26]. The product of oxidation of *NN'*-dibenzoyldeoxystreptamine with periodate was shown to be αγ-dibenzamido-β-hydroxyadipaldehyde, which could not have been formed if the methylene group in deoxystreptamine were in the 2- or 5-position. The linkage of streptidine to streptobiosamine must therefore be through position 4 leading to the following complete structural formula (XXI) for streptomycin:



Streptomycin (XXI)

Other Streptomycins

Mannosidostreptomycin (*Streptomycin B*). By the use of the counter current distribution technique of Craig[27] for the examination of streptomycin concentrates Titus and Fried[28] obtained evidence of the presence of a second maltol-producing substance which they were able to isolate as a crystalline reineckate[29]. The new substance possessed antibiotic activity and was named streptomycin B. The name was later changed

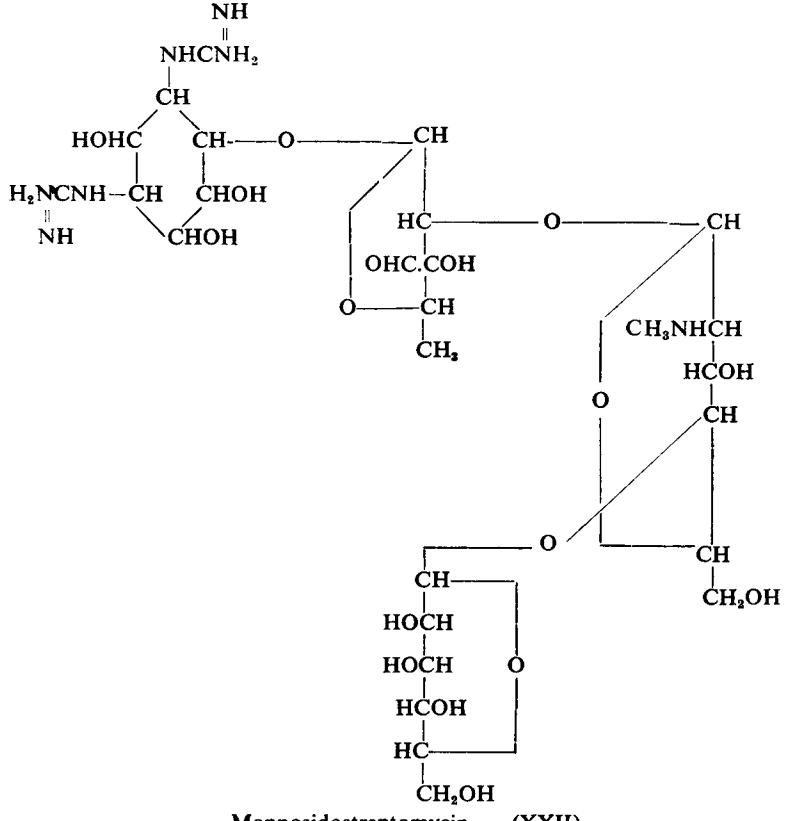
to mannosidostreptomycin when its nature became apparent. It could be differentiated from streptomycin by paper chromatography as shown by Winsten and Eigen[30]. Other methods of separation of mannosidostreptomycin from streptomycin using the counter current technique have been proposed. Plant and McCormack[31] used an aqueous phase containing sodium bicarbonate and sodium chloride with a solvent phase consisting of mixed amyl alcohols containing 5 per cent. of stearic acid. O'Keefe, Dolliver and Stiller[32] employed lauric acid in amyl alcohol as the solvent phase with aqueous phosphate and borate buffer.

Like streptomycin, mannosidostreptomycin is inactivated by carbonyl reagents and it forms a dihydro compound by reduction of the carbonyl group. The free base has the molecular formula $C_{27}H_{49}O_{17}N_7$. The difference in composition $C_6H_{10}O_5$ between mannosidostreptomycin and streptomycin appeared to be due to one hexose unit attached to the latter with loss of one molecule of water. Fried and Stavely[33] were able to show that degradation of mannosidostreptomycin gave rise to derivatives of streptidine, streptobiosamine and D-mannose. Stavely and Fried[34] further showed that in mannosidostreptomycin the mannose is attached glycosidically to the streptobiosamine moiety through one of the three free hydroxyl groups of *N*-methyl L-glucosamine. The reactions employed in various fissions of streptomycin, methanolysis, mercaptolysis, acetolysis were again put to good use in the elucidation of the still more complex mannosidostreptomycin. From their data Stavely and Fried by applying the Hudson rule deduced that the glycosidic linkage between the D-mannose and *N*-methyl-L-glucosamine moieties in mannosidostreptomycin was of the α_D type and further that mannosidostreptomycin is a D-mannosido-*N*-methyl-α-L-glucosaminide-L-streptoside of streptidine. By analogy with streptomycin the structure proposed for mannosidostreptomycin was (XXII).

Perlman and Langlykke[35] found that certain growing cultures of *Streptomyces griseus* are able to cleave hydrolytically added mannosidostreptomycin with the production of streptomycin. This acticity, ascribed to the presence of an enzyme termed mannosidostreptomycinase was found only in cultures which produce streptomycin. The enzyme has a *p*H optimum of 7·5 to 8. Reducing conditions and copper and mercury ions inhibit hydrolysis. Active cell-free preparations also appear to induce hydrolysis of dihydromannosidostreptomycin. This is a matter of considerable interest since, during the production of streptomycin by fermentation, the first active product obtained is mannosidostreptomycin, but this under the influence of the enzyme present is almost completely converted in a few hours to streptomycin in the normal fermentation.

Hydroxystreptomycin. A new species of Streptomyces isolated from Japanese soil, and which was named *Streptomyces griseo-carneus*, was recently found by Benedict *et al.*[36] to produce a new antibiotic. This new substance gave an antibacterial spectrum typical of streptomycin and when extracted and purified by the usual methods it appears to resemble streptomycin more closely in composition, optical rotation and

biological potency at each successive stage of purification. Only by strip chromatography with the technique of Winsten and Eigen[30] was a complete separation achieved of the new antibiotic from mannosidiostrepto-

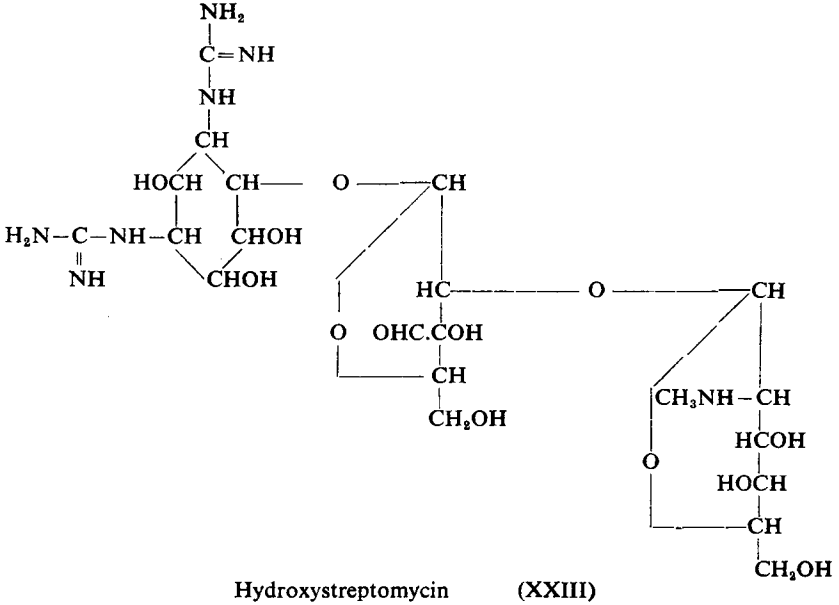


Mannosidostreptomycin (XXII)

mycin and streptomycin in artificially prepared mixtures. Analysis of the crystalline helianthate and trihydrochloride indicated that the new base had the empirical formula $C_{20}H_{39}O_{13}N_7$, and thus contained one atom of oxygen more than streptomycin. From its composition and the nature of the degradation products the new antibiotic was named hydroxystreptomycin.

When dihydrohydroxystreptomycin was hydrolysed by methanolic hydrogen chloride it afforded streptidine and a disaccharide fragment isolated as the hexa-acetate, m.pt. 124° to 125°C. Under identical conditions dihydrostreptomycin gave streptidine and α-methylpenta-acetydihydrostreptobiosaminide, m.pt. 194°C. Drastic acid hydrolysis of hydroxystreptomycin followed by acetylation yielded penta-acetyl *N*-methyl-α-L-glucosamine identical with the product from streptomycin. The additional oxygen atom is therefore located in the streptose portion of the molecule. Closer examination of the alkali degradation product of hydroxystreptomycin revealed that it was not maltol as at first

thought but a hydroxymaltol, presumably 2-hydroxymethyl-3-hydroxy-1:4-pyrone, since methylation of its ring hydroxyl with diazomethane gave a crystalline methyl ether in which the presence of a remaining hydroxyl group could be demonstrated by the formation of a crystalline derivative with *p*-phenylazobenzoyl chloride. On the assumption that the component parts of the molecule are linked in the same way as in streptomycin the formula (XXIII) in which a CH_2OH replaces the CH_3 of the streptose moiety, is tentatively assigned to hydroxystreptomycin.



Hydroxystreptomycin (XXIII)

Antibiotic and Biological Properties

Activity of streptomycin. Streptomycin exerts antibacterial activity against a wide range of organisms. These include both Gram-positive and Gram-negative species. In this respect streptomycin is complementary to penicillin which is active in general only against Gram-positive organisms. Owing to its low toxicity and its retention of activity in the animal body it has been used with success in the treatment of a number of diseases, and perhaps its most interesting application has been its use in tuberculosis in which it has proved a valuable adjunct to medical treatment.

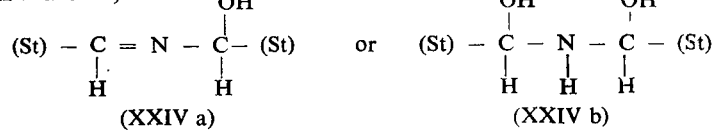
Dihydrostreptomycin shows an activity very similar to that of streptomycin against a number of species of bacteria. Mannosidostreptomycin has in general a lower activity against bacteria than streptomycin. On a weight basis Rake *et al.*[37] found that the ratio of streptomycin to mannosidostreptomycin activity against various organisms *in vitro* varied from 1·23 to 7·06. The relative activities *in vivo* were of the same order. Thus any substantial amount of mannosidostreptomycin in the commercial product would reduce its activity.

A disturbing feature of the use of antibiotics in chemotherapy is the fact that micro-organisms may develop resistance to the drug. This is particularly marked in the case of streptomycin where the acquirement of resistance by many species of pathogenic organisms has been investigated. This resistance is acquired both *in vitro* and *in vivo* and once established appears to be permanent. To cite one example; Williston and Youmans[38] found that on repeated subculture of 18 strains of *Mycobacterium tuberculosis* (15 human, 2 bovine, 1 avian) in increasing concentration of streptomycin the initial inhibition exhibited at a streptomycin concentration of 0·095 to 3·125 μg./ml. was increased in 9 strains to 1000 μg./ml., in 2 strains to 500 μg./ml. and in 1 to 50 μg./ml. The other 6 strains developed only a 2- to 8-fold increase in resistance.

In some cases streptomycin in small doses has been observed to exert a stimulating effect on pathogenic organisms *in vitro* and suggestions of a similar effect have been obtained *in vivo*. Even more interesting is the evidence for the functioning of streptomycin as an essential growth factor in the case of certain organisms. Thus Miller and Bohnhoff[39] found that when strains of meningococcus were cultivated on solid media containing graded amounts of streptomycin two types of variant appeared. Type A was very resistant to streptomycin both *in vitro* and *in vivo*. Type B would not grow on medium free from streptomycin. It failed to kill mice unless they were injected with 500 to 5000 μg. of streptomycin. These observations have been found to apply to other micro-organisms, e.g., *Myco. ranæ*.

Toxicity. When the chemotherapeutic possibilities of streptomycin were first investigated certain unpleasant toxic manifestations became apparent. Some of these were due to impurities present in the earliest samples employed, but even when streptomycin has been rigorously purified it is not entirely free from toxic action which seems to be an inherent property of the drug itself when given in large doses. Its most important effect appears to be to cause damage to the eighth cranial nerve, but this effect can be compensated when therapy is discontinued. Dihydrostreptomycin was earlier thought to be less toxic than streptomycin and was for this reason often preferred for administration. The consensus of opinion now has veered to the view that the toxicities of the two substances are equal. Hydrogenation may have had the effect of eliminating from the earlier samples of streptomycin some of the toxic impurities.

The efficiency of the present processes of manufacture and purification have virtually eliminated these toxic impurities of the nature of which little is known. One such impurity has however been studied. Solomons and Regna[40] isolated from streptomycin sulphate residues a toxic product, bis(α-hydroxystreptomycyl)amine to which is assigned the constitution (XXIV a or b)



where (St) is streptomycin less –CHO. This compound is obtained by the interaction of streptomycin in aqueous methanol with ammonium chloride. The *p*H is adjusted to 7·5 with triethylamine and the mixture is heated for 3 hours at 50° to 60°C. Dihydrostreptomycin on the other hand does not react with ammonia in this way. The bis(α-hydroxystreptomycyl)amine is highly toxic to mice when injected intravenously and can be produced under the ordinary conditions of fermentation, particularly at alkaline *p*H.

Site of action of streptomycin. Although various observers have found that streptomycin inhibits the metabolic activities of non-proliferating cells of susceptible strains of bacteria, there is only scanty evidence to show at what stage in the metabolic chain this inhibition is accomplished. Geiger[41], however, demonstrated with a particular strain of *Escherichia coli* that there was a marked stimulation of serine oxidation by the previous oxidation of fumarate. When streptomycin was present during the oxidation of the fumarate this stimulation was prevented. Umbreit[42] followed up this observation and found that with the Gratia strain of *E. coli* the oxidation of threonine was likewise stimulated by the previous oxidation of fumarate but that this stimulation was also prevented by streptomycin. He found that under suitable conditions the oxidation of fumarate and pyruvate could be inhibited by streptomycin and that this inhibition affects the terminal respiration process. He concluded that in particular the pyruvate-oxalacetate condensation is involved. Oginsky, Smith and Umbreit[43] further examined the effect and found that the susceptible reaction, when inhibited, prevents a variety of substances from entering the terminal respiration system that resembles the citric acid cycle. Resistant and dependent variants of the streptomycin-sensitive *E. coli* do not apparently possess the ability to effect the oxalacetate-pyruvate condensation. This is the reaction inhibited by streptomycin in the sensitive strains and it appears that ability to grow in presence of streptomycin depends on the development of an unknown alternative pathway, which can by-pass this particular condensation[44]. It was further found[45] that streptomycin will inhibit the oxalacetate-pyruvate condensation in animal tissue as well as in the bacterial cell. That this does not normally occur is attributed to permeability barriers existing not only at the cell wall but also at the mitochondria.

Streptomycin, depite its minor drawbacks, is still the drug of choice in a number of infections such as tularæmia, *Hæmophilus influenzæ* infections and urinary tract infections due to susceptible Gram-negative organisms. It is also used in bacteriæmia, bacterial endocarditis and meningitis, tuberculosis, *Klebsiella pneumoniæ* and *Salmonella* infections and peritonitis when due to susceptible organisms. Dr. Hinshaw, who was responsible for much of the earlier clinical work in assessing the value of streptomycin in tuberculosis has stated:—" Streptomycin is the only effective remedy in miliary tuberculosis and in tuberculous meningitis and while it has not cured all it is a very great remedy offering the first real hope of treatment of a disease which was formerly almost

universally fatal. . . . Streptomycin has very great value in some of the most fulminating types of lung tuberculosis."

In the prevention and cure of tuberculosis it has frequently been observed that certain combinations of drugs have a synergistic action, the combined effect being better than could be anticipated by a simple summation of the effects of each drug administered singly. Such combinations as streptomycin and sulphetrone and streptomycin and *p*-aminosalicylic acid show synergism to a marked degree. These observations are indicative of recent trends in the employment of streptomycin in chemotherapy.

A very full and well-documented account of the earlier work (up to 1948) on all except the purely clinical aspects of streptomycin has been compiled by Florey and his group at Oxford[46].

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PARASYMPATHOMIMETICS AND ANTICHOLINESTERASES

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This Journal, July, 1951

Corrections:

Page 387, line 14, for " stability, or drugs . . . ", read " stability of drugs . . .";

page 391, in formula 20 the P should carry, in addition to the other groups, a $\longrightarrow$ O (double bonded oxygen); in formula 21a & b the NO_2 group should be in the *para* position to the phosphate group;

page 396, second line from bottom, for " kinetics[39] " read " kinetics[38] ";

page 397, first line of second paragraph, for " cholinesterases[31] " read " cholinesterases[38] "